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The *Saccharomyces cerevisiae* ∑1278b strain is sensitive to NaCl because of mutations in its *ENA1* gene

David Engelberg [1,2,3,*], Alexey Baskin[1], Shelly Ben Zaken[1], Irit Marbach[1]

[1]Department of Biological Chemistry, The Institute of Life Science, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[2]Singapore–HUJ Alliance for Research and Enterprise, Mechanisms of Liver Inflammatory Diseases Program, National University of Singapore, Singapore 138602
[3]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117456
*Corresponding author. David Engelberg, Department of Biological Chemistry, The Institute of Life Science, The Hebrew University of Jerusalem, Jerusalem 91904, ISRAEL. E-mail: engelber@mail.huji.ac.il
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Abstract

Most laboratory strains of the yeast *Saccharomyces cerevisiae* are incapable of invading agar, to form large colonies (mats), and to develop filament-like structures (pseudohyphae). A prominent strain that manifests these morphologies is ∑1278b. While induced transcription of the *FLO11* gene is critical for executing invasive growth, mat formation, and pseudohyphal growth, downregulation of the 'general stress response' also seems to be required. As this response is weak in ∑1278b cells, we assumed that they may be sensitives to stresses. We report, however, that they are resistant to various stressors, but severely sensitive specifically to NaCl. We found that this sensitivity is a result of mutations in the single ∑1278b's *ENA* gene, encoding P-type sodium ATPase. Other laboratory strains harbor three to five copies of *ENA*, suggesting that ∑1278b was selected against Ena activity. Obtaining ∑1278b cells that can grow on NaCl allows checking its effect on colony morphologies. In the presence of NaCl, ∑1278b/*ENA1*$^+$ cells do not invade agar, and do not form pseudohyphae or mats. Thus, we have found the following: (i) The ∑1278b strain differs from other laboratory strains with respect to sensitivity to NaCl, because it has no active Na^+ ATPase exporter. (ii) NaCl is a suppressor of invasiveness, filamentous growth, and mat formation.

Keywords: Invasive growth; Filamentous growth; pseudohyphae; mat formation; ∑1278b; ENA1

Introduction

Laboratory strains of the yeast *Saccharomyces cerevisiae* differ from each other in various properties. The dissimilarities are consequences of genetic polymorphism, including mutations, different copy numbers of particular genes, and even missing parts of the genome (Liu et al. 1996, Roberts et al. 1997, Takagi et al 2000, Cohen and Engelberg 2007, Schacherer et al. 2007, Dowell et al. 2010, Honigberg 2011, Chin et al. 2012, Matheson et al. 2017, Hou et al. 2019). Although a unicellular organism, *S. cerevisiae* has served as a model for aspects of multicellular organization (Madhani and Fink 1998, Reynolds and Fink 2001, Palecek et al. 2002, Honigberg 2011, Brückner and Mosch 2012, Cullen and Sprague 2012, Váchová and Palkova 2018, Kumar 2021), and the differences between strains were found to be most prominent in the ability to modify multicellular organization. When plated on solid media, i.e. supplemented with 2% agar, rich in nitrogen and fermentable carbon sources (e.g. glucose), *S. cerevisiae* cells form typical rounded concentric colonies, a consequence of bipolar budding. The diameter and height of the colony are determined by a combination of environmental and genetic factors (Scherz et al. 2001). If nitrogen concentration is particularly low (0.05 mM ammonium, instead of 3.5 mM in commonly used synthetic dextrose (SD) medium), then the rounded appearance is lost and cells, mainly diploids, operate a unipolar budding process that results in formation of elongated filaments, known as pseudohyphae (Gimeno and Fink 1992, Gimeno et al. 1992, Kron et al. 1994). If glucose concentration drops, cells, mainly haploids, invade agar so that they cannot be washed off the agar by a water current (Roberts and Fink 1994, Palecek et al. 2002). When agar concentration is low (~0.3%), colonies, commonly haploids, become extremely enlarged (termed mats) (Reynolds et al. 2008, Váchová et al. 2011, Reynolds 2018, Kumar 2021). Finally, when exposed to UV radiation on plates supplemented with high agar concentrations, surviving cells may form tall colonies (>1 cm high) termed stalk-like structures (Engelberg et al. 1998).

Filamentous growth, mat formation, and invasiveness are manifested by a very few laboratory strains, primarily of the ∑1278b genetic background. The reason for the inability of some strains (e.g. S288c) to form these morphologies is mutations in components required for inducing these phenotypes (Liu et al. 1996). For most strains, the reason is not clear.

The biochemical basis for inducing the changes in colony morphologies is activation of transcription of *FLO11*, encoding a GPI-anchored cell surface glycoprotein (flocculin) (Bouyx et al. 2021). *FLO11* is induced by the Ras/cAMP and the MAPK Kss1 cascades (Roberts and Fink 1994, Mösch et al. 1999, Rupp et al. 1999, Palecek et al. 2002, Cullen and Sprague 2012, Reynolds 2018, Kumar 2021). In addition to activating *FLO11* transcription, the yeast Ras/cAMP cascade inhibits the 'general stress response', and this inhibition seems to also be required for imposing invasive

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growth (Stanhill et al. 1999). Accordingly, activation of the 'general stress response' in response to stress is weaker in ∑1278b cells compared to other strains (reflected by induction of the stress-responsive transcription activators Msn2 and Msn4 (Stanhill et al. 1999). The original impetus of this study was, therefore, to test the resistance/sensitivity of ∑1278b cells to stress, assuming that they should be more stress-sensitive than other laboratory strains.

As we show below, this presumption was proved wrong, as ∑1278b cells were found to be resistant to most stresses tested. Yet, they were highly sensitive, very specifically, to NaCl. We report that the underlying cause of this sensitivity is non-functionality of the ATPase sodium pump Ena1 (Ruiz and Ariño 2007). The ∑1278b *ENA1* gene was found to encode a protein that differs from the S288c's Ena1 in 14 residues. Expressing the S288c *ENA1* gene in ∑1278b cells rescues their NaCl sensitivity. The availability of ∑1278b cells that proliferate in the presence of NaCl (∑1278b/*ENA1*$^+$ cells) allowed assessing whether this salt has any effect on the unique ability of ∑1278b cells to undergo developmental switches. It was observed that when exposed to NaCl, ∑1278b/*ENA1*$^+$ cells cannot invade agar or form pseudohyphae and mat.

Materials and methods

Yeast strains and media

Yeast strains used and the relevant genotypes are listed in Table S1. Cultures were commonly grown on yeast extract peptone dextrose (YPD) (2% glucose, 1% yeast extract, and 2% bacto peptone). To maintain library plasmids or single plasmids, cultures were grown on SD (0.17% yeast nitrogen base without amino acid and ammonium sulfate, 0.5% $NH_4(SO_4)_2$, 2% glucose and the required amino acids and nitrogen bases, but with no uracil [SD(-URA)] or no leucine [SD(-LEU)]. Low ammonium medium (SLAD) was SD, but with 50 μM $NH_4(SO_4)_2$. To grow cultures on a solid surface, media were supplemented with 2.5% bacto agar, except for YPD plates used to monitor mat formation that were supplemented with 0.3% agar. Sensitivity/resistance to NaCl was monitored under various NaCl concentrations, as described in each experiment. Sensitivity to stressors was tested on YPD plates supplemented with either 14 mM caffeine, 1 M KCl, 1 M sorbitol, or 5 mM H_2O_2, or by incubating YPD plates at 37 °C for 3 days.

Monitoring invasiveness, filamentous growth, and mat formation

To induce filamentous growth, cells were streaked on SLAD plates and allowed to grow at 30 °C for 5 days. To induce mat formation, ∼800 cells of cultures at the logarithmic phase were plated at the centre of a plate containing YPD supplemented with 0.3% agar. Plates were sealed with parafilm to avoid drying, and incubated for 9 days at 30 °C. To test for invasive growth, cultures were plated to cover a large area of the YPD plate (about one-third of the plate area), allowed to grow for 5 days, and then washed under a gentle water current.

Yeast transformation with plasmid DNA (individual or plasmids composing a genomic library) was performed as described in Gietz and Schiestl (2007).

Genomic library screening and sub-cloning of the *ENA1* gene

For the screening of genes that may rescue the NaCl sensitivity of ∑1278b cells, a genomic library, prepared from S288c cells, was used. The library was obtained from G. R. Fink (Whitehead Institute, Cambridge, MA). It was created by partial digestion of the genomic DNA with the Sau3A restriction enzyme and insertion of fragments to the BamHI site of pRS426. The library was introduced into ∑L5527 cells. Transformants were plated on SD(-URA) plates and colonies obtained were replica-plated to plates supplemented with YPD + 0.8 M NaCl (see also the 'Results' section). Plasmids were isolated from colonies that grew on YPD + 0.8 M NaCl after growing each colony overnight on SD(-URA) (5 ml) and using the 'Zymoprep Yeast Plasmid Miniprep I' (Zymo Research, Irvine, CA).

Construction of chimeric S288c/∑1278b *ENA1* genes

Chimeras that combine the seven N-terminal polymorphic residues of *ENA1* from one strain (S288c or ∑1278b) with the seven C-terminal polymorphic residues of the other strain (i.e. ENA1–∑7WT7 and ENA1–WT7∑7) were obtained by swapping the Pst1–Bln1(Avr2) fragment between the genes. Chimeras containing the 3 N-terminal polymorphic residues of *ENA1* of one strain with 11 C-terminal polymorphic residues of the other were obtained by swapping the Sac1–Spe1 fragment between the S288c or ∑1278b genes, cloned on plasmids pAES305-HA-*ENA1* (harbouring either S288c's or ∑1278b's *ENA1*). Other chimeras were obtained by swapping the Sac1–Spe1 fragment of the ENA1–∑7WT7 and ENA1–WT7∑7 chimeras.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange kit (Stratagene) with the following primers.

To change A754 to T: 5′-GTAGGAAAAAGTTCTGCACAATGACGG GTGATGGTG-3′ and 5′-CACCATCACCCGTCATTGTGCAGAACTTT TTCCTAC-3′. To change F860 to S: 5′-GGATTATTGTCGTCACCTCTTGTTTTCCTGCTATGGG-3′ and 5′-CCCATAGCAGGAAAAGAGGTGACGACAATAATCC-3′. To change N882 to H: 5′-GATTTGATGGATAGACCTCCTCATGATTCAGAGGTT GG-3′ and 5′-CCAACCTCTGAATCATGAGGTCTATCCATCAAATC-3′.

Cloning genomic ∑1278b and S288c *ENA1* genes

The *ENA1* genes were cloned from genomic DNA by PCR using the following primers: 5′-CCTCTTTCTTTGCCTCGAGAG-3′ and 5′-GGATTTAGCGTGCACGAAAGG-3′. The coding sequences alone were cloned via PCR using the primers 5′-TCCCCCCGGGCTTCGTACACAGAATTG-3′ and 5′-TCCCCCCGGGAGCACTTAATAGGCC-3′, which contain recognition sites for the restriction enzyme Xma1. The PCR products were sub-cloned into the Xma1 site of pAES426, pAES305, and pAES306 (Levin-Salomon et al. 2009).

RT–PCR analysis

Total RNA was isolated using the Qiagen RNeasy kit. cDNA was prepared and analysed by PCR using the iScript kit (BioRad) and product levels were normalized to the levels of RDN18. Primers used for monitoring *FLO11* were 5′-CAAGGTAGTGCCGCTCAATATG-3′ and 5′-TGAGAGCCTTGATTGTCAT-3′ and primers used for monitoring *RDN18* were 5′-ACGATACAGGGCCCATTCG-3′ and 5′-ACTTGCCCTCCAATTGTTCCT-3′.

β-Galactosidase assay

The Yep365-*FLO11*::LacZ plasmid, containing 3 kb of the *FLO11* promoter fused to the gene encoding β-galactosidase was

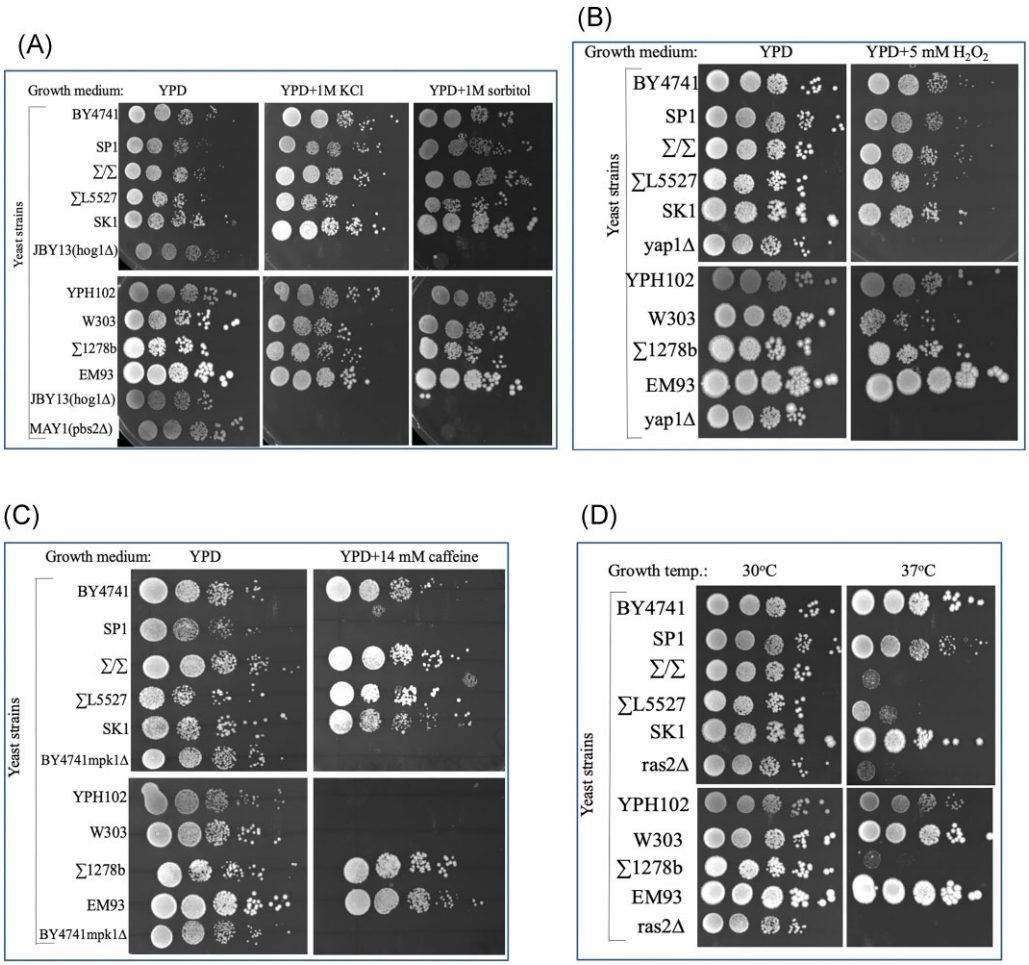

Figure 1. ∑1278b cells are resistant to high osmotic pressure, oxidative stress, and caffeine, but are sensitive to elevated temperature. Cells of the indicated strains, removed from cultures at the logarithmic phase, were plated in decimal dilutions on YPD plates supplemented with 1 M KCl, 1 M sorbitol (A), 5 mM H_2O_2 (panel b), or 14 mM caffeine (C), and incubated at 30 °C. Other YPD plates were incubated at 37 °C (D). Plates were photographed after 3 days of incubation. The JBY13(*hog1*Δ) and MAY1(*pbs2*Δ) strains (Bell et al. 2001, Brewster et al. 1993) were used as a control for the osmotic pressure stress (A), the SP1*yap1*Δ strain was used as a control for the oxidative stress (B), the BY4741*mpk1*Δ strain served as a control for caffeine stress (C), and SP1*ras2*Δ cells served as a control for temperature sensitivity (D). Note that the YPH102 strain (Sikorski and Hieter 1989) is of the S288c genetic background, isogenic to JBY13 and MAY1.

obtained from G. R. Fink (Rupp et al. 1999). For monitoring β-galactosidase activity, cells were collected, disrupted, and assayed as described previously (Grably et al. 2002).

Determination of the putative 3D structure of Ena1

The 3D models of the ∑1278b and S288c Ena1 proteins were computationally generated from their amino acid sequences using the AlphaFold 3 Server (Abramson et al. 2024). The server was accessed on 5 January 2025. Superimposition of the AlphaFold 3 models was done using the PyMOL align command (The PyMOL Molecular Graphics System, Version 3.1, Schrödinger) and RMSD was calculated using the Super command.

Results

∑1278b cells are specifically sensitive to sodium ions

To test whether ∑1278b cells may be more sensitive to stresses than other laboratory strains, haploid and diploid ∑1278b cells were exposed, along with several commonly used laboratory strains, to oxidative stress (H_2O_2), caffeine, high concentrations of sorbitol, NaCl and KCl (1.0 M), and elevated temperature (37 °C). ∑1278b cells were resistant to all stresses inflicted (Fig. 1) except for NaCl (Fig. 2). They were also sensitive to some extent to elevated temperature (Fig. 1d). Another of the tested strains, SP1 (Broek et al. 1987), was also found to be sensitive to NaCl, but not as severely as ∑1278b (Fig. 2). Thus, although ∑1278b cells do not efficiently activate the 'general stress response' (Stanhill et al. 1999), they are resistant to an array of stresses. They are, however, sensitive, very specifically, to NaCl.

To determine the degree of their sensitivity to NaCl, ∑1278b cells were exposed to several concentrations, from 100 to 700 mM. Three strains, an original ∑1278b culture, and two derivatives were tested, and all found to be similarly sensitive to a low concentration of 200 mM (Fig. 2). Notably, SP1 cells, and even cells of the *hog1*Δ and *pbs2*Δ strains, known to be sensitive to NaCl as they are defective in the pathway that controls the response to osmostress, still proliferate on medium supplemented with 200 mM NaCl, emphasizing the severe sensitivity of ∑1278b cells (Fig. 2).

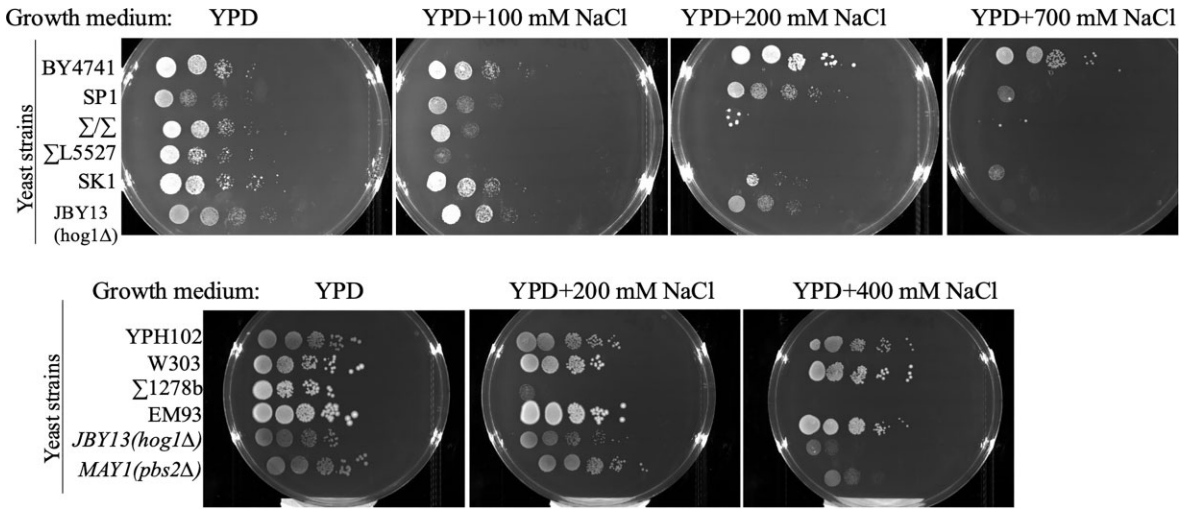

Figure 2. ∑1278b cells are sensitive to NaCl. Cells of the indicated strains, removed from cultures at the logarithmic phase, were plated in decimal dilutions on YPD plates supplemented with the indicated concentrations of NaCl. About 50 000 cells were spotted on the first point. Plates were photographed after 3 days of incubation. JBY13(*hog1*Δ) and MAY1(*pbs2*Δ) strains were used as a control.

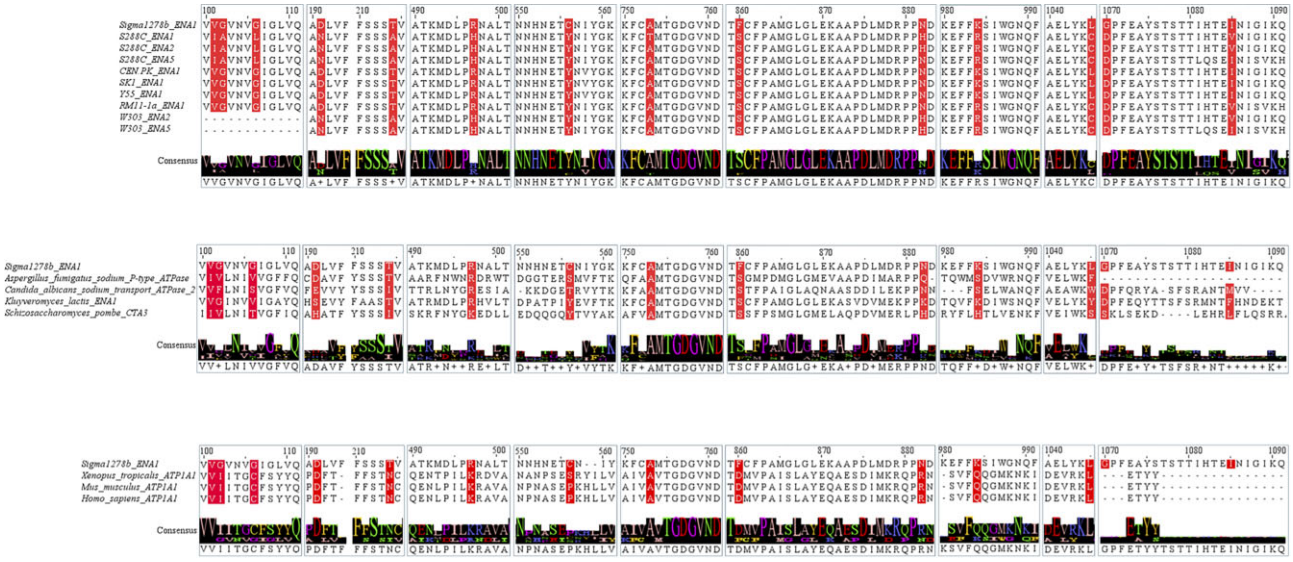

Figure 3. The *ENA1* genes of the ∑1278b and S288c strains differ in 14 positions. Some of the polymorphic residues are evolutionary conserved. Upper panel: sequence alignment of polymorphic regions of the *ENA1* gene of ∑1278b, with the *ENA1* genes of the indicated strains as well as with *ENA2* and *ENA5* of S288c. Middle panel: sequence alignment of the *ENA1* gene of ∑1278b, with the *ENA1* genes of the indicated yeast species and the fungus *Aspergillus fumigatus*. Lower panel: sequence alignment of the *ENA1* gene of ∑1278b, with the *ENA1* genes of the indicated vertebrates.

The very specific sensitivity to NaCl, and not to KCl, H_2O_2, caffeine, or sorbitol, shows that ∑1278b cells are sensitive to the toxicity of sodium ions and not to the osmotic pressure imposed by NaCl.

The ∑1278b strain is sensitive to NaCl because it possesses a defective *ENA1* gene

To decipher the basis for the sodium sensitivity of ∑1278b cells, we introduced into these cells a genomic library, cloned in a 2μ-based plasmid, prepared from the DNA of the S288c strain, which is NaCl-resistant (Fig. 2). Note that BY4741 and YPH102 are derivatives of S288c. About 50 000 transformants were obtained on Y-nb(-URA) plates. Transformants were then replica plated onto plates supplemented with 0.8 M NaCl to screen for transformants in which NaCl sensitivity might have been rescued and were rendered able to proliferate under this condition. A total of 17 such colonies appeared. They are presumed to carry a genomic fragment of S288c carrying the gene(s) that rescued the NaCl sensitivity of ∑1278b cells. Isolation of the library plasmid from all colonies revealed that each of the 17 colonies harbours either of the two plasmids. The two plasmids were found to carry a different genomic fragment, but with a large overlapping region. The

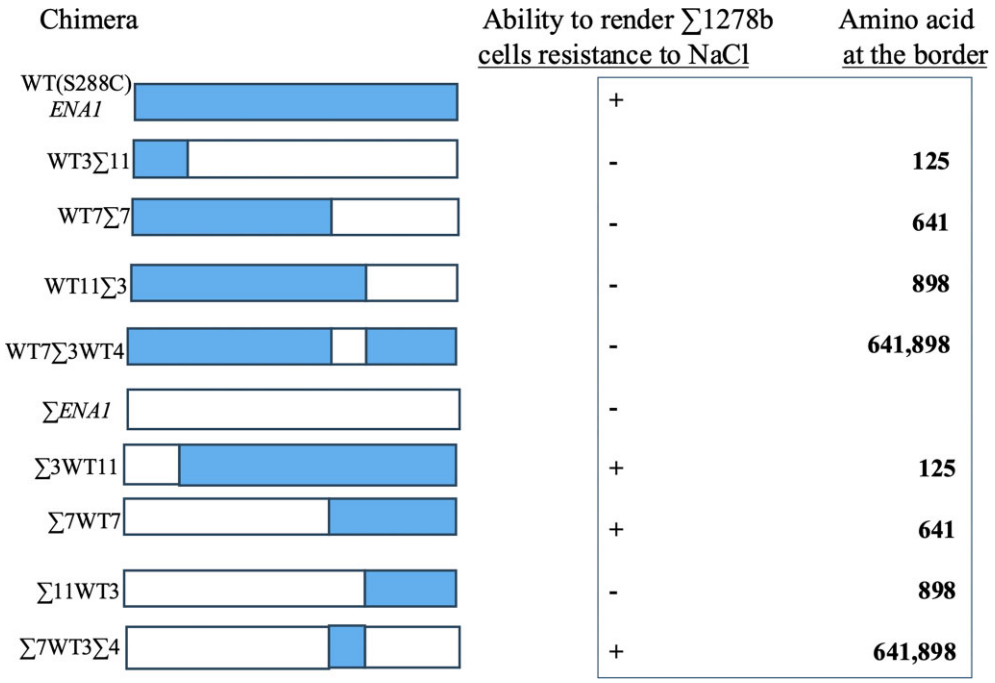

Figure 4. A fragment of the ∑1278b *ENA1* gene containing differences between the S288c and ∑1278b genes at positions encoding residues 753, 860, and 882 is responsible for the lack of function of the ∑1278b *ENA1* gene product. A series of chimeric *ENA1* genes, composed of the indicated parts of the S288c and the ∑1278b genes, was constructed and introduced into ∑1278b cells. Transformants were tested for their ability to proliferate on plates containing YPD supplemented with 0.8 M NaCl.

genomic region that is common to the two rescuing plasmids contains the *ENA1* gene, encoding an ATPase sodium pump (Wieland et al. 1995, Ruiz and Ariño 2007).

The Ena proteins consist of one of the two NaCl extrusion systems that exist in *S. cerevisiae*. The other system is the Na^+/H^+ antiporter Nha1 machinery. Nha1 seems to be less critical than the Ena enzymes, as mutations in the latter render cells highly sensitive to sodium (Ariño et al. 2019, Ruiz and Ariño 2007, Cyert and Philpott 2013, Serra-Cardona et al. 2014). Therefore, probably as a backup for this critical activity, most *S. cerevisiae* genetic backgrounds evolved three to five copies of the gene encoding it (Wieland et al. 1995). That being the case, the fact that ∑1278b genetic background possesses a single *ENA* gene is puzzling, and is raising the possibility that ∑1278b cells are sensitive to NaCl simply because the Ena levels in these cells are low. The CEN.PK strain, for example, possesses a single *ENA1* gene and is sensitive to NaCl, but overexpression of the gene rescues the sensitivity (Daran-Lapujade et al. 2009). Notably, our screen was performed with a 2μ-based library. To check whether ∑1278b's *ENA1* could rescue sodium sensitivity of the strain when overexpressed, we cloned the gene and overexpressed it in ∑1278b cells. We observed that the overexpressed gene could not rescue NaCl sensitivity. This experiment suggests that the sensitivity of the ∑1278b genetic background does not stem from low expression levels but probably from the fact that its Ena1 protein is not active. Indeed, a single integrated copy of S288c's *ENA1* gene could rescue ∑1278b cells from sodium stress (Fig. 6). This result also strongly suggests that the ∑1278b strain is defective in *ENA1* itself, and not in some upstream regulator of Ena1 (so that overexpression of *ENA1* overcomes its defect).

Ena1 of ∑1278b differs from Ena1 of S288c in 14 residues

To check whether the ∑1278b *ENA1* may indeed carry a mutation that renders the encoded protein inactive, we sequenced the ∑1278b *ENA1* and the S288c genes that we cloned from the strains available in our laboratory, as well as the S288c's *ENA1* gene from the library plasmid. All sequences were found to be identical to those appeared in the Saccharomyces Genome Database (SGD). Comparing the sequences at the amino acid level revealed that the Ena1 of S288c and ∑1278b differ from each other in 14 positions (Fig. 3; upper panel, the first two rows). We further compared both sequences to those of Ena2 and Ena5 of S288c and W303, and to that of Ena1 of the CEN.PK strain (known as Pmr/Ena6) and the SK1, Y55, and RM11-1a strains (Fig. 3, upper panel).

Interestingly, of the 14 residues that the defective Ena1 of ∑1278b does not share with the intact Ena1 of S288c, 11 are shared with Ena1 of the CEN.PK, SK1, and Y55 strains (i.e. V101, G102, G106, D191, T204, R497, A753, N882, L1045, I1085; Fig. 3) and some with RM11-1a too. Only C556, F860, and G1070 are unique to Ena1 of ∑1278b (Fig. 3, upper panel). In spite of the differences in these residues, the overall similarity of the *ENA1* coding sequences between *Saccharomyces cerevisiae* strains is ~98% (Table S2). Sequence alignment of the *ENA1* gene of ∑1278b and S288c with the *ENA1* of *Kluyvemyces lactis* revealed 77.6% similarity, but comparison with *ENA1* of more distant yeasts, *Schizosaccharomyces pombe*, *Candida albicans*, and the fungus *Aspergillus fumigatus*, revealed lower degrees of similarity (60.5%–70.3%; Table S2; Fig. 3, middle panel), and similarity to respective mammalian proteins is in the range of 40% (Table S2; Fig. 3, lower panel).

Table 1. Capability of the mutated *ENA1* of the ∑1278b strain to rescue Na^+ sensitivity of ∑1278b cells.

ENA1 gene	Ability to render ∑1278b cells resistant to NaCl
***ENA1* of ∑1278b (∑*ENA1*)**	-
∑ENA1^{A753T}	-
∑ENA1^{F860S}	+
∑ENA1^{N882H}	-
∑ENA1 $^{A753T+N882H}$	-
∑ENA1 $^{F860S+N882H}$	+

The Phe at position 860 in the Ena1 of ∑1278b is responsible for non-functionality of the protein

To identify the residues responsible for the non-functionality of ∑1278b Ena1, we first constructed a series of ∑1278b–*ENA1*/S288c–*ENA1* chimeras (Fig. 4). We found that when a fragment containing three differences between the S288c and the ∑1278b Ena1 sequences (T753A, S860F, H382N) was swapped between the genes, ∑1278b became functional while the S288c gene became inactive Fig. 4). We therefore changed systematically these three residues in the ∑1278b gene to those that appear in the S288c gene. As shown in Table 1, changing F860 in the ∑1278b protein to Ser, the residue found at this position in all yeast Ena proteins (Fig. 3, upper and middle panels) makes the protein capable of supporting proliferation in the presence of NaCl. The Phe residue at position 860 is thus the major component that renders the ∑1278b Ena1 protein inactive.

The hydrophobic Phe at position 860 of ∑1278b Ena1 may interfere with ion movement

Why does the Phe at position 860 renders the ∑1278b Ena1 inactive? AlphaFold 3-based structures of the ∑1278b and S288c Ena1 show that although the structures are almost identical, with high degree of overlapping (Fig. 5a), the phenylalanine at position 860 could interfere with sodium movement (Fig. 5b). Position 860 is located within a putative transmembrane α-helix, which is one of the helices that form a hydrophilic region, a potential channel for the sodium ions. This channel is clearly shown in the cryogenic electron microscpy (cryo-EM) structure of the human Na^+K^+ ATPase (PDB:7E1Z; Fig. 5c and d). It seems that in the ∑1278b Ena1, Phe860 is oriented towards the channel, possibly interfering with ion movement (Fig. 5c).

The fact that a single residue is responsible for the non-functionality of the ∑1278b Ena1 protein raises the question of the role of the other 13 differences between the S288c and ∑1278b proteins. These differences in sequence must cause some differences in the biochemical and physiological properties of the proteins. Based on the overlapping of the predicted structures (Fig. 5a), these differences are probably not very dramatic, but the question regarding the significant polymorphism of this protein between yeast strains remains open.

∑1278b cells expressing intact *Ena1* protein do not form pseudohyphae or mats, nor invade agar, when grown on media supplemented with NaCl

The generation of ∑1278b/*ENA1*$^+$ cells, which can proliferate on media supplemented with NaCl, allows testing whether intact Ena1 and/or NaCl affects invasiveness and/or formation of pseudohyphae and mats. When grown on YPD (with no addition of NaCl), ∑1278b/*ENA1*$^+$ cells did manifest invasiveness, but it was less efficient than the invasiveness of parental ∑1278b cells, harbouring an 'empty' plasmid (Fig. 6, plates on the right-hand side). When grown under the relevant conditions (with no NaCl), ∑1278b/*ENA1*$^+$ cells were able to form pseudohyphae (Fig. 7, photos in the left-hand side) and mats (Fig. 8, photos at the left hand-side), suggesting that an intact and active Ena1 *per se* does not affect these morphologies (except for affecting invasiveness to some degree). We then monitored the capability of ∑1278b/*ENA1*$^+$ cells to invade agar and to form mats and pseudohyphae on media supplemented with 0.8 M NaCl. It was observed that the presence of NaCl suppressed the ability of ∑1278b/*ENA1*$^+$ cells to manifest any of these growth patterns (Fig. 6, photos at the left, Figs 7 and 8, photos at the right-hand side).

Why does NaCl block the morphological changes? All three growth patterns, invasive and filamentous growth, and mat formation, require elevated expression of Flo11, induced at the transcriptional level (Rupp et al. 1999, Reynolds 2018, Bouyx et al. 2021). We therefore checked whether the presence of NaCl inhibits *FLO11* expression. This was done by directly measuring *FLO11* mRNA levels (Fig. 9) and by monitoring the activity of a *Flo11–LacZ* reporter. Both assays suggest that *FLO11* expression is not affected by NaCl. mRNA levels were similar in ∑1278b and ∑1278b/ENA1$^+$ cells (Fig. 9).

β-Galactosidase activity measured in lysates prepared from ∑1278b/*Flo11–LacZ* and ∑1278b/ENA1$^+$/*Flo11–LacZ* cells grown on YPD was very low (3 and 8 units, respectively) and did not change much after 2 h on YPD supplemented with 0.1 M or 0.4 M NaCl (4.2 and 4.5 units for ∑1278b/*Flo11–LacZ* and 5.4 and 5.2 units for ∑1278b/ENA1$^+$/*Flo11–LacZ*; results are averages of two repeats that were performed in triplicates). On the basis of these measures, it seems that neither Ena1 nor NaCl affects *FLO11* levels and therefore the inability of *S. cerevisiae* cells to change colony morphology in the presence of NaCl is not a consequence of down-regulating *FLO11* levels. As upregulation of the general stress response seems to be an inhibitor of morphological changes (Stanhill et al. 1999), perhaps the induction of various stress-responsive genes in response to NaCl (Rep et al. 2000, Saito and Posas 2012, de Nadal and Posas 2015) is the mechanism that blocks the changes in growth patterns.

Discussion

Although sodium ions have been abundant in nature throughout the course of evolution, living organisms do not tolerate high concentrations and developed ways to maintain low intracellular sodium levels. Malfunctioning of this machinery could be deleterious, particularly when external sodium concentration is high. Several mechanisms were developed to constantly extrude sodium from the cell against the electrochemical gradient. While two such extrusion mechanisms exist in *S. cerevisiae*, the Na^+/H^+ antiporter Nha1 and the Ena ATPases, mutations in the latter render cells highly sensitive to sodium (Ruiz

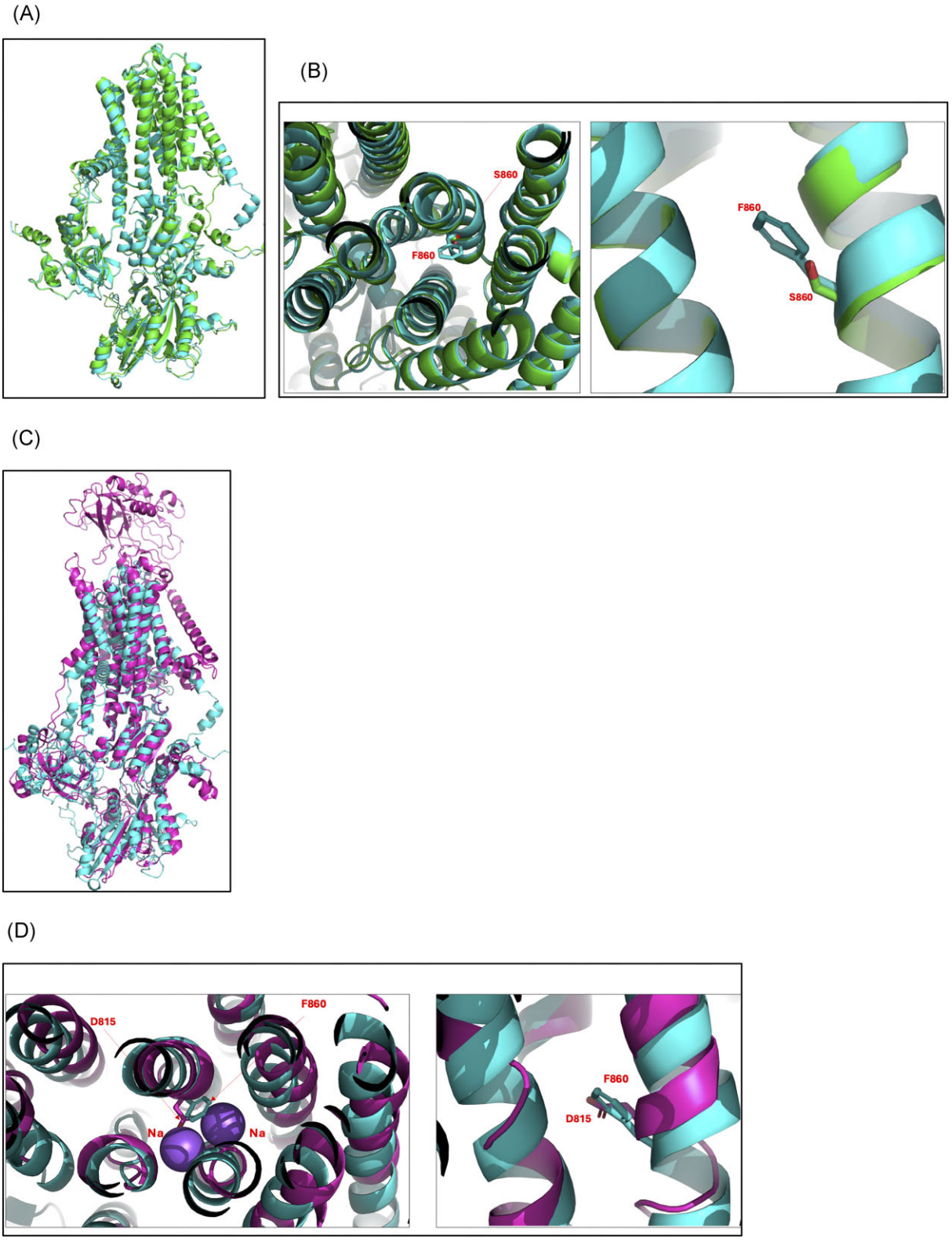

Figure 5. Phe860 of ∑1278b Ena1 seems to point at the sodium pathway. (A) Superimposition of AlphaFold 3-predicted structures of Ena1 of ∑1278b (teal) and of S288C (green). The RMSD is 0.6 (for more parameters, see Table S2). (B) Same superimposition as shown in A, but zoomed in on position 860. (C) Superimposition of AlphaFold 3-predicted structures of Ena1 of ∑1278b (teal) and the cryo-EM structure of the Na^+-bound human Na^+K^+-ATPase (PDB:7E1Z). (D) Same superimposition as in (C), but zoomed in on 860's position of ∑1278b Ena1 and 815's position of the human protein.

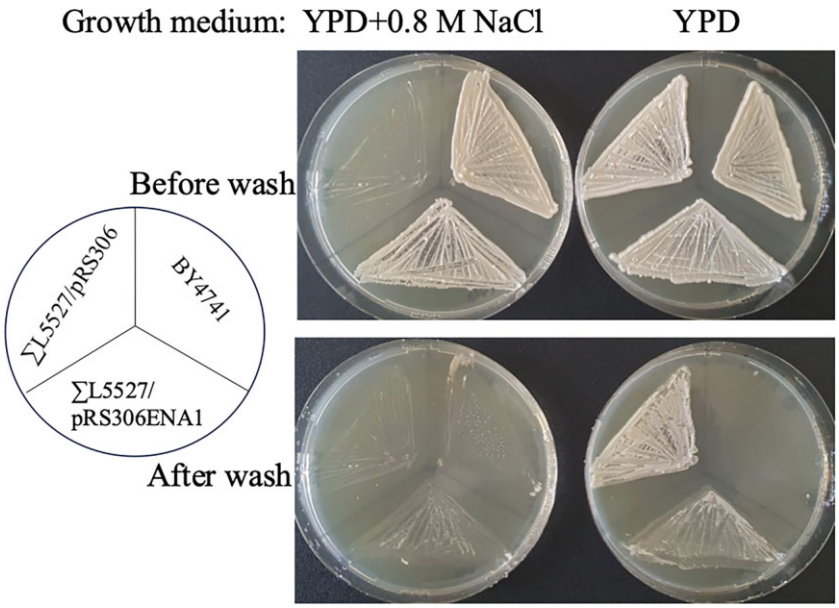

Figure 6. ∑1278b/*ENA1*$^{+}$ cells manifest a reduced agar invasiveness on YPD and do not invade agar at all when grown on YPD supplemented with 0.8 M NaCl. Cells of the indicated strains were plated on plates containing the indicated media and incubated at 30 °C for 3 days. Plates were then photographed (upper plates), washed under water current, and photographed again (lower panels).

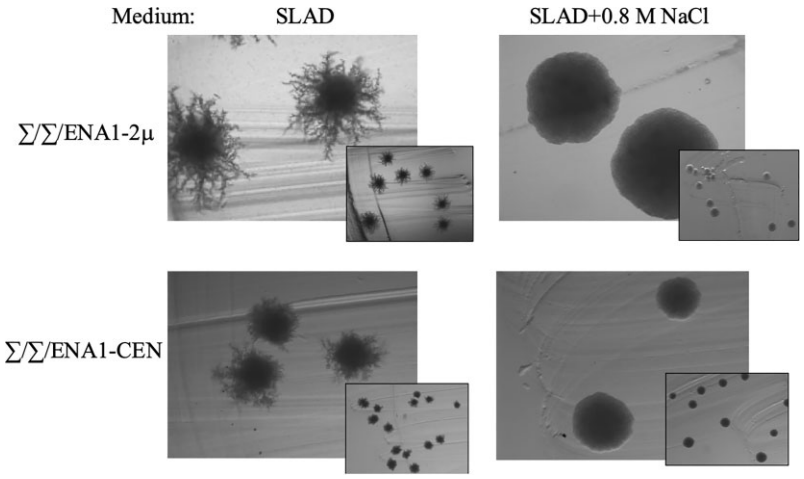

Figure 7. Diploid ∑1278b/*ENA1*$^{+}$ cells do not form pseudohyphae when grown on SLAD medium supplemented with 0.8 M NaCl. Diploid ∑1278b cells, harbouring either a 2μ-based or a CEN-based plasmid carrying the *ENA1* of the S288c strain, were plated on plates containing the indicated media, allowed to proliferate for 4 days, and photographed.

and Ariño 2007, Cyert and Philpott 2013, Serra-Cardona et al. 2014, Ariño et al. 2019). Therefore, probably as a backup for the critical Ena activity, most *S. cerevisiae* genetic backgrounds evolved three to five copies of the gene encoding it (Wieland et al. 1995). That being the case, the fact that the ∑1278b genetic background possesses a single *ENA* gene is puzzling. The inactivation mutation this single gene acquired adds to the riddle.

∑1278b cells are popular as an experimental model for multicellular organization. Our results suggest a weak linkage between the inactive Ena1 in this genetic background and its ability to invade agar because reconstituting Ena1 activity in ∑1278b cells resulted in a somewhat reduced invasiveness. But, filamentous growth and mat formation seem unaffected. This suggests that the severe sodium sensitivity and the ability of ∑1278b cells to form pseudohyphae and mats are not strongly linked. Yet,

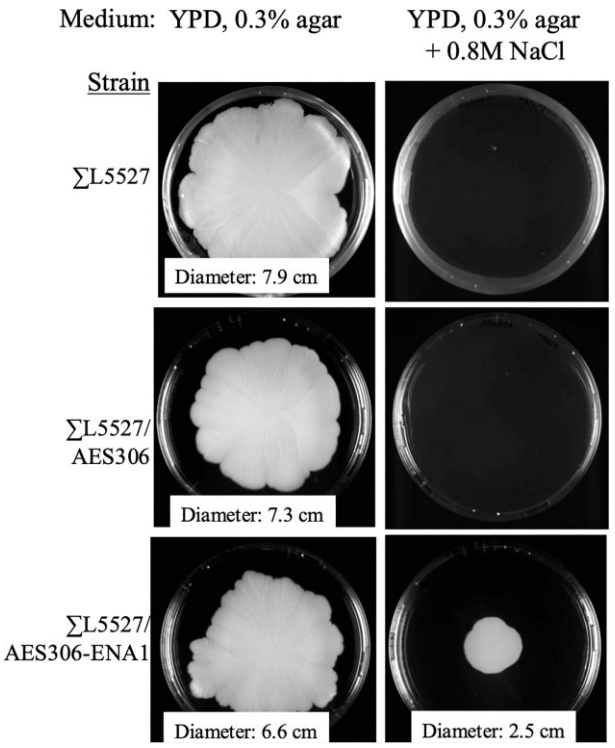

Figure 8. ∑1278b/*ENA1*$^{+}$ cells do not form mats when grown on medium supplemented with 0.3% agar and 0.8 M NaCl. Cells of the indicated strains were plated on plates containing the indicated media, incubated at 30 °C for 6 days, and photographed.

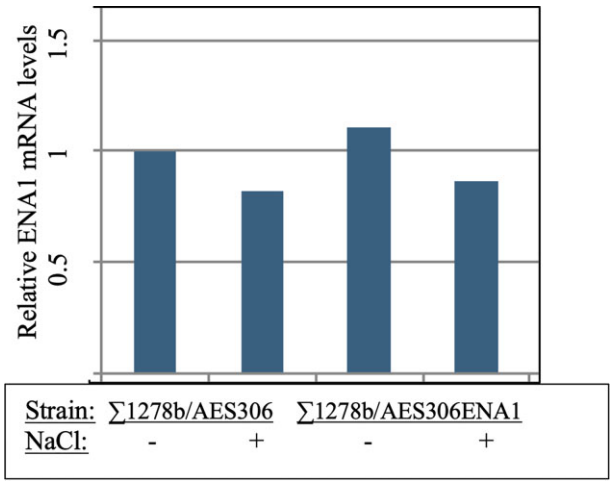

Figure 9. Expression of the *ENA1* gene or exposure to NaCl does not affect levels of *FLO11* mRNA. *FLO11* mRNA levels were monitored via quantitative reverse transcription polymerase chain reaction (qRT–PCR), and normalized to levels of *RDN18* (see the 'Materials and methods' section) in total RNA samples prepared from the indicated strains, grown on YPD to OD_{600} = 0.3, split into two, with one half exposed (+) and the other not exposed (–) to 0.4 M NaCl for 3 h.

NaCl was found here to be a suppressor of invasiveness, pseudohyphal growth, and mat formation, probably due to the induction of the cellular stress response. What could be the biological explanation behind NaCl-imposed suppression of colony morphological organization? The answer to this question will be obtained once the biological roles of these morphologies will be deciphered. It was proposed that colonies develop long filaments in response to low ammonium levels in order to reach regions with better nutrition. But, the need to seek nitrogen when scarce still holds when NaCl is present, and yet regular concentric colonies are formed on SLAD medium supplemented with NaCl. Perhaps the biological function of filamentous growth, and of invasiveness and mats, is not connected with nutrient availability. Revealing the connection between invasiveness, mat formation, and filamentous growth, and the general stress response may assist in deciphering the puzzle. The notion that these morphologies of yeast colonies may model developmental and pathological processes in higher eukaryotes (Botstein and Fink 2011, Cáp et al. 2012, Engelberg et al. 2014) strengthens the need to understand the phenomenon.

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Supplementary data

Supplementary data is available at *FEMSYR Journal* online.

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