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Molecular Components of Nitrate and Nitrite Efflux in Yeast

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Some eukaryotes, such as plant and fungi, are capable of utilizing nitrate as the sole nitrogen source. Once transported into the cell, nitrate is reduced to ammonium by the consecutive action of nitrate and nitrite reductase. How nitrate assimilation is balanced with nitrate and nitrite efflux is unknown, as are the proteins involved. The nitrate assimilatory yeast *Hansenula polymorpha* was used as a model to dissect these efflux systems. We identified the sulfite transporters Ssu1 and Ssu2 as effective nitrate exporters, Ssu2 being quantitatively more important, and we characterize the Nar1 protein as a nitrate/nitrite exporter. The use of strains lacking either *SSU2* or *NAR1* along with the nitrate reductase gene *YNR1* showed that nitrate reductase activity is not required for net nitrate uptake. Growth test experiments indicated that Ssu2 and Nar1 exporters allow yeast to cope with nitrite toxicity. We also have shown that the well-known *Saccharomyces cerevisiae* sulfite efflux permease Ssu1 is also able to excrete nitrite and nitrate. These results characterize for the first time essential components of the nitrate/nitrite efflux system and their impact on net nitrate uptake and its regulation.

The yeast *Hansenula polymorpha* is able to use nitrate as the sole nitrogen source. Nitrate is transported into the cell and then reduced to ammonium by the consecutive action of nitrate and nitrite reductase (NR) (1–3). Nitrate assimilation genes are induced by nitrate (4, 5) and repressed by preferred nitrogen sources (6). High-affinity nitrate and nitrite transport is mainly mediated by Ynt1, which also is posttranslationally regulated in response to nitrogen source quality (7, 8). In algae and yeast, nitrate acts as an inducer once it enters the cell, and therefore, intracellular nitrate levels play a key role in regulating nitrate assimilation genes (9). In this framework, nitrate and nitrite effluxes from the cell could play an important role in net nitrate/nitrite uptake and also in keeping nitrite below toxic levels. Nitrite efflux has been observed in most organisms, including *H. polymorpha*, growing in nitrate (1, 10–13), indicating a clear imbalance between nitrate uptake and reduction to nitrite and its further transformation to ammonium. In contrast, nitrate efflux has not been found in fungi. However, in plants, nitrate efflux can even exceed nitrate uptake in various stress situations. A nitrate excretion transporter, NAXT1, belonging to the NRT1/PTR family has been found in the root plasma membrane of *Arabidopsis thaliana*, although its role is scarcely understood (14). Moreover, it has been reported that *Arabidopsis* NRT1.1 (CHL1) is a bidirectional transporter involved in root-to-shoot nitrate translocation (15).

In *Saccharomyces cerevisiae*, Ssu1 is involved in sulfite efflux (16). It belongs to the tellurite resistance/dicarboxylate transporter (TDT) family, which includes the *Escherichia coli* tellurite transporter TehAp and the *Schizosaccharomyces pombe* malate transporter Mae1 (17). Upregulation of *SSU1* and *YHB1* has been found in *S. cerevisiae* and *Candida albicans* in response to nitric oxide (NO)-generating compounds (18, 19). *YHB1* encodes a flavohemoglobin that presents NO dioxygenase activity, which catalyzes the transformation of NO to nontoxic nitrate and thereby protects against nitrosylation of cellular targets and inhibition of cell growth, under both aerobic and anaerobic conditions (20). However, the role of Ssu1 in NO detoxification is unknown, although it has been suggested that besides transporting sulfite, Ssu1 may also transport NO-derived metabolites, such as nitrite or nitrate, out of the cell (19).

Aspergillus nidulans NitA (AnNitA), belonging to the formate-nitrite transporter family (FNT), mediates specific high-affinity transport of nitrite in *A. nidulans* and also has some role in nitrite efflux in that fungus (13). FNT members have been found in bacteria, archaea, fungi, algae, and protozoan parasites. In *E. coli*, FocA and NirC have been characterized and implicated in the transport of formate and nitrite, respectively (21, 22). Moreover, NirC is also involved in nitrite efflux (11). The structure of FocA strongly suggests that it is a channel rather than a transporter (23). In *Chlamydomonas reinhardtii*, some of the NAR1 genes are clearly regulated by carbon or nitrogen (24) and involved in nitrite transport in the chloroplast (25).

In this study, we aimed to explore at a molecular level the nitrate and nitrite extrusion systems in the nitrate-assimilatory yeast *H. polymorpha*. The rationale of our approach was to search the *H. polymorpha* genome database for genes encoding membrane proteins with similarity to nitrate/nitrite transporters. Ssu1/2, encoding a sulfite permease, were included because of the structural resemblance between sulfite and nitrite and also since *SSU1* is induced by NO precursor donors in *S. cerevisiae* (19). We have uncovered some of the molecular entities involved in nitrate/nitrite efflux in fungi. Ssu2 and to a lesser extent Ssu1 extrude

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nitrate, while Nar1 extrudes nitrate and nitrite. We also have shown that *S. cerevisiae* Ssu1 extrudes nitrite and nitrate, in addition to sulfite.

MATERIALS AND METHODS

Strains and growth conditions. The *H. polymorpha* strains used in this work are listed in Table S1 in the supplemental material. All strains are derivatives of the NCYC495 *leu2 ura3* strain. Yeast cells were grown with shaking at 37°C in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] glucose) or synthetic medium containing 0.17% (wt/vol) yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% (wt/vol) glucose, and the nitrogen source indicated in each case. Nitrogen deprivation medium (nitrogen-free medium) contains 0.17% (wt/vol) yeast nitrogen base without amino acids and ammonium sulfate (Difco) and 2% (wt/vol) glucose (YG). Whenever necessary, media were supplemented with 30 μg/ml L-leucine, 20 μg/ml uracil, or 100 μg/ml Zeocin (Invitrogen). Sulfite plates were made as described previously (26). To test yeast chlorate sensitivity, potassium chlorate was added to medium before sterilization at the concentration indicated in each case. One OD_{660} (optical density at 660 nm) unit was about 3.5 mg cells · ml^{-1} (approximately 7×10^7 cells · ml^{-1}).

Plasmids. All of the primers for gene disruption, tagging, or quantitative real-time PCR (qRT-PCR) are described in Table S2 in the supplemental material. All vectors used in this work are listed in Table S3. The *pHPI 359* vector (27) was used to fuse the promoter of the *SSU2* or *NAR1* gene to the *lacZ* gene to obtain pP_{SSU2}-*lacZ* or *pNAR1-lacZ*, respectively. The region from −1006 to +45 relative to ATG of *SSU2* was amplified by PCR from genomic DNA, using the primers SSU2Prom-F and SSU2Prom-R. To obtain the promoter of *NAR1*, the region from −900 to +31 was amplified with the primers proNAR1-F and proNAR1-R. Both constructs were linearized at BstEII in *LEU2* before yeast transformation. pP_{SSU2I}-Sc*SSU1LEU2* was generated to express *S. cerevisiae SSU1* (Sc*SSU1*) (NC_001148.4, NCBI reference sequence) under the *H. polymorpha SSU2* (Hp*SSU2*) gene promoter and was obtained by inserting a 1,883-bp DNA fragment containing the Sc*SSU1* open reading frame into the plasmid $pGEMT\text{-}P_{SSU2I}LEU2$. This last vector was constructed by inserting a 1,200-bp DNA fragment containing the Hp*SSU2* gene promoter into the plasmid pGEM-T Easy (Promega) and by inserting the *LEU2* gene marker. To transform yeast, DNA was linearized at BstEII in *LEU2*. *pSSU2-GFP* and *pNAR1-GFP* carry the *SSU2* or *NAR1* C-terminal region fused in frame to GFP (green fluorescence protein) by inserting each open reading frame without its stop codon into the BglII site of *pANL31* (28). *pSSU2-GFP* was linearized at *SSU2* with BclI while *pNAR1-GFP* was linearized at *NAR1* with KpnI to transform strains bearing *SSU2* or *NAR1*. *pSSU2-YFP* contains the *SSU2* open reading frame without a stop codon fused to the 5′-end cDNA of the enhanced yellow fluorescent protein (eYFP) in EcoRI-SalI sites of *pEYFP-N1* (BD Biosciences Clontech) and subcloning into EcoRI-SalI sites of *pGEMHE*. A 1,200-bp DNA fragment containing *SSU2-YFP* was subcloned into *pGEMHE*, which contains 5′ and 3′ untranslated regions of the *Xenopus laevis* β-globin gene (29) to enhance protein expression in *X. laevis* oocytes.

Disruption of *H. polymorpha SSU1*, *SSU2*, and *NAR1* genes. To disrupt *SSU1*, the region from −905 to +1947 relative to the ATG start codon was amplified by PCR using *Pfu* from genomic DNA by using the oligonucleotides SSU1-F and SSU1-R. This fragment was cloned into the plasmid pGEM-T Easy (Promega), obtaining the vector *pGEMT-SSU1*. A 1,642 bp-internal region from *SSU1* (from nucleotide −427 to +1215) was removed with XhoI and BglII and replaced by the *URA3* gene marker to generate the vector *pssu1ΔURA3*. *ssu1Δ* strains were then generated by transforming the wild type (WT) with the 3,134-bp fragment amplified from *pssu1ΔURA3* with the SSU1-F and SSU1-R oligonucleotides. Transformants bearing the disrupted target gene were identified by PCR. The region from −999 to +1828 relative to ATG of the *SSU2* gene was amplified as above using the oligonucleotides SSU2-F and SSU2-R. This fragment was cloned into the plasmid pGEM-T Easy (Promega), obtaining the vector *pGEM-SSU2 int*. A 1,599-bp internal region from *SSU2* (from nucleotides −174 to +1425) was removed with NruI and replaced by the *zeocin* resistance gene (*ble* gene) as a selective marker from *pREMIZ* (30) to generate the vector *pssu2Δble*. *ssu2Δ* strains were then generated by transforming the WT with the 3,217-bp fragment amplified from *pssu2Δble* with the SSU2-F and SSU2-R oligonucleotides. Transformants bearing the disrupted target gene were identified by PCR and by sulfite sensitivity. To disrupt *NAR1*, the region from −777 to +2096 relative to ATG was amplified by PCR using *Pfu* from genomic DNA by using the oligonucleotides 334int-F and 334int-R. This fragment was cloned into the plasmid pGEM-T Easy (Promega), obtaining the vector *pNAR1*. A 347-bp internal region from *NAR1* (from nucleotides +430 to +777) was removed with BamHI and KpnI and replaced by the *URA3* gene marker to generate the vector *pnar1Δ*. The *nar1Δ*::*URA3* strain was generated by transforming the WT with the 4,499-bp fragment amplified from *pnar1Δ* with the 334int-F and 334int-R oligonucleotides. Transformants bearing the disrupted target gene were identified by PCR.

n*SSU2* and n*NAR1* strains. Strains bearing several copies of the *SSU2* gene (n*SSU2*) were obtained by transforming the WT strain with the plasmid *pSSU2-URA3* or *pSSU2-LEU2* linearized at the *URA3* gene with BglII or at *LEU2* with BstEII. Strains bearing multiple integrations of *pSSU2URA3* or *pSSU2LEU2* were screened for increased sulfite resistance and low nitrate uptake. The n*NAR1* strain was obtained by transforming *nar1Δ* with the plasmid *pNAR1-LEU2* linearized at *LEU2* with NarI. n*NAR1* strains were screened for increased nitrite resistance and nitrite excretion.

Determination of intracellular nitrate and nitrite. Cells grown in ammonium were resuspended at 10 mg/ml (wet weight) in YG and incubated with shaking for 120 min, and then nitrate and nitrite were added to the cells at the concentration indicated in each case. Cells (250 mg [wet weight]) were collected over 25 ml cold water by centrifugation for 5 min at 4,863 × *g* at 4°C, washed with cold water, and kept below −20°C until use. Cells were resuspended in 1 ml of a boiling solution made of 75% ethanol (vol/vol) buffered with 70 mM HEPES, pH 7.5, and incubated 5 min at 80°C, as described previously (31). After cooling down on ice for 5 min, samples were centrifuged for 15 min at 20,500 × *g* at 4°C to remove the cells. Volume was reduced to 500 μl by evaporation at 40°C using a vacuum concentrator (Heto). Nitrate and nitrite uptake activity was measured as described in a previous report (32) as extracellular nitrate or nitrite depletion. Purified *H. polymorpha* nitrate reductase enzyme (NECi) was used to determine the nitrate concentration. Nitrite was colorimetrically measured as described previously (33). Nitrate uptake is expressed as nmol of NO_3^-/NO_2^- transported · min^{-1} · mg of $cell^{-1}$. Results are reported as mean values ± standard deviations (SD) from at least three independent experiments. Sulfite up to 240 μM does not interfere with nitrate determination assays using purified *H. polymorpha* nitrate reductase (NECi) (data not shown).

Functional expression of Hp*SSU2* in *Xenopus* oocytes. Capped mRNA was transcribed *in vitro* from linearized *pSSU2-YFP* by using mMESSAGE mMACHINE kits (Ambion). All procedures involving *Xenopus laevis* were approved by the University of La Laguna Research Ethics Committee in agreement with local and national legislation. Oocytes were harvested from adult females under benzocaine anesthesia by partial ovariectomy and collagenase IA dispersion. Stage V to VI oocytes were selected and microinjected with 20, 10, and 5 ng of *SSU2* cRNA. Cells were then incubated for 7 days at 18°C in oocyte Ringer's medium (containing [mmol/liter] NaCl [82.5], KCl [2], $CaCl_2$ [2], $MgCl_2$ [2], Na_2HPO_4 [1], and HEPES [10], at pH 7.5). *SSU2-YFP* protein expression was detected by Western blot analysis of *Xenopus* oocyte extracts using an anti-GFP monoclonal antibody (Roche) as previously described (34). Cell surface expression of fluorescently labeled *SSU2* was detected from whole oocytes using a laser scanning confocal microscope (Olympus FluoView 1000). Background fluorescence was assessed by imaging noninjected or water-injected oocytes (35). Nitrate efflux was measured by microinjecting oocytes expressing or not expressing Ssu2 with 30 nl of 30 mM KNO_3 or

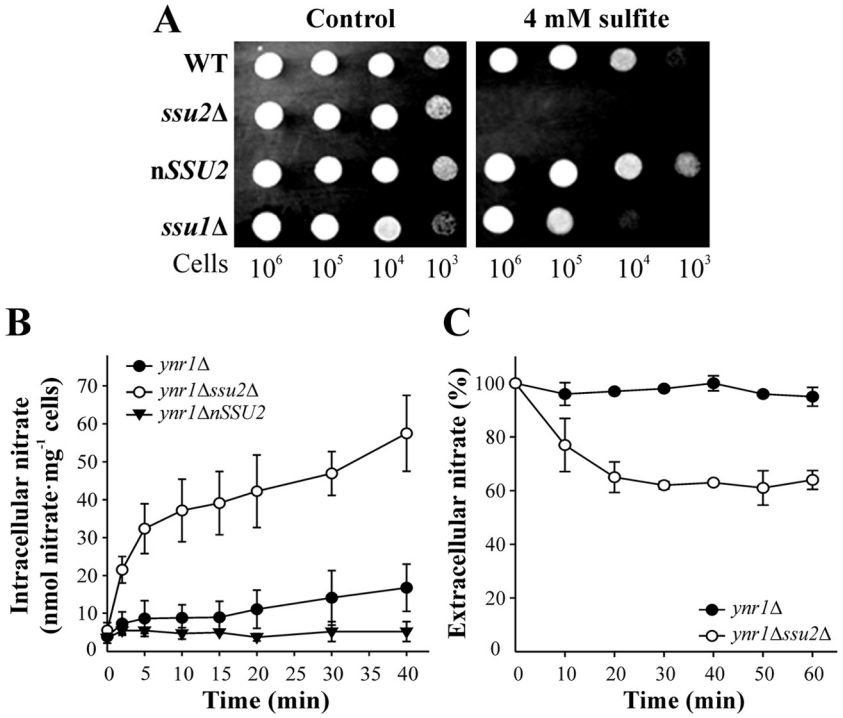

FIG 1 Ssu1 and Ssu2 are involved in sulfite sensitivity and nitrate efflux. (A) Deletion of the *SSU1* or *SSU2* gene produces sulfite sensitivity. The *ssu1*Δ and *ssu2*Δ strains were grown in YPD. Serial 10-fold dilutions were spotted on pH 3.5 buffered synthetic medium containing 5 mM ammonium chloride plus sodium sulfite at the concentration indicated. Plates were incubated at 37°C for 2 days. (B) The *ssu2*Δ strain accumulates nitrate. Ammonium-grown cells were resuspended in synthetic medium at an OD_{660} of 2 to 3 and then nitrogen starved for 120 min. Nitrate accumulation assays were triggered with 1 mM nitrate. Intracellular nitrate was determined in ethanolic cell extracts. (C) Net nitrate uptake increases in the *ssu2*Δ *ynr*Δ strain. Ammonium-grown cells were resuspended to an OD_{660} of 10 in nitrogen-free medium buffered at pH 5.5 for 60 min. Nitrate uptake assays were triggered with 0.1 mM nitrate. Nitrate uptake was determined as extracellular nitrate depletion for 60 min. Data ± SE from three independent experiments are shown.

30 mM $NaNO_2$, followed by a 15-min incubation in Ringer's medium. Afterwards, nitrate in the medium was measured as described above. Nitrate efflux is expressed as nmol of nitrate determined in the medium after 15 min of nitrate microinjection.

Cell viability. WT, *ssu2*Δ, *nar1*Δ, *ynr1*Δ, and *ynr1*Δ *ssu2*Δ strains were grown in ammonium and resuspended at 10 mg/ml (wet weight) in 10 mM nitrate, 5 mM ammonium, and 1 mM nitrite. This point represented 100% of viability. To calculate the percentage of viable cells, approximately 100 cells were plated over YPD in triplicate. These experiments were repeated at least three times.

Miscellaneous methods. Electrotransformation of yeast cells was performed as described previously (36). β-Galactosidase activity was determined as described in reference 27. Yeast cell extract preparation, SDS-PAGE, and immunoblotting were done as described in reference 9. Fluorescence microscopy of *SSU2-GFP* was performed as described previously (8). RNA extraction and qRT-PCR were done as described in reference 6.

Nucleotide sequence accession numbers. The sequences of *SSU1*, *SSU2*, and *NAR1* have been deposited in GenBank under accession numbers HF585084, HF585085, and HF585083, respectively.

RESULTS

***H. polymorpha* Ssu1 and Ssu2 are involved in nitrate efflux.** The *S. cerevisiae* sulfite efflux permease *SSU1* gene is induced by NO-generating compounds, and its involvement in NO-derived metabolite efflux has been suggested (19). This prompted us to study the role of Sc*SSU1* orthologs in nitrate efflux in the yeast *H. polymorpha*. Two open reading frames (ORFs), termed Hp*SSU1* and Hp*SSU2*, encoding proteins similar to ScSsu1, are present in the *H. polymorpha* genome database. Strains bearing disrupted *SSU1* or *SSU2* showed sensitivity to sulfite, which was much greater in the *ssu2*Δ strain. In contrast, the n*SSU2* strain, bearing several copies of *SSU2*, was more resistant (Fig. 1A).

To analyze whether Ssu2 is involved in nitrate efflux, we measured intracellular nitrate in the *ssu2*Δ strain bearing disrupted *YNR1* (nitrate reductase) to avoid nitrate reduction to nitrite. Nitrate accumulated in the *ynr1*Δ *ssu2*Δ strain at a higher level than in the *ynr1*Δ strain, while in the n*SSU2* strain, no intracellular nitrate accumulation was detected (Fig. 1B). Consistently, we also observed greater net nitrate uptake in the *ynr1*Δ *ssu2*Δ strain than in the *ynr1*Δ strain (Fig. 1C). We also measured intracellular nitrite in the *ssu2*Δ strain lacking nitrite reductase (*yni1*Δ *ssu2*Δ), showing that Ssu2 was not involved in nitrite efflux (data not shown). These results strongly suggest that Ssu2 plays a role in nitrate efflux.

The ability of Ssu2 to extrude nitrate was also studied using a heterologous system. As expected for a permease, *Xenopus* oocytes expressed Ssu2 at the plasma membrane (Fig. 2A), with a good correlation between the amount of cSSU2 injected and the Ssu2 levels (Fig. 2B). Oocytes preloaded with nitrate or nitrite showed nitrate efflux levels according to the Ssu2 levels (Fig. 2C), but this was not the case with nitrite (data not shown).

Involvement of Ssu2 in nitrate and sulfite extrusion was also found in cells previously incubated in nitrate plus sulfite. Under these conditions, a transitory nitrate accumulation in the *ynr1*Δ strain was observed only when sulfite was present (Fig. 3). This

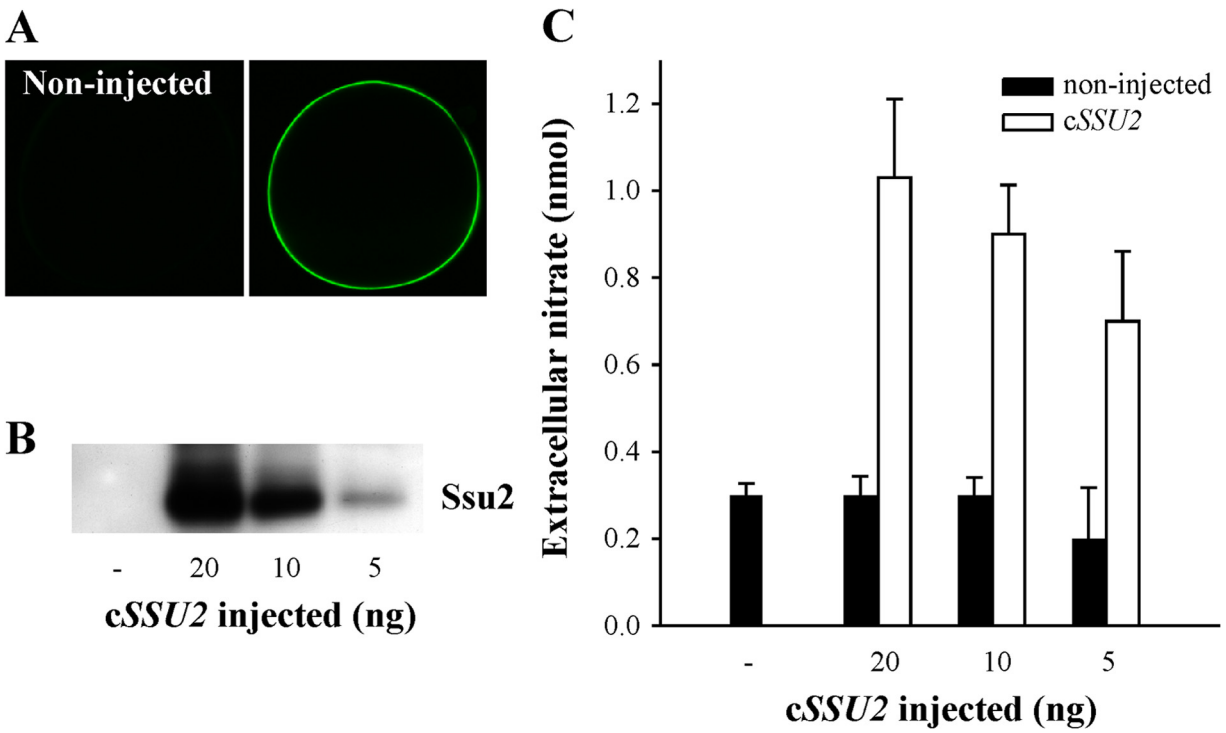

FIG 2 Ssu2 is involved nitrate efflux in oocytes from *Xenopus laevis*. Oocytes were injected with different amounts of *SSU2* cRNA and incubated for 7 days at 17°C. (A) Ssu2-YFP is localized in injected oocytes at the cell surface. (B) The amount of Ssu2-YFP is directly proportional to the amount of cRNA injected. (C) Oocytes injected or not with *SSU2* cRNA were preloaded with 0.9 nmol of nitrate and incubated for 15 min. Afterward, nitrate was determined in the extracellular medium. Nitrate (nmol) excreted in 15 min ± SE from 5 independent experiments is shown.

increase in intracellular nitrate could be explained by a decrease in nitrate efflux or an increased uptake. Considering the fact that Ssu2 extrudes sulfite as well as nitrate, competition of sulfite with nitrate for Ssu2 and Ssu1 would be expected in the *ynr1*Δ strain, leading to the observed nitrate accumulation. Once sulfite is metabolized, nitrate is extruded from the cell. Consistent with our results, on solid medium the presence of nitrate increased the sensitivity of the WT and *ssu1*Δ strains to sulfite. This was hard to observe in the *ssu2*Δ strain, probably due to higher sensitivity of this strain to sulfite (see Fig. S1 in the supplemental material). The fact that nitrate accumulation in the *ynr1*Δ strain in the presence of sulfite (Fig. 3) is higher than that in the *ynr1*Δ *ssu2*Δ strain (Fig. 1B) suggested that sulfite could inhibit other nitrate efflux transporters apart from Ssu1 and Ssu2.

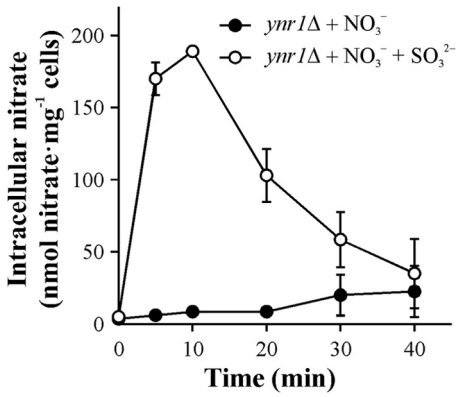

FIG 3 Sulfite raises intracellular nitrate levels. The *ynr1*Δ strain, lacking nitrate reductase, grown in ammonium, was transferred to nitrogen-free synthetic medium buffered at pH 3.5 for 120 min. Nitrate accumulation assays were triggered with 0.75 mM sodium nitrate or 0.75 mM sodium nitrate plus 1.5 mM sodium sulfite. Intracellular nitrate was determined in ethanolic cell extracts. Data ± SE from three independent experiments are shown.

Involvement of Ssu2 in net nitrate uptake and nitrate-induced gene expression. Next, we evaluated the role of Ssu1 and particularly Ssu2 in nitrate uptake. We found that net nitrate uptake was almost negligible in the strain overexpressing *SSU2* (n*SSU2*) (Fig. 4A). Both the *ssu1*Δ and *ssu2*Δ strains present a significant increase in nitrate uptake with respect to the WT, that of the *ssu2*Δ strain being higher. The *ssu1*Δ *ssu2*Δ and *ssu2*Δ strains presented equal nitrate uptake rates, indicating the lower participation of Ssu1 in nitrate extrusion than of Ssu2. These findings clearly show that Ssu2 is affecting net nitrate uptake levels, acting directly on nitrate efflux and indirectly on nitrate induction, which is mediated by intracellular nitrate. Indeed the lag phase preceding nitrate uptake is shorter in the *ssu1*Δ strain than in the WT and even shorter in the *ssu2*Δ strain. This suggests that due to the lesser efflux of nitrate in the *ssu2*Δ strain and consequently the higher nitrate accumulation, nitrate induction becomes quicker and nitrate uptake is triggered sooner. To test the role of Ssu2 in nitrate induction, we measured nitrate reductase gene expression (*YNR1-lacZ*) as a readout of nitrate-induced gene expression in the n*SSU2* and *ssu2*Δ strains. Indeed, *YNR1-lacZ* expression was about 50% lower in the n*SSU2* strain than in the WT. In the *ssu2*Δ strain, however, *YNR1-lacZ* expression was higher and took place earlier than in in the WT (Fig. 4B). The nitrate *YNR1-lacZ* induction time course is essentially the same in

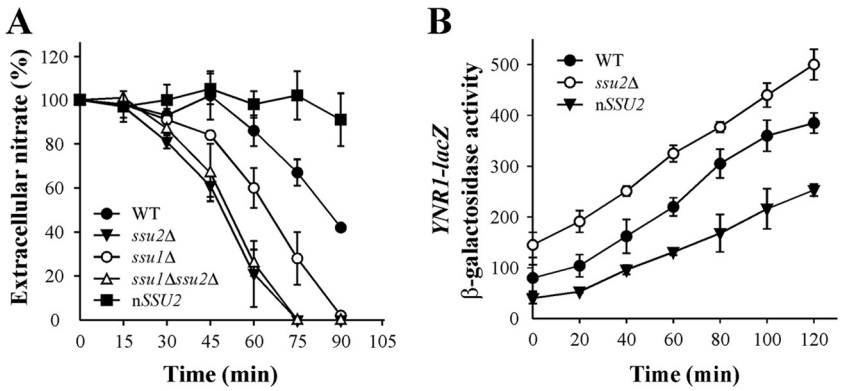

FIG 4 *SSU2* deletion raises nitrate uptake and nitrate induction. (A) Nitrate uptake. Cells grown in synthetic medium plus 5 mM ammonium were resuspended in the same medium at an OD_{660} of 2 to 3 but without a nitrogen source and incubated for 90 min. Nitrate uptake was determined as extracellular nitrate depletion for 90 min. Assays were triggered with 0.5 mM nitrate. Data ± SE from three independent experiments are shown. (B) Nitrate induction. Strains bearing *YNR1-lacZ* grown in synthetic medium plus 5 mM ammonium were resuspended in nitrogen-free medium for 90 min. *YNR1-lacZ* induction in response to 1 mM nitrate was determined as β-galactosidase activity and expressed as nmol *o*-nitrophenol · min^{-1} (mU) · mg^{-1} of protein. Data ± SE from three independent experiments are shown.

both the WT and the *ssu2*Δ strain. However, in the *ssu2*Δ *YNR1-lacZ* strain, levels are higher at time zero, after the cells have been depleted of nitrogen for 90 min. This can be explained if nitrate traces present in a nitrogen-free medium are still able to slightly induce *YNR1-lacZ* expression (7). These traces are not excreted to the medium because of *SSU2* deletion. We also measured *YNR1-lacZ* expression in the *ynr1*Δ *ssu2*Δ and *ynr1*Δ strains in very low nitrate (micromolar level of nitrate). The induction of *YNR1-lacZ* expression was faster and its levels higher in the *ynr1*Δ *ssu2*Δ strain than in the *ynr1*Δ strain. This indicates that nitrate traces are not excreted once entering *ynr1*Δ *ssu2*Δ cells, unlike the case with *ynr1*Δ and WT cells, increasing the levels of intracellular nitrate and as a result the rate of nitrate assimilation gene induction (see Fig. S2 in the supplemental material). Likewise, in the presence of nitrate, nitrite efflux peaked earlier in the *ssu2*Δ strain than in the WT, while in the n*SSU2* strain it remained very low (see Fig. S3). We conclude that Ssu2 plays key roles in net nitrate uptake and also in modulating the response of nitrate-induced genes to nitrate.

Nar1 is involved in nitrite and nitrate efflux. The levels of intracellular nitrate in the *ynr1*Δ strain incubated in nitrate plus sulfite are higher than in those in the *ynr1*Δ *ssu2*Δ strain This pointed to the presence in *H. polymorpha* of nitrate efflux system components other than Ssu2 and Ssu1. To check this, we searched for genes encoding proteins with similarity to nitrate and nitrite transporters in the *H. polymorpha* genome database. We found two genes encoding putative nitrate/nitrite transporters with similarity to *A. thaliana* CHL1 and *Chlamydomonas reinhardtii* NAR1. Chl1 belongs to the nitrate transporter family (NRT1/PTR), as does *A. thaliana* nitrate transporter CHL1 (37). We disrupted *CHL1* but could not find any involvement of Chl1 in nitrate efflux or influx in *H. polymorpha* (data not shown). Nar1 belongs to the formate nitrite transporter family (FNT). *H. polymorpha* Nar1 (HpNar1) showed about 20% identity with different members of the FNT family, such as NirC from *E. coli* and NAR1.1 from *C. reinhardtii*, all involved in nitrite transport (11, 22, 38).

To study the role of Nar1, we determined nitrite excretion in the WT, *nar1*Δ and n*NAR1* strain (bearing several copies of *NAR1*). We observed that nitrite excretion was higher in the WT than in the *nar1*Δ strain when they were incubated in 5 mM nitrate, while in the n*NAR1* strain it increased strongly (Fig. 5A). This suggests that Nar1 could be involved in nitrite efflux. Therefore, we analyzed nitrite accumulation in the WT, *nar1*Δ, and n*NAR1* strains. There was a clear accumulation of nitrite in the *nar1*Δ strain, unlike the case with the n*NAR1* strain, where intracellular nitrite was almost nil (Fig. 5B). This allows us to conclude that Nar1 is involved in nitrite efflux.

We then asked if Nar1 excretes nitrate. To address this, intracellular nitrate was determined for different strains lacking nitrate reductase (*ynr1*Δ) incubated in nitrate. The highest accumulation was in the *ynr1*Δ *nar1*Δ *ssu2*Δ strain, followed by the *ynr1*Δ *nar1*Δ, *ynr1*Δ *ssu2*Δ, and *ynr1*Δ strains Therefore, Nar1 has a high capacity to extrude nitrate, even higher than that of Ssu2 (Fig. 5C). Short-term nitrate accumulation was analyzed in the same strains for 20 min (Fig. 5D) and found to be higher in the *ynr1*Δ *ssu2*Δ strain than in the *ynr1*Δ *nar1*Δ strain. In contrast, nitrate accumulation after 20 min was higher in the *ynr1*Δ *nar1*Δ strain than in the *ynr1*Δ *ssu2*Δ strain (Fig. 5C). This suggests that Ssu2 is the high-affinity transport system that copes with nitrate excretion at low intracellular nitrate levels, while Nar1 seems to be important at high intracellular nitrate levels.

We also explore the role of Nar1 in nitrate and nitrite uptake in the WT and *nar1*Δ strains, finding that nitrate (Fig. 5E) and nitrite (Fig. 5F) uptake was less in the *nar1*Δ strain. This could be explained by the accumulation of nitrite in the *nar1*Δ strain, which leads to a downregulation of nitrate assimilation genes. Indeed, *YNR1-lacZ* levels decrease in the *nar1*Δ strain incubated in nitrate (see Fig. S4 in the supplemental material). However, we cannot rule out that Nar1 could be also involved in nitrate and nitrite influx.

***SSU2* is upregulated by nitrite, unlike *NAR1*.** To monitor *SSU2* and *NAR1* gene expression, *SSU2-lacZ*, *NAR1-lacZ*, and qRT-PCR were used. We measured *SSU2* expression bearing in mind that Sc*SSU1* is induced by NO-generating compounds (19) and that NO could be also produced by NR from nitrite (39). The WT and *ynr1*Δ strains were incubated in either ammonium or nitrate so as to focus on the role of NR. As depicted in Fig. 6A, we observed that nitrate induced Ssu2 levels about 4-fold, although this induction was abolished in strains lacking nitrate reductase

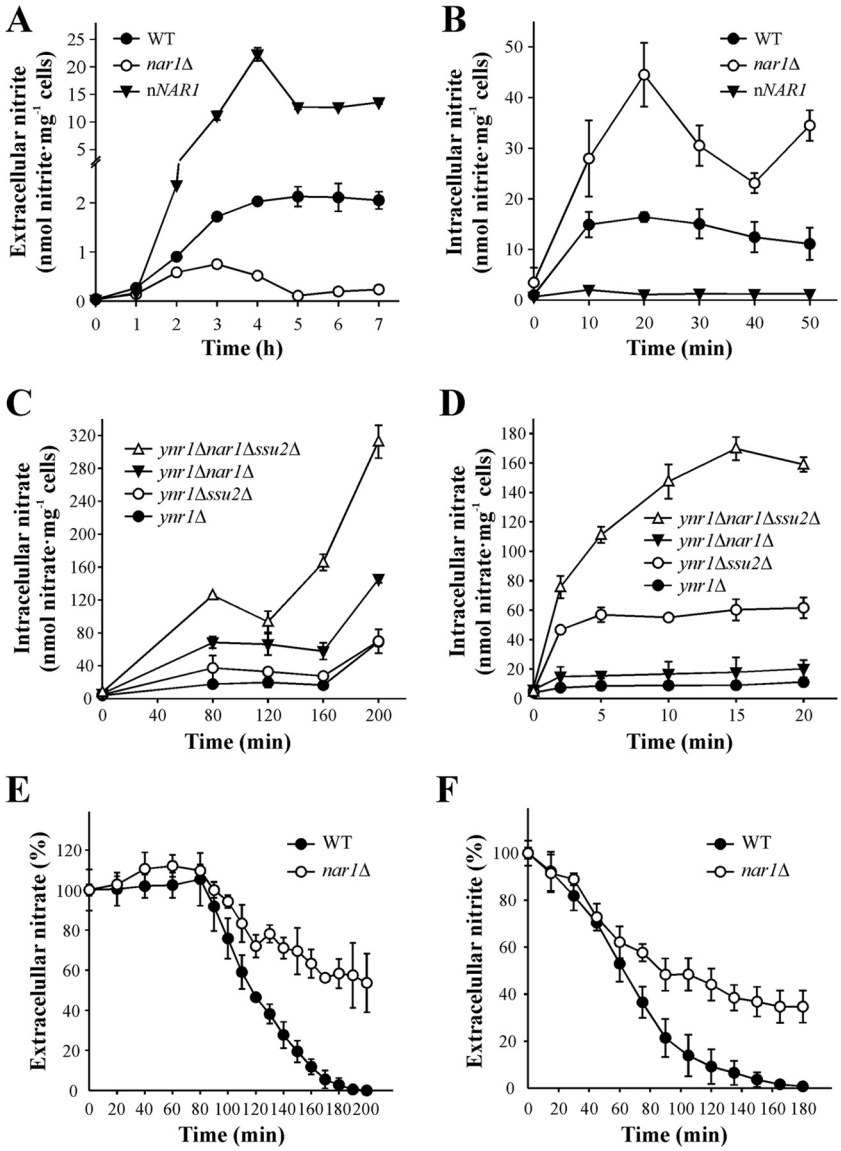

FIG 5 Nar1 is involved in nitrite and nitrate efflux. (A) Nitrite excretion. Ammonium-grown cells were resuspended at an OD_{660} of 2 to 3 in synthetic medium plus 5 mM nitrate. Appearance of nitrite in the medium was determined for 7 h. (B) Intracellular nitrite. Ammonium-grown cells were nitrogen starved in synthetic medium at an OD_{660} of 2 to 3 for 120 min. Intracellular nitrite was determined in ethanolic cell extracts in assays triggered with 1 mM nitrite. (C and D) Intracellular nitrate is highest in the *ynr1*Δ *nar1*Δ *ssu2*Δ strain. Ammonium-grown cells were nitrogen starved in synthetic medium for 60 min at an OD_{660} of 2 to 3. Nitrate accumulation assays were triggered with 1 mM nitrate. Intracellular nitrate was determined in ethanolic cell extracts from long (C) or short (D) assays. (E and F) The *nar1*Δ strain shows lower net nitrate and nitrite uptake. Ammonium-grown cells were nitrogen starved on pH 5.5 buffered synthetic medium at an OD_{660} of 2 to 3 for 90 min. Net nitrate (E) or nitrite uptake (F) assays were triggered with 1 mM nitrate or 0.5 mM nitrite. Net nitrate or nitrite uptake was determined as extracellular nitrate or nitrite depletion. Data ± SE from three independent experiments are shown.

(*ynr1*Δ). This suggests that *SSU2* upregulation is due to the nitrite from nitrate reduction or NO generated from nitrite by NR. Further experiments showed that nitrite induced *SSU2* expression whether NR was present or not (Fig. 6B). Therefore, nitrite is clearly involved in *SSU2* upregulation, even though further transformations of nitrite to NO independently of NR (39) cannot be excluded. Other enzymes, such as xanthine oxidase, mitochondrial cytochromes, or even nonenzymatic reduction, could account for this (40–42). We also measured Ssu2 levels in a strain bearing Ssu2-GFP, in different nitrogen sources, finding them well correlated with *SSU2* expression (Fig. 6C). This was confirmed by epifluorescence microscopy, which also showed that Ssu2 is localized mainly at the plasma membrane (Fig. 6D), consistent with data obtained for *Xenopus* oocytes (Fig. 2A). Interestingly, we observed a small proportion of Ssu2-GFP intracellular retention in some yeast cells, which could be transporting some nitrate into an internal compartment. However, this does not affect our total cell nitrate content measurements after complete disruption of cell membranes (31).

Unlike the case with *SSU2*, no significant differences were observed in the response of *NAR1* or Nar1 to different nitrogen sources (data not shown). Epifluorescence microscopy showed that Nar1-GFP was localized mainly at the cell surface in nitrate and ammonium (data not shown).

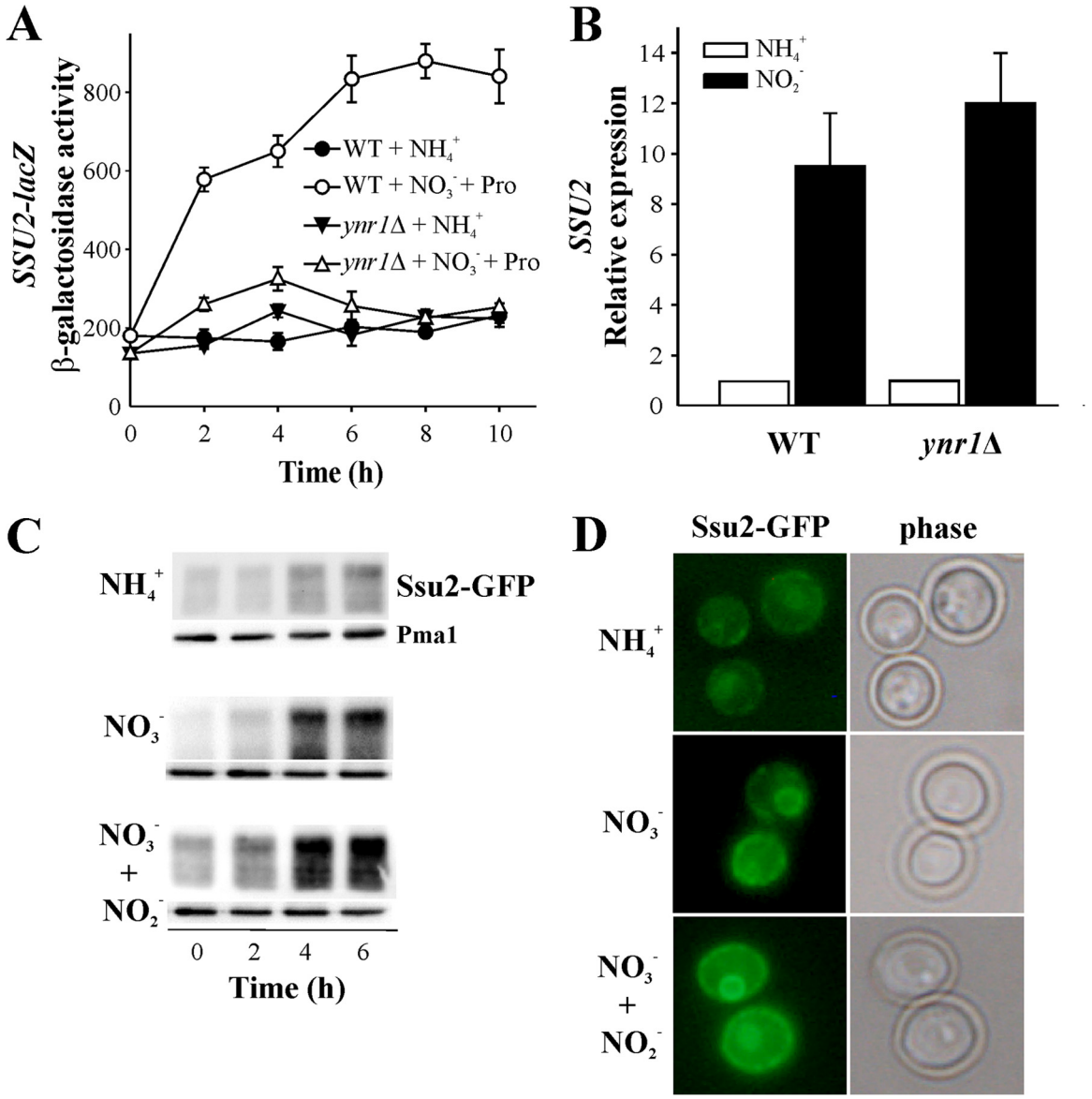

FIG 6 *SSU2* is upregulated by nitrite. (A) *SSU2* expression levels. *SSU2* expression was followed by assaying β-galactosidase activity in the WT and the *ynr1*Δ strain bearing *SSU2-lacZ*. Strains grown in YPD were transferred to synthetic medium plus 5 mM nitrate and 0.5 mM proline or 5 mM ammonium at an OD_{660} of 3. Data ± SE from three independent experiments are shown. (B) Effect of nitrite on *SSU2* expression levels in WT and *ynr1*Δ strains. Ammonium-grown cells were incubated at an OD_{660} of 3 in synthetic medium plus 5 mM ammonium or 2 mM nitrite for 120 min. Relative expression was determined by qRT-PCR. Data ± SE from three independent experiments are shown. The expression is normalized to cells incubated in ammonium. Hp*ACT1* was used as a reference gene. (C) Nitrate and nitrite raise Ssu2 levels. Ssu2 was determined by immunoblot analysis of Ssu2-GFP. Strains grown in YPD were resuspended at an OD_{660} of 0.8 in synthetic medium plus 5 mM ammonium, 10 mM nitrate, or 10 mM nitrate plus 2 mM nitrite. Pma1 was used as a loading control. (D) Ssu2 is located mainly at the cell surface, and its levels increased in nitrate or nitrate plus nitrite. Cells bearing Ssu2-GFP fusion grown in YPD were transferred to synthetic medium plus 5 mM ammonium, 10 mM nitrate, or 10 mM nitrate plus 2 mM nitrite at an OD_{660} of 2 to 3. Ssu2-GFP was monitored by fluorescence microscopy.

Involvement of Ssu2 and Nar1 in growth and cell viability. We further studied the role of Ssu2 and Nar1 in cell growth and viability. A liquid medium assay in 5 mM nitrate showed that the *ssu2*Δ strain grew more slowly than the WT (Fig. 7A). In contrast, under the same conditions, *nar1*Δ strain growth was slightly less than that of the WT. However, in 1 mM nitrite, the *nar1*Δ strain was unable to grow, while growth of the *ssu2*Δ strain was about 50% less than that of the WT (Fig. 7B).

We also studied the effects of Ssu2 and Nar1 on cell viability in nitrate and nitrite. Cells grown in ammonium up to an OD_{660} of 3 were resuspended at the same cell density in 5 mM ammonium, 10 mM nitrate, or 1 mM nitrite. Cell suspensions were incubated with shaking for 8 h. The *ssu2*Δ strain showed lower viability in nitrate (Fig. 7C). These results raise the question of whether nitrate, or nitrite from nitrate reduction, was responsible for cell viability reduction of the *ssu2*Δ strain in nitrate. However, to avoid nitrite production, strains lacking NR, the *ynr1*Δ *ssu2*Δ and *ynr1*Δ strains, were incubated in nitrate and did not show any difference

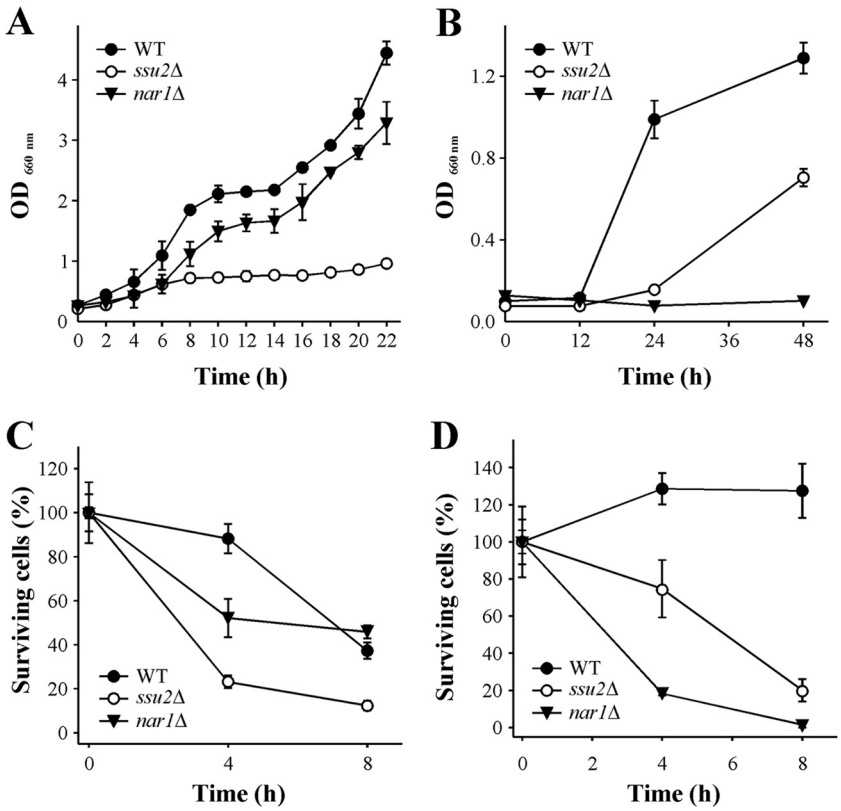

FIG 7 The *ssu2*Δ strain presents reduced cell viability in nitrate while the *nar1*Δ strain does so in nitrite. (A) Growth in nitrate. Strains grown in YPD were transferred to synthetic medium plus 5 mM nitrate at an OD_{660} of 0.2. Cell growth was determined by measuring the OD_{660}. (B) Growth in nitrite. The procedure was the same as that for panel A except that the medium contained only 1 mM nitrite. (C) Cell viability in nitrate. Ammonium-grown cells were resuspended at an OD_{660} of 3 in synthetic medium plus 10 mM nitrate and incubated with shaking at 37°C for 8 h. (D) Cell viability in nitrite. Ammonium-grown cells were resuspended at an OD_{660} of 3 in synthetic medium plus 1 mM nitrite and incubated with shaking at 37°C for 8 h. Cell viability was determined after different incubation times in nitrate or nitrite by measuring growth on YPD plates at 37°C for 2 days. Data ± SE from three independent experiments are shown.

in cell viability (data not shown). This suggests that nitrite, and not nitrate, was involved in the lower cell viability of the *ssu2*Δ strain in nitrate. In the case of Nar1 (Fig. 7C), the differences between the *nar1*Δ and WT strains are noticeable only after 4 h of incubation in nitrate. The fact that after 8 h no differences were observed could be due to a weaker nitrate assimilation gene induction in the *nar1*Δ strain due to nitrite accumulation. Nitrite toxicity was further confirmed in the WT, *ssu2*Δ, and *nar1*Δ strains. The *nar1*Δ strain was almost killed after 4 h in nitrite, while unexpectedly, the *ssu2*Δ strain presented a moderate sensitivity to nitrite (Fig. 7D). We conclude that Ssu2 is as essential for cell growth and viability in nitrate as is Nar1 in nitrite.

Besides sulfite, *S. cerevisiae* Ssu1 is able to mediate the efflux of nitrite and nitrate. We studied whether Sc*SSU1*, in addition to sulfite, is involved in nitrate and nitrite efflux. This was tackled by expressing Sc*SSU1* in the *H. polymorpha ssu2*Δ strain under the Hp*SSU2* promoter to avoid misinterpretations due to expression level alterations. The *ssu2*Δ strain expressing Sc*SSU1* was able to recover sulfite tolerance, confirming the suitability of this construct to functionally render Sc*SSU1* in *H. polymorpha* (see Fig. S5A in the supplemental material). Nitrate uptake experiments revealed that the *ssu2*Δ strain expressing Sc*SSU1* presented a lower net nitrate uptake, almost nil net nitrite uptake (see Fig. S5B and S5C), and incapacity to grow in nitrate and nitrite (data not shown). However, determination of intracellular nitrate in the *ynr1*Δ *ssu2*Δ Sc*SSU1*, *ynr1*Δ *ssu2*Δ, and *ynr1*Δ strains showed that the *ynr1*Δ *ssu2*Δ Sc*SSU1* strain does not accumulate nitrate, unlike the *ynr1*Δ *ssu2*Δ strain (Fig. 8A). This indicates that Sc*SSU1* is involved in nitrate efflux, since nitrate is not accumulated in the presence of ScSsu1 in the *ssu2*Δ strain. Likewise, we measured intracellular nitrite in the WT, *ssu2*Δ, and *ssu2*Δ Sc*SSU1* strains. As shown in Fig. 8B, the latter strain does not accumulate nitrite, indicating that ScSsu1 is also involved in nitrite efflux. To obtain further confirmation of this, we measured nitrite efflux in the *ssu2*Δ Sc*SSU1*, s*su2*Δ, and WT strains incubated in 1 mM nitrate. The ability of the *ssu2*Δ Sc*SSU1* strain to efflux nitrite was about 10-fold higher than that of the WT and *ssu2*Δ strains (Fig. 8C). This did not decrease after 4 h, unlike the case with the *ssu2*Δ and WT strains, where nitrite efflux disappears after about 2 h. Further evidence on this was obtained by transforming the WT with Sc*SSU1*. The resultant strains were better able to extrude nitrite and lower the intracellular nitrite (see Fig. S6). This indicates that ScSsu1 presents high nitrite efflux activity. We also proposed that the incapacity of the *ssu2*Δ Sc*SSU1* strain to grow in nitrate is due to the very high nitrite efflux activity of this strain. The high activity of *ssu2*Δ Sc*SSU1* strain suggests that Sc*SSU1* preferentially excretes nitrite instead of nitrate, since in the latter case nitrite excretion would be lower, but it is not. In addition to sulfite efflux, we concluded that Sc*SSU1* also extrudes nitrite and nitrate.

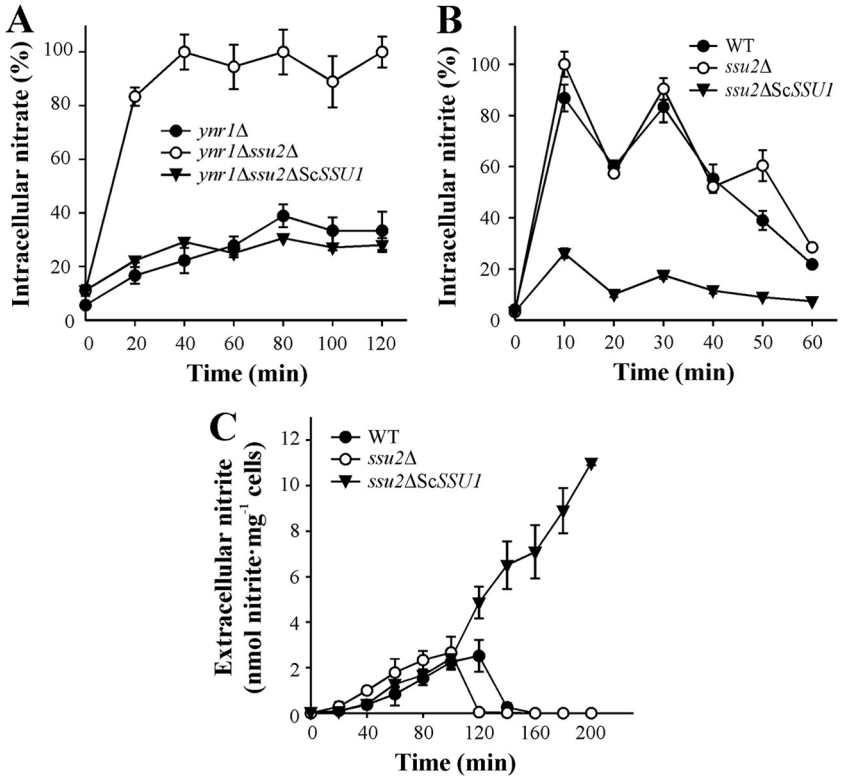

FIG 8 Sc*SSU1* is involved in nitrate and nitrite efflux. Ammonium-grown cells were resuspended at an OD_{660} of 2to 3 in synthetic medium and then nitrogen starved for 120 min. Intracellular nitrate (A) or nitrite (B) assays were triggered with 1 mM nitrate or 1 mM nitrite. Nitrate and nitrite were determined in ethanolic cell extracts. (C) Nitrite excretion increased in the *ssu2*Δ Sc*SSU1* strain. Ammonium-grown cells were resuspended at an OD_{660} of 2 to 3 in synthetic medium buffered at pH 5.5 and then nitrogen starved for 120 min; afterward, 1 mM nitrate was added. Nitrite excretion was determined in the medium. Data ± SE from three independent experiments are shown.

DISCUSSION

Using the yeast *H. polymorpha* as a model system, we have characterized the molecular components of nitrate and nitrite efflux and its impact on nitrate assimilation. We wish to suggest here an essential role for nitrate and nitrite efflux in nitrate assimilation in terms of net nitrate transport, growth, and viability. We found that the sulfite efflux permease Ssu1 and especially Ssu2 were also able to extrude nitrate, while Nar1 extrudes nitrite and nitrate.

Intracellular nitrate accumulation and net nitrate uptake show that deletion of *SSU2* led to higher levels of intracellular nitrate and net nitrate uptake than were seen with the control strains (the WT and the *ynr1*Δ strain). In contrast, in the n*SSU2* strain, bearing several copies of *SSU2*, intracellular nitrate accumulation and net nitrate uptake were almost nil. We reasoned that the high net nitrate uptake in the *ssu1*Δ, *ssu2*Δ, and *ssu1*Δ *ssu2*Δ strains was due to these strains presenting lower nitrate efflux than the WT and therefore higher net nitrate uptake (Fig. 4A). Furthermore, in these mutants, nitrate accumulation is greater, and as a result, nitrate assimilation gene induction rises, also contributing to the increased net nitrate uptake. Indeed, we found that deletion of *SSU2* increased nitrate assimilation gene expression while *SSU2* overexpression downregulated these genes (Fig. 4B). These results suggest that Ssu2 negatively regulates nitrate assimilation gene expression by acting on intracellular nitrate levels, since nitrate acts as an inducer once inside the cell (7). In this framework of regulation, we also found that Ssu2 levels are positively regulated by nitrate (Fig. 6C).

Experiments with *Xenopus* oocytes preloaded with nitrate or nitrite also confirmed that Ssu2 was able to efflux nitrate but not nitrite. This inability is also seen from the scarce accumulation of nitrite in the *yni1*Δ *ssu2*Δ strain (data not shown).

Ssu2 appears to be more important in sulfite efflux than Ssu1, since the *ssu2*Δ strain was more sensitive to sulfite than the *ssu1*Δ strain (Fig. 1A). Accordingly, under the conditions used, our results indicate that the contribution of Ssu1 to nitrate efflux was much lower than that of Ssu2 (Fig. 4A).

In our search for genes involved in nitrate and nitrite transport, we also found that deletion of one ORF, encoding a protein termed Nar1, belonging to the FNT family, led to lower nitrite excretion. In contrast, a strain bearing multiple copies of *NAR1* (n*NAR1*) increased it dramatically. Consistent with this, the *nar1*Δ strain presents high intracellular nitrite accumulation (Fig. 5B), and this is even more the case with the *yni1*Δ *nar1*Δ strain (data not shown). However, contrary to what was expected for a member of the FNT family, Nar1 was also involved in nitrate extrusion. Thus, the *ynr1*Δ *nar1*Δ strain accumulated more nitrate than the *ynr1*Δ strain and even more than the *ynr1*Δ *ssu2*Δ strain. Moreover, the *ynr1*Δ *nar1*Δ *ssu2*Δ triple mutant yielded the highest intracellular levels of nitrate under the tested conditions. The time courses of nitrate accumulation in the *ynr1*Δ *ssu2*Δ, *ynr1*Δ *nar1*Δ, and *ynr1*Δ *nar1*Δ *ssu2*Δ strains suggest that Ssu2 was responsible for short-term nitrate extrusion, while intracellular nitrate levels remained lower (60 nmol mg^{-1} of cells). In accordance with this, in the *ynr1*Δ *nar1*Δ strain, unlike the case with the *ynr1*Δ

*ssu2*Δ strain, no nitrate accumulation is observed in the short term. In contrast, once intracellular nitrate levels increase, Nar1 seems to be mainly responsible for nitrate excretion. This conclusion is also supported by the fact that the *ynr1*Δ *nar1*Δ strain accumulates nitrate only after 80 min in nitrate, when intracellular levels of nitrate rise (Fig. 5C). These results also suggest that Ssu2 presents greater affinity for nitrate than Nar1. Indeed, Nar1 belongs to the FNT family, whose members are involved in nitrite transport and efflux (11, 21–23, 25). Consistent with this, in the *yni1*Δ *nar1*Δ strain, nitrite is quickly accumulated (data not shown), while nitrate is slowly accumulated in the *ynr1*Δ *nar1*Δ strain, suggesting again that Nar1 presents a higher affinity for nitrite than for nitrate (Fig. 5B and D).

Uptake assays showed a lower net uptake of nitrate and nitrite by the *nar1*Δ strain. This is explained because nitrite, coming from nitrate reduction or from the medium, is intracellularly accumulated in the *nar1*Δ strain and represses the nitrate assimilation gene (1). Indeed, decreased net uptake of nitrate and nitrite is observed after a 40-min incubation. Nevertheless, the involvement of Nar1 in their influx cannot be absolutely ruled out.

Our results challenge the idea that NR plays an important role in net nitrate uptake in yeast and filamentous fungi. This had been concluded from uptake assays using the tracer $^{13}NO_3^-$, which showed that net nitrate uptake is negligible in an NR deletion mutant yeast (43). However, nitrate efflux could also account for the absence of intracellular nitrate accumulation in mutants lacking NR. Thus, we observed that the *ynr1*Δ *ssu2*Δ strain and particularly the *ynr1*Δ *nar1*Δ *ssu2*Δ strain were able to accumulate nitrate, unlike the *ynr1*Δ strain. Therefore, nitrate influx was operative in mutants lacking NR. The accumulation of nitrate in the *ynr1*Δ *ssu2*Δ and *ynr1*Δ *nar1*Δ strains is therefore consistent with the absence of a nitrate efflux system (Fig. 5C and D).

The role of nitrite excretion seems to be a response of the cell to cope with the toxicity of nitrite. However, this could be regulated at the nitrate uptake step to avoid the imbalance between nitrate transported into the cell and that reduced to ammonium by NR. Since nitrite is toxic for most organisms (44–47), this could contribute to the success of nitrate-assimilating microorganisms in colonizing nitrate-containing media in competition with non-nitrate assimilators. In contrast, the precise role(s) of nitrate efflux is difficult to explain, since nitrate appears not to be toxic for cells, because strains lacking NR, the *ynr1*Δ *ssu2*Δ and *ynr1*Δ strains, were viable in nitrate (data not shown). Nitrate uptake takes place against an electrochemical gradient, and therefore its efflux apparently seems to be a waste of energy for the cell. Nevertheless, we observed that the *ssu2*Δ strain grew poorly in nitrate (Fig. 7A) and also presented lower cell viability after incubation in it (Fig. 7C). This suggests that intracellular nitrate levels must also be tightly regulated. In this regard, we have observed in the *ssu2*Δ strain that nitrate induction of nitrate assimilation genes is quicker and higher. This could produce an imbalance between the capacity of the cells to take up nitrate and its reduction to nitrite and then ammonium, as a result increasing intracellular nitrite. In any case, the question of whether nitrate itself is toxic for the cells or the toxicity is due to nitrite was also addressed. We observed that when the *ssu2*Δ strain is incubated in nitrate, nitrite efflux is higher than that in the WT, suggesting that nitrite could actually be the main cause of the low growth and viability of this mutant in nitrate. In fact, when we compared growth and viability in nitrate of the *ssu2*Δ strain with those of the *ynr1*Δ *ssu2*Δ strain, we observed that the latter becomes more viable since it is unable to produce nitrite (data not shown). We conclude that Ssu2 contributes to nitrate homeostasis, avoiding nitrite accumulation. Unlike the *ssu2*Δ strain, the *nar1*Δ strain is unviable in nitrite due to its capacity to accumulate it. These results show that Nar1 is crucial for growth and cell viability in nitrite. In contrast, Ssu2 is essential in nitrate. However, the lower growth of the *ssu2*Δ strain in nitrite could be due to the conversion of nitrite to NO via NR. Nitric oxide could be oxidized to nitrate by flavohemoglobin, as shown with FhbA from *A. nidulans* (48). So, in the *ssu2*Δ strain, the intracellular nitrate from flavohemoglobin activity is not excreted into the medium at the same levels as in the WT. This would increase nitrite accumulation, which would be toxic for the cell and limit growth. However, the ability of Ssu2 to extrude some nitrite cannot be excluded. The capacity of Ssu2 to excrete nitrate, the inducer of the nitrate assimilation genes, also acts to balance nitrate levels by downregulating these genes. Thus, *SSU2* was induced about 5-fold by the presence of nitrate, although this induction almost disappears in mutants lacking nitrate reductase. Furthermore, several experiments showed a correlation between nitrite and *SSU2* upregulation. This again suggests that nitrite, or nitric oxide from nitrite, could be involved in the induction of *SSU2*, as seen in the Sc*SSU1* strain. Unlike that of *SSU2*, *NAR1* expression was not significantly modified in response to nitrate or ammonium (data not shown). The apparent lack of *NAR1* regulation in nitrate and ammonium does not rule out other mechanisms of regulating Nar1. However, Nar1 levels determined by Western blotting confirm that the regulation of protein levels seems unimportant (data not shown).

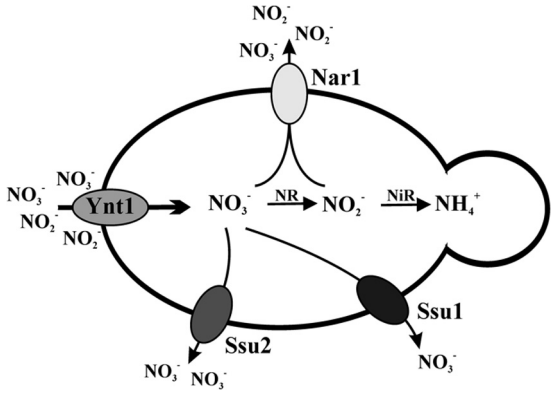

FIG 9 Nitrate and nitrite efflux systems in *H. polymorpha*. The high-affinity nitrate transporter (Ynt1) is involved in nitrate and nitrite influx. The nitrate is reduced by nitrate reductase (NR) to nitrite, which is catalyzed to ammonium by nitrite reductase (NiR). Ssu2 and to a lesser extent Ssu1 are involved nitrate efflux. Nar1 is involved in nitrite and nitrate efflux. Nar1-dependent nitrate efflux is observed when intracellular nitrate reaches higher levels. The nitrate and nitrite influx systems allow cells to cope with intracellular nitrite in cells growing in nitrate or nitrite as the sole nitrogen source.

We also addressed the question of whether an Sc*SSU1* strain was able to efflux nitrate/nitrite. Sc*SSU1* expressed in the Hp*ssu2*Δ strain under the *SSU2* gene promoter restores sulfite tolerance. Our experiments clearly show that ScSsu1 extrudes nitrate and nitrite (Fig. 8). It is difficult to reach a conclusion regarding the affinity of ScSsu1 for nitrate and nitrite, but it seems that ScSsu1 possesses a high capacity to extrude nitrite. This capacity was clearly shown in a WT strain expressing Sc*SSU1*. We suggested

that ScSsu1 could have an important role when *S. cerevisiae*, a non-nitrate-assimilating yeast, interacts with mammals. ScSsu1 appears to detoxify NO, extruding it from the cell as nitrite and nitrate.

In conclusion, net nitrate transport in yeast is a balance between nitrate influx and nitrate/nitrite efflux (Fig. 9). This is crucial in coping with nitrite toxicity. In nonassimilatory microorganisms, nitrate and nitrite extrusion could be involved in detoxifying nitrate and nitrite derived from NO.

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