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Authors

Higgins, Douglas A

Young, Megan KM

Tremaine, Mary

et al.

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Natural Variation in the Multidrug Efflux Pump *SGE1* Underlies Ionic Liquid Tolerance in Yeast

Douglas A. Higgins,*,†,1 Megan K. M. Young,‡ Mary Tremaine,‡ Maria Sardi,‡,§ Jenna M. Fletcher,‡ Margaret Agnew,‡ Lisa Liu,‡ Quinn Dickinson,‡ David Peris,‡,§,2 Russell L. Wrobel,‡,§ Chris Todd Hittinger,‡,§ Audrey P. Gasch,‡,§ Steven W. Singer,*, Blake A. Simmons,*,† Robert Landick,‡,‡‡,§§ Michael P. Thelen,*,†,3 and Trey K. Sato‡,3**

*Deconstruction Division, Joint BioEnergy Institute, Emeryville, California 94608, †Biosciences and Biotechnology Division, Lawrence Livermore National Laboratory, California 94550, ‡Department of Energy Great Lakes Bioenergy Research Center, §Laboratory of Genetics, ‡‡Department of Biochemistry, and §§Department of Bacteriology, University of Wisconsin-Madison, Wisconsin 53726, **Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, California 94720, and ††Sandia National Laboratories, Livermore, California 94550

ORCID IDs: 0000-0001-9912-8802 (D.P.); 0000-0002-5309-6718 (R.L.W.); 0000-0001-5088-7461 (C.T.H.); 0000-0002-1332-1810 (B.A.S.); 0000-0002-2479-5480 (M.P.T.); 0000-0001-6592-9337 (T.K.S.)

ABSTRACT Imidazolium ionic liquids (IILs) have a range of biotechnological applications, including as pretreatment solvents that extract cellulose from plant biomass for microbial fermentation into sustainable bioenergy. However, residual levels of IILs, such as 1-ethyl-3-methylimidazolium chloride ($[C_2C_1im]Cl$), are toxic to biofuel-producing microbes, including the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* strains isolated from diverse ecological niches differ in genomic sequence and in phenotypes potentially beneficial for industrial applications, including tolerance to inhibitory compounds present in hydrolyzed plant feedstocks. We evaluated >100 genome-sequenced *S. cerevisiae* strains for tolerance to $[C_2C_1im]Cl$ and identified one strain with exceptional tolerance. By screening a library of genomic DNA fragments from the $[C_2C_1im]Cl$-tolerant strain for improved IIL tolerance, we identified *SGE1*, which encodes a plasma membrane multidrug efflux pump, and a previously uncharacterized gene that we named *ionic liquid tolerance 1* (*ILT1*), which encodes a predicted membrane protein. Analyses of *SGE1* sequences from our panel of *S. cerevisiae* strains together with growth phenotypes implicated two single nucleotide polymorphisms (SNPs) that associated with IIL tolerance and sensitivity. We confirmed these phenotypic effects by transferring the *SGE1* SNPs into a $[C_2C_1im]Cl$-sensitive yeast strain using CRISPR/Cas9 genome editing. Further studies indicated that these SNPs affect Sge1 protein stability and cell surface localization, influencing the amount of toxic IILs that cells can pump out of the cytoplasm. Our results highlight the general potential for discovering useful biotechnological functions from untapped natural sequence variation and provide functional insight into emergent *SGE1* alleles with reduced capacities to protect against IIL toxicity.

KEYWORDS *Saccharomyces cerevisiae*; yeast; ionic liquid; natural variation; major facilitator superfamily; biofuels; toxin tolerance

IONIC liquids are neutral salts that attain a liquid state at temperatures mostly <100° (reviewed in Welton 1999) and have a broad range of biological applications from biomedicine (reviewed in Dias *et al.* 2017; Egorova *et al.* 2017) to production of biochemicals and bioenergy. In renewable bioenergy applications, ionic liquids, particularly imidazolium ionic liquids (IILs) such as 1-ethyl-3-methylimidazolium chloride ($[C_2C_1im]Cl$), 1-ethyl-3-methylimidazolium acetate ($[C_2C_1im][OAc]$), and 1-butyl-3-methylimidazolium chloride ($[C_4C_1im]Cl$) are effective in solubilizing plant biomass for purification of cellulose through a process called pretreatment (Binder and Raines 2010; Li *et al.* 2010; Elgharbawy

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[1]Present address: Pivot Bio, 2929 Seventh Street Suite 120, Berkeley, CA 94710.
[2]Present address: Department of Food Biotechnology, Institute of Agrochemistry and Food Technology, Consejo Superior de Investigaciones Cientfficas, 46980, Valencia, Spain.
[3]Corresponding authors: Lawrence Livermore National Laboratory, 7000 East Ave., L-452, Livermore, CA 94550. E-mail: thelen1@llnl.gov; and Department of Energy Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, 1552 University Ave., Madison, WI 53726. E-mail: tksato@glbrc.wisc.edu

et al. 2016). After pretreatment, cellulose is highly accessible to cellulase enzymes that hydrolyze it into monomeric glucose, which is then fermented into bioethanol or other biofuels by industrial microbes. However, two disadvantages of these solvents are their high cost (Blanch *et al.* 2011; Konda *et al.* 2014) and toxicity to biofuel-producing microbes, both of which impose a demand for IIL recovery after pretreatment. *Escherichia coli* strains used in the industrial production of biofuels and biochemicals are growth impaired in laboratory media containing 200–270 mM $[C_2C_1im]Cl$ (Khudyakov *et al.* 2012; Ruegg *et al.* 2014). The dominant biofuel-producing microbe, *Saccharomyces cerevisiae*, is even more sensitive; 30–60 mM $[C_2C_1im]Cl$ can inhibit growth (Ouellet *et al.* 2011; Dickinson *et al.* 2016). After biomass pretreatment and hydrolysis, up to 270 mM IIL may persist during fermentation (Datta *et al.* 2010); IILs at these concentrations severely impair both yeast growth and biofuel production. Thus genetically modified yeasts that better tolerate inhibitory IIL concentrations are highly desirable to improve the production of lignocellulosic biofuels and bioproducts.

To circumvent IIL toxicity, gene sequences from IIL-tolerant microbes can be inserted into biofuel-producing microbes to improve tolerance to IILs. For example, two genes, *eilA* and *eilR*, are determined to be primarily responsible for IIL tolerance in *Enterobacter lignolyticus* (Ruegg *et al.* 2014), an IIL-tolerant rain forest bacterium (Khudyakov *et al.* 2012). A member of the major facilitator superfamily (MFS) of proteins, the inner membrane transporter EilA exports quaternary ammonium cations and is transcriptionally regulated by EilR, which is induced by the $[C_2C_1im]^+$ cation. Because of this, when expressed in *E. coli*, the *eilAR* gene cassette increases both cell growth and biofuel production in media containing IILs (Ruegg *et al.* 2014).

In yeast, chemical genomics screening of *S. cerevisiae* mutants determined that deletion of *PTK2* increases cell fitness and sugar metabolism in the presence of inhibitory IIL concentrations (Dickinson *et al.* 2016). *PTK2* encodes a putative serine/threonine protein kinase that activates the plasma membrane H^+-ATPase Pma1 (Eraso *et al.* 2006), and it was suggested that deletion of *PTK2* blocks Pma1 proton-coupled import of IILs into the cytoplasm (Dickinson *et al.* 2016), where IILs appear to affect mitochondrial function (Mehmood *et al.* 2015; Dickinson *et al.* 2016). Although deletion of *PTK2* improves IIL tolerance, the resulting reduction in Pma1 activity and altered ion homeostasis also causes decreased strain fitness in other conditions (Giaever *et al.* 2002; Qian *et al.* 2012).

Functional screening of homologous DNA libraries has been an effective means to identify overexpressed genes in yeast that confer tolerance to industrially relevant inhibitors, such as ethanol (Anderson *et al.* 2012) and toxins in molasses fermentations (Ness and Aigle 1995). As an alternative approach, we explored the genetic variation in natural *S. cerevisiae* isolates to identify additional genes or sequence variants that enable IIL tolerance. The growth and fermentation phenotypes of numerous wild and domesticated *S. cerevisiae* strains have been examined across a wide range of media conditions (Fay and Benavides 2005; Liti *et al.* 2009; Schacherer *et al.* 2009; Strope *et al.* 2015), including media that contained various inhibitory compounds generated from biomass pretreatment (Parreiras *et al.* 2014; Sato *et al.* 2014; Wohlbach *et al.* 2014; Kong *et al.* 2018). Individual strains exhibited a wide range of growth tolerances, indicating that some natural isolates contain genetic differences that are protective against toxins present in hydrolyzed plant biomass. Here, we combined phenotypic and genotypic analyses with functional screening to identify the MFS transporter *SGE*1 and an uncharacterized open reading frame (ORF) *YDR090C* with important roles in IIL tolerance. Our results uncovered the impact of natural genetic variation in IIL tolerance and identified an *SGE1* allele that offers a clear technological application for biofuel production.

Materials and Methods

Media

Standard yeast laboratory media were prepared as described elsewhere (Sherman 2002), with modifications. YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) and synthetic complete (SC) media were adjusted to pH 5.0 with HCl. For experiments described in Supplemental Material, Figure S4, the pH was adjusted to the indicated values with HCl or NaOH accordingly. Cationic compounds were purchased from the following vendors: $[C_2C_1im]Cl$ (catalog #272841, Sigma Aldrich; or catalog #AC354080250, Fisher Scientific, Pittsburgh, PA), $[C_4C_1im]Cl$ (catalog #94128; Sigma Aldrich), $[C_2C_1im][OAc]$ (catalog #689483; Sigma Aldrich), and Crystal Violet (CV) (catalog #NC9002731; Fisher Scientific). Cationic compounds were added directly to YPD or SC media and sterilized by passing through 2 μm filters. The following concentrations were used to select for plasmid and PCR product transformations in yeast and *E. coli*: 200 μg/ml Geneticin (catalog #10131027; Life Technologies), 100 μg/ml nourseothricin sulfate (catalog #RC-187; G-Biosciences), 200 μg/ml hygromycin B (catalog #10687010; GIBCO, Grand Island, NY), 200 μg/ml Zeocin (catalog #R25001; GIBCO), and 100 μg/ml carbenicillin (catalog #00049; Chem-Impex).

Yeast strain construction

Genotypes and sources of *S. cerevisiae* strains used in this study are described in File S1 and Table S2. Deletion mutant strains from the Yeast Knockout (YKO) Collection (Winzeler *et al.* 1999) were obtained from Open Biosystems/Dharmacon. Deletion of *SGE1* and *ionic liquid tolerance 1* (*ILT1*) were performed by integration of PCR products generated from *LoxP-KanMX-LoxP* and *LoxP-hphMX-LoxP* templates (Guldener *et al.* 1996; Gueldener *et al.* 2002), primers containing 50–60 bp of homology flanking the *SGE1* or *ILT1* ORF, and Phusion DNA polymerase (New England Biolabs, Beverly, MA). For deletion of *ILT1* with *KanMX4*, strain 4025 from the YKO Collection was used as the PCR template. PCR

products were purified (PCR Purification Kit, QIAGEN, Valencia, CA) and transformed into the appropriate strains (Gietz and Schiestl 2007). Antibiotic selection markers flanked by *LoxP* sequences were excised by Cre recombinase as published (Guldener *et al.* 1996). Both GLBRCY412 and GLBRCY490 were generated in the homozygous diploid 378604X (hereafter named 378) strain background, thus requiring two gene deletions to create the complete null mutants. To generate GLBRCY412, one copy of *ILT1* was deleted by replacement with the *bleMX4* selection marker, followed by a subsequent replacement of the second *ILT1* copy with *KanMX*. Construction of GLBRCY490 was conducted by deleting the first copy of *SGE1* with *LoxP-KanMX-LoxP* and the second copy with *LoxP-hphMX-LoxP*, followed by Cre-mediated excision of both *KanMX* and *hphMX* selection markers. *SGE1* and *ILT1* deletions were confirmed by Sanger sequencing (University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility) of purified PCR products generated from purified genomic DNA (gDNA) (Epicentre MasterPure Yeast DNA Purification kit) and primers that annealed outside of the homologous sequences were used for gene deletion. In-frame, genomic insertion of MYC or green fluorescence protein (GFP) (S65T) at the 3′ ends of *SGE1* or *ILT1* were performed by transformation of a PCR product generated from the pFA6a-13Myc-KanMX6 or pFA6a-GFP(S65T)-KanMX (Bähler *et al.* 1998) plasmid templates and the following primer pairs:

For *SGE1* fusions: SGE1MycFOR, CTTTGGAATATTCACTTC GAGTAAGAAAACAACAATATCAGCCAAAAAGCAACAA cggatccccgggttaattaa;
SGE1MycREV, GTACTGTCTAGTTTTATCGAACTACGATAAG TTAATTTATACGTTGGAAAATTGT gaattcgagctcgtttaaac.
For *ILT1* fusions: YDR090MYCfor, TGTCCATGGAGTTGT GGTTAGAACAGATCCTGATCGTTATTCGAGGCTAAGTGTG cggatccccgggttaattaa;
YDR090MYCrev,
AAGCGTGCTATCAAAAAGAGATGAAAACGTGCTAACTAAAA AGGACTCAGATTCG gaattcgagctcgtttaaac.

The uppercase nucleotides correspond to the sequences used for homologous recombination at 3′ ends of *SGE1* and *ILT1*. The lowercase nucleotides correspond to the annealing sequences for the pFA6a plasmids. Sanger sequencing of PCR products confirmed proper construction of all *MYC*- and GFP-tagged strains. Strains are available upon request.

Plasmid construction

E. coli strains E. cloni (Lucigen), DH10B (New England Biolabs), and EPI300 (Epicentre) were used for bacterial transformation, plasmid amplification, and assembly. Yeast genes were amplified by PCR of gDNA or fosmid DNA and primer pairs that annealed 1137 bp 5′ and 180 bp 3′ of the *SGE1* ORF or 885 bp 5′ and 96 bp 3′ of the *ILT1* ORF. *SGE1* and *ILT1* PCR products were cloned into pRS416 and pRS415 plasmids (Christianson *et al.* 1992), respectively, by sequence- and ligation-independent cloning (SLIC) (Li and Elledge 2007). Gene splicing by overlap extension (SOE) (Horton 1995) and SLIC cloning were used to generate mutant $SGE1^{PLL}$ plasmids. For experiments in YPD medium, *SGE1* and *ILT1* plasmids containing *KanMX* or *hphMX* antibiotic selection markers in place of *URA3* or *TRP1* auxotrophic markers, respectively, were used. Additionally, empty vector controls (lacking *SGE1* or *ILT1*) with *KanMX* and *hphMX* selection markers were also generated. To generate doxycycline-inducible *SGE1* expression plasmids, the *SGE1* ORF was amplified by PCR with primers containing 60 bp of flanking sequence that were homologous to the *CYC1* minimal promoter and 6× glycine linker/16× MYC tag in pBM5155 (Alexander *et al.* 2016). Purified PCR product was then cotransformed with *Not*I-digested pBM5155 into BY4741 (BY) yeast for gapped plasmid repair (Muhlrad *et al.* 1992). Plasmids were rescued from nourseothricin-resistant yeast colonies as described elsewhere (Mülleder *et al.* 2016) with modifications: cells were resuspended in 200 μl 1 M sorbitol with 20 units of zymolyase (Zymo Research) and incubated at 37° for 1 hr. Zymolyase-treated cells were then centrifuged at 3000 relative centrifugal force for 3 min, supernatant was aspirated, and then they were resuspended in P1 buffer for glass bead lysis. Rescued plasmids were then transformed into *E. coli*, miniprepped (QIAGEN), and fully sequenced to confirm proper in-frame insertion of *SGE1* into the pBM5155 plasmid. Plasmids are available upon request.

CRISPR/Cas9-mediated genome editing

CRISPR/Cas9 editing was performed by modification of plasmids published elsewhere (Kuang *et al.* 2018). In brief, a protospacer adjacent motif single guide RNA (sgRNA) sequence (TTTCATTTTCTGTCATTATC) that targeted adjacent to the *SGE1 SLS* site along with an HDV ribozyme were cloned between the SNR52 promoter and terminator. This sgRNA expression cassette was then amplified by PCR and cloned into the *Not*I site of the pXIPHOS vector (accession MG897154; GenBank), which contains a codon-optimized Cas9 gene driven by the constitutive *RNR2* promoter and the *NatMX* selection marker by gapped plasmid repair. *SGE1* repair templates were generated by PCR amplification of 378 $sge1^{SLS}$, BY $SGE1^{PLL}$ plasmid, or BY $SGE1^{PLS}$ and BY $SGE1^{SLL}$ sequences generated by gene SOE. Purified repair templates were cotransformed in 20-fold molar excess with the pXIPHOS-*SGE1* sgRNA plasmid into the BY or 378 yeast strain. Single nourseothricin-resistant colonies were restreaked two times on YPD agar plates. *SGE1* was amplified by PCR of gDNA from single colonies, purified, and sequenced to confirm the *SGE1* allele swap. For the 378 $sge1^{SLS}/sge1^{SLS}$ homozygous mutant strain, Sanger sequencing only identified the presence of the *SLS* allele. Confirmed strains were also tested for loss of nourseothricin resistance, which indicates that the strains also lost the pXIPHOS-*SGE1* sgRNA plasmid.

Fosmid library construction and screening

A yeast fosmid library vector was prepared by adding the yeast replicative origin and *URA3* gene from the pRS416 yeast shuttle vector into the pCC1FOS fosmid vector (Epicentre Biotechnologies). Yeast maintenance regions were amplified from pRS416 by PCR using the following primer pair: GACGGGCGGCCACCTGGGTCCTTTTCATCA and GACGGGC GGCTCTGTGCGGTATTTCACACC. The resulting ~1.9-kb fragment and the pCC1FOS fosmid vector were digested with *Kas*I restriction enzyme and ligated together using T4 ligase (Thermo Fisher). Plasmids was transformed into *E. coli* and sequence verified. The resulting plasmid, pDH219, was further digested with *Pml*I to release a small unnecessary fragment bordered by *Pml*I sites. The backbone was religated together, transformed into *E. coli* EPI300 cells, and verified by Sanger sequencing. The resulting plasmid (pDH241) was digested with *Pml*I to yield a blunt linear vector for fosmid library construction.

S. cerevisiae 378 gDNA was isolated as described elsewhere (Hoffman and Winston 1987). The metagenomic library was constructed following the manufacturer's protocol for the pCC1FOS fosmid vector (Epicentre), with the modification that *Pml*I-linearized pDH241 was substituted for the pCC1FOS vector. *E. coli* transductions were plated on LB supplemented with 12.5 μg/ml chloramphenicol (henceforth cm 12.5), resulting in ~3600 *E. coli* colonies. *E. coli* cells were swabbed up, diluted into LB cm 12.5 with CopyControl Fosmid Autoinduction Solution (Epicentre), grown, and purified by plasmid miniprepping (QIAGEN).

BY yeast-competent cells were prepared and transformed (Gietz and Schiestl 2007) with the fosmid library. Yeast transformants were selected on SC (pH 5) agar plates lacking uracil and supplemented with 0 or 125 mM [C_2C_1im]Cl. A total of 19 colonies that grew to large size on 125 mM [C_2C_1im]Cl were restreaked to confirm IIL tolerance. Fosmids were harvested from confirmed transformant yeast cells and transformed into electrocompetent *E. coli* EPI300 cells. Fosmid preparations were recovered from *E. coli* and sequenced from the fosmid backbone into the chromosome inserts, which were subsequently mapped to the S288c yeast genome sequence (*Saccharomyces* Genome Database).

Yeast growth assays

To assess IIL tolerance of wild and domesticated yeast isolates, total cell growth for each strain was determined as previously described (Parreiras *et al.* 2014) with modifications. Individual strains were cultured aerobically at 30° in 96-well plates containing YPD (pH 5), YPD (pH 5) with 250 mM [C_2C_1im]Cl and 250 mM [C_2C_1im][OAc]. Total cell growth for each strain was determined by subtracting the OD_{600} measurement after 24 hr of growth from the initial OD_{600} value. Relative total cell growth was calculated by dividing the total cell growth in YPD (pH 5) with 250 mM [C_2C_1im]Cl or [C_2C_1im][OAc] by the total cell growth in YPD (pH 5) alone. For BY strains transformed with pRS415-*ILT1* or pRS416-*SGE1* plasmids (Figure 1, B and C), triplicate cultures of *S. cerevisiae* were inoculated and grown to stationary phase in SC − leucine or SC − uracil medium, respectively. Cells were diluted to an OD_{600} of 0.05 in the appropriate SC medium containing 0–250 mM [C_2C_1im]Cl or 125 mM [C_2C_1im][OAc]. Cell growth was monitored by OD_{600} measurements every 20 min at 30° with shaking using an Infinite F-200, F-200 PRO, or Safire multimode reader (Tecan).

Growth assays in 24-well plates were similarly carried out as described for 96-well plates with modifications. Yeast strains were cultured overnight at 30° in tubes containing 5 ml of YPD medium and any appropriate antibiotic for plasmid selection. The following morning, cultures were diluted in fresh medium and regrown to log phase (OD_{600} = 0.8, as measured in a 1-cm path length cuvette with a Beckman Coulter spectrophotometer). Cells were centrifuged, washed with sterile water, and inoculated into 24-well plates at an OD_{600} of 0.1 in 1.5 ml YPD (pH 5) media containing serial dilutions of [C_2C_1im]Cl, [C_2C_1im][OAc], [C_4C_1im]Cl, or CV and any appropriate antibiotics. Cell densities were determined from OD_{600} measurements taken every 10–40 min for 24–70 hr with a Tecan Infinite M200Pro multimode reader. Relative total cell growth was determined by dividing the total cell growth after 18–24 hr for a strain in media containing specified concentrations of CV, [C_4C_1im]Cl, or [C_2C_1im]Cl by the cell growth for the same strain in media lacking those compounds in the same time frame. For pH-dependency experiments, relative total cell growth was determined by dividing the total cell growth after 24 hr for each strain in 125 mM [C_2C_1im]Cl by the total cell growth for the same strain in media lacking [C_2C_1im]Cl at a specific pH. For experiments using tetracycline-inducible *SGE1-MYC*, BY *sge1*Δ yeast transformed with empty pBM5155, pBM5155-*SGE1*^{SLS}, or pBM5155-*SGE1*^{PLL} were cultured in 1.5 ml YPD (pH 5), 0 or 125 mM [C_2C_1im]Cl, 100 μg/ml nourseothricin, and 0–625 ng/ml doxycycline hydrochloride (BP26535; Fisher Scientific). Relative total cell growth was determined by dividing the total cell growth after 24 hr for each strain by the total cell growth for yeast with empty pBM515 in 0 ng/ml doxycycline hydrochloride.

Aerobic and anaerobic growth experiments were performed with tubes and flasks, respectively, as previously described (Parreiras *et al.* 2014) with some modifications. Cells grown to log phase in 10–30 ml YPD (pH 5) medium containing the appropriate antibiotic for plasmid selection were washed with sterile water and inoculated to a concentration of OD_{600} = 0.1 in YPD (pH 5) medium containing 0–250 mM [C_2C_1im]Cl and the appropriate antibiotic for plasmid selection. Cell densities were determined by OD_{600} spectrophotometer (Beckman Coulter) measurements as described above. Extracellular glucose and ethanol concentrations were determined by high performance liquid chromatography and refractive index detection (Keating *et al.* 2014).

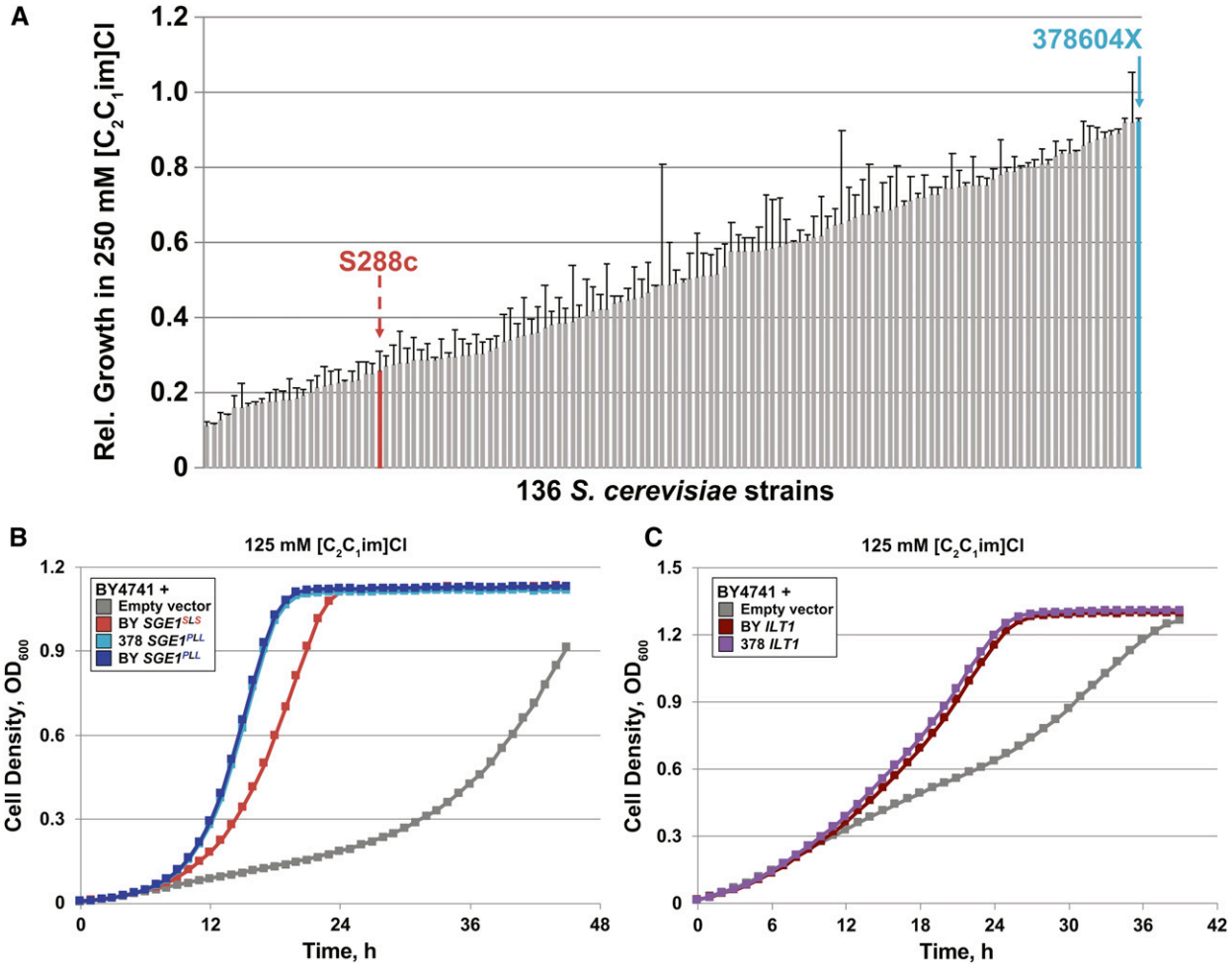

Figure 1 *SGE1* and *YDR090C/ILT1* function in $[C_2C_1im]^+$ tolerance. (A) Relative total cell densities for 136 wild or domesticated *S. cerevisiae* strains cultured aerobically in 96-well plates containing YPD (pH 5) + 250 mM $[C_2C_1im]Cl$ medium, relative to total cell growth in YPD (pH 5) alone. Red and blue ↓'s indicate the locations of the S288c laboratory and 378 wild strains, respectively. Average values and SEM were determined from independent biological triplicates. (B and C) Representative aerobic growth of BY cells transformed with the indicated low-copy plasmids and cultured in SC (pH 5) medium + 125 mM $[C_2C_1im]Cl$ for 40–46 hr. Vector-specific growth differences were observed in (B and C). Rel., relative.

SGE1 sequence analysis

Single nucleotide polymorphisms (SNPs) for the *SGE1* gene were extracted from a whole genome variant data set for the strains phenotyped in this study (Sardi *et al.* 2018). Briefly, whole genome Illumina sequences for *S. cerevisiae* strains from publicly available sequencing projects (Skelly *et al.* 2013; Bergström *et al.* 2014; Hose *et al.* 2015; Strope *et al.* 2015) were mapped to reference genome S288c [NC_001133 version 64 (Engel *et al.* 2014)] using bwa-mem. Variants were identified using the GATK pipeline for Unified Genotyper (McKenna *et al.* 2010) using default parameters with a -mbq of 25 to reduce false positives. Annotation of variants was performed with SNPEff (Cingolani *et al.* 2012).

Additional genome sequences from *S. cerevisiae* and non-*S. cerevisiae* yeast strains were obtained as described in previous publications (Scannell *et al.* 2011; Liti *et al.* 2013; Almeida *et al.* 2014; Bing *et al.* 2014; Gayevskiy and Goddard 2016; Gonçalves *et al.* 2016; Peris *et al.* 2016; Yue *et al.* 2017). *SGE1* sequences from additional *S. cerevisiae* and non-*S. cerevisiae* strains (File S3) were retrieved by using two approaches: (1) BLASTing the S288c *SGE1* gene sequence to a local database (Altschul *et al.* 1990), and/or (2) downloading the Illumina reads and mapping them to the reference *SGE1* nucleotide sequence to extract and assemble the *SGE1* alleles using the HybPiper wrapper (Johnson *et al.* 2016). Sequence alignment and amino acid comparisons were performed in Geneious version 6.1.6 (Kearse *et al.* 2012).

Western blotting

Protein from whole cell lysates were prepared as previously described (Zhang *et al.* 2011) with modifications. Yeast cells were grown in 24-well plates as described above. After 24 hr of growth, 1.5 ml of cell culture was transferred to tubes, centrifuged, and the supernatant was aspirated. Cell pellets were washed with sterile water and then resuspended in

67 μl of 2 M lithium acetate per OD_{600} of cells. LiAc-treated cells were then centrifuged, the supernatant was aspirated, and the cells were resuspended in 67 μl/OD_{600} of cells in 0.4 M NaOH and placed on ice for 5 min. Treated cells were then centrifuged, the supernatant was aspirated, and protein was extracted by lysing cells in 1× Laemmli sample buffer with β-mercaptoethanol at 100° for 5 min.

Protein electrophoresis and Western blotting were performed using the Mini-Protean system according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Total protein (30 mg) from each cell sample was loaded on the 4–15% acrylamide gels, along with 5 μl chemiluminescent protein standards (Precision Plus Protein WesternC Standards; Bio-Rad). Blots were cut into two separate pieces along the 50-kD protein standard, with the top half used to for detection of Sge1-Myc, and the bottom half used for actin. Sge1-Myc and actin proteins were detected by incubation with anti-Myc (9E10; Sigma Aldrich) or anti-actin (mAbGEa; Thermo Fisher Scientific) mouse monoclonal primary antibodies at dilutions of 1:1000 in TBST (Tris buffered saline + 0.2% Tween-20; Bio-Rad) and 5% nonfat dried milk (TBSTM) or 1:5000 in TBST with 3% bovine serum albumin (TBSTB), respectively. After primary incubation, washed blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Bio-Rad) and StrepTactin-HRP conjugate (Bio-Rad) in corresponding TBSTM or TBSTB buffer. Sge1-Myc and actin protein bands were visualized by enhanced chemiluminescence (Clarity Western ECL; Bio-Rad) and quantified by densitometry (Quantity One Software; Bio-Rad).

Quantification of chemiluminescent signals for Sge1-Myc, actin, and an ~130-kD nonspecific (NS) band was performed by densitometry (Quantity One Software; Bio-Rad). Volume intensities for each were measured using identical areas for each protein across replicate experiments. Actin and Sge1-Myc/NS band signals were captured from 5 or 30–60 sec exposures, respectively. Each Sge1-Myc chemiluminescent signal was normalized relative to the NS or actin bands from the same sample for three to five biological replicates. For correlations, Sge1-Myc protein levels normalized to NS band intensities were paired to relative total cell growth (see above) from the same sample. Paired values were analyzed by Spearman's rank correlation in Spotfire (TIBCO).

Fluorescence microscopy

Yeast strains were cultured to exponential growth phase in YPD at 30°. Cells were harvested by centrifugation and resuspended at a ratio of 1:5 in fresh YPD media. A total of 3 μl of cells were then spotted onto a poly-L-lysine-coated glass slide and covered with an 8 × 8 mm coverslip. GFP fusions were visualized at 100× magnification using fluorescence (with an EVOS GFP LED cube) or transmitted light sources, an EVOS FL Auto 2 microscope (Invitrogen, Carlsbad, CA), and EVOS FL Auto 2 Imaging System (Invitrogen). Contrast and brightness for whole images were adjusted uniformly using Adobe Photoshop in accordance with the journal's image-manipulation policy.

Data availability

All yeast strains and plasmids used in this study are available upon request. Table S2 contains a list of the strains described in this study. Data necessary to confirm findings of this article are present within the article text and figures as well as supplemental files, figures, and tables. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6731201.

Results

Environmental isolates of S. cerevisiae display a range of growth abilities in IIL media

To identify strains with innate tolerance to IILs, we measured the growth of 136 wild and domesticated *S. cerevisiae* strains cultured in media containing 250 mM $[C_2C_1im]Cl$ (Figure 1A and File S1), $[C_2C_1im][OAc]$ (Figure S1 and File S1), or lacking IILs. Strains displayed a wide range of maximum cell growth in IIL-containing media relative to the control medium lacking IILs, with more strains growing to lower cell densities in $[C_2C_1im][OAc]$ than $[C_2C_1im]Cl$ (File S1). Weak correlation ($R^2 = 0.27$) in relative cell growth between the different IILs indicated that the Cl^- and $[OAc]^-$ anions synergize differently with $[C_2C_1im]^+$ to inhibit yeast growth, consistent with an earlier report (Ouellet *et al.* 2011). The strain with highest relative growth in both $[C_2C_1im]Cl$ and $[C_2C_1im][OAc]$ was 378, a clinical isolate from Newcastle, United Kingdom. In contrast, the common laboratory strain, S288c, achieved significantly lower relative cell growth in $[C_2C_1im]Cl$ and $[C_2C_1im][OAc]$, suggesting that the 378 strain contains different genetic sequences that permit greater cell growth in the presence of $[C_2C_1im]^+$.

SGE1 and YDR090C/ILT1 function in ionic liquid tolerance

Reasoning that genetic factors in the 378 strain are responsible for its IIL tolerance, we used a selection scheme adapted from our previous work with bacteria (Ruegg *et al.* 2014). Specifically, we generated a fosmid library containing large (~30–40 kb) fragments of gDNA from this IIL-tolerant strain and transformed the library into an IIL-sensitive laboratory derivative of S288c, BY (Figure S2). We then selected transformants that grew normally on solid medium containing 125 mM $[C_2C_1im]Cl$. Fosmid inserts from selected transformants were recovered and partially sequenced onto the *S. cerevisiae* genome. We identified nine distinct inserts that clustered in two genome regions. Five of these nine DNA segments coincided in a core 14-kb region of chromosome XVI that contained four genes: *HPA2*, encoding a histone acetyl transferase (Angus-Hill *et al.* 1999); *OPT2*, an oligopeptide transporter (Wiles *et al.* 2006) that may also function in drug detoxification (Aouida *et al.* 2009); *SKI3*, involved in exosome-mediated messenger RNA decay (Anderson and Parker 1998); and *SGE1*, an MFS multidrug efflux pump that exports toxic cationic dyes out of the cytoplasm (Amakasu

et al. 1993; Ehrenhofer-Murray *et al.* 1994, 1998; Jacquot *et al.* 1997). Because we previously found that a bacterial MFS efflux pump, EilA, functions in $[C_2C_1im]^+$ tolerance (Ruegg *et al.* 2014), we were immediately drawn to investigate *SGE1*.

The remaining four fosmid inserts coincided in a core 18-kb region of chromosome IV containing nine genes: *STN1*, *RRP8*, *TVP23*, *AFR1*, *SSS1*, *RRP1*, *SLU7*, *YDR089W*, and *YDR090C* (Figure S2). Unlike *SGE1*, none of these genes had immediately obvious functions predicted to be relevant to IIL tolerance. Considering that deletions of IIL-tolerance genes would sensitize yeast to $[C_2C_1im]Cl$, we examined five available strains from the BY deletion library (Winzeler *et al.* 1999) containing individual deletions in genes within this region of chromosome IV; only *ydr090c*Δ displayed reduced growth in medium with a subtoxic 31 mM $[C_2C_1im]Cl$ concentration compared to medium without IIL (Table S2). Based on these results, we propose *ILT1* as the standard name for the *YDR090C* ORF. Protein structure and homology analyses (Altschul *et al.* 1990; Claros and von Heijne 1994) indicated that *ILT1* encodes a membrane protein with seven transmembrane helices and has a putative PQ-loop motif found in Ypq1, Ypq2, and Rtc2/Ypq3, which are putative vacuolar membrane transporters of cationic amino acids (Jézégou *et al.* 2012).

Next, we investigated whether specific expression of *SGE1* or *ILT1* could explain the tolerance phenotypes of the fosmid-carrying strains. The individual promoters, ORFs, and terminators for *SGE1* and *ILT1* from the tolerant 378 and sensitive BY strains were cloned into a low-copy plasmid and expressed in the BY strain (Figure 1, B and C, and Figure S3). In media containing 125 mM $[C_2C_1im]Cl$, BY transformants expressing the *SGE1* and *ILT1* ORFs from the tolerant 378 strain grew faster than BY containing the same plasmid but lacking *SGE1* (empty vector), providing evidence that these specific genes contributed to IIL-tolerance effects from the fosmid constructs. Expression of BY *SGE1* and *ILT1* alleles also increased growth in 125 mM $[C_2C_1im]Cl$ over the empty vector control, indicating that additional copies of the identical BY *SGE1* and *ILT1* alleles granted IIL protection. However, expression of the 378 *SGE1* gene conferred faster growth than BY *SGE1* in $[C_2C_1im]Cl$-containing media, suggesting that differences in the BY and 378 *SGE1* sequences affect IIL tolerance. In contrast, there were no growth differences between cells expressing *ILT1* from BY or 378.

The *SGE1* sequences from the sensitive BY and tolerant 378 strains were examined for coding differences that could explain the IIL-tolerance phenotypes. Two nonsynonymous SNPs corresponding to amino acid positions Ser 282 and Ser 284 (hereafter denoted as the "*SLS*" sequence), which flank Leu 283, in BY were found to encode Pro 282 and Leu 284 (the "*PLL*" sequence) in 378. Transmembrane prediction models place these amino acid residues in the fifth cytoplasmic loop between the eighth and ninth transmembrane helices of Sge1p. To exclude any effects from promoter and terminator sequence differences or silent mutations between the BY and 378 strains, we generated site-directed S282P and S284L mutations in our low copy plasmid-borne *SGE1* from the sensitive BY strain (BY $SGE1^{PLL}$). Expression of the BY $SGE1^{PLL}$ mutant in the BY strain enabled an equivalent growth rate as 378 $SGE1^{PLL}$ (Figure 1B), indicating that the Pro 282 and Leu 284 amino acid differences were specifically responsible for the increased $[C_2C_1im]Cl$ tolerance. There were no differences in the *ILT1* coding sequence between the BY and 378 strains, explaining why no growth differences were seen for strains expressing BY or 378 *ILT1* (Figure 1C).

We next examined the requirements for *SGE1* and *ILT1* in IIL tolerance by deleting *SGE1* or *ILT1* genes in the haploid BY and homozygous diploid 378 strains. Both *sge1*Δ and *ilt1*Δ deletion mutants were transformed with an empty vector or plasmids containing the various *SGE1* and *ILT1* sequences and examined for growth in media containing $[C_2C_1im]Cl$ (Figure 2). The BY *sge1*Δ and 378 *sge1*Δ/*sge1*Δ null mutant strains grew to significantly lower cell densities in 125 mM $[C_2C_1im]Cl$ than strains expressing their corresponding wild-type *SGE1* sequences (Figure 2, A and B). Expression of the BY $SGE1^{PLL}$ or 378 $SGE1^{PLL}$ alleles resulted in significantly faster growth and higher cell densities than the BY $SGE1^{SLS}$ allele in both BY *sge1*Δ and 378 *sge1*Δ/*sge1*Δ mutant strains. The BY *ilt1*Δ strain transformed with the empty control vector grew significantly slower than the deletion strain complemented with the *ILT1* sequence (Figure 2C). In contrast, no significant differences in cell growth were seen between 378 *ilt1*Δ/*ilt1*Δ mutants transformed with empty or *ILT1* plasmids (Figure 2D), suggesting that *ILT1* has strain-specific functions. Together, these results indicate that both *SGE1* and *ILT1* function in $[C_2C_1im]^+$ tolerance, and that natural sequence differences in *SGE1* alleles can influence tolerance levels.

Since some biofuels, such as ethanol, are industrially produced under anaerobic, fermentative conditions, we assessed whether *SGE1* and *ILT1* were important for ionic liquid tolerance in the absence of oxygen. Consistent with the aerobic results, both BY *sge1*Δ and *ilt1*Δ strains transformed with empty plasmids grew more slowly and fermented less glucose to ethanol anaerobically than strains expressing their native *SGE1* or *ILT1* sequences in media containing 250 mM $[C_2C_1im]Cl$ (Figure S4). BY *sge1*Δ mutants expressing the *PLL* allele grew to higher cell densities and fermented more glucose into ethanol in the presence of 250 mM $[C_2C_1im]Cl$ than the BY strain expressing the native $SGE1^{SLS}$ allele (Figure S4, A–C). Thus, swapping the $SGE1^{PLL}$ allele into the BY genome enabled greater anaerobic bioethanol production in the presence of $[C_2C_1im]Cl$.

SGE1 and ILT1 confer resistance to multiple cationic compounds

Previously, *SGE1* was identified for its role in resistance to the toxic cationic dyes CV, 10-N-nonyl acridine orange, and ethidium bromide in other *S. cerevisiae* strain backgrounds (Ehrenhofer-Murray *et al.* 1994; Jacquot *et al.* 1997). We wanted to determine if *ILT1* functioned similarly in cationic

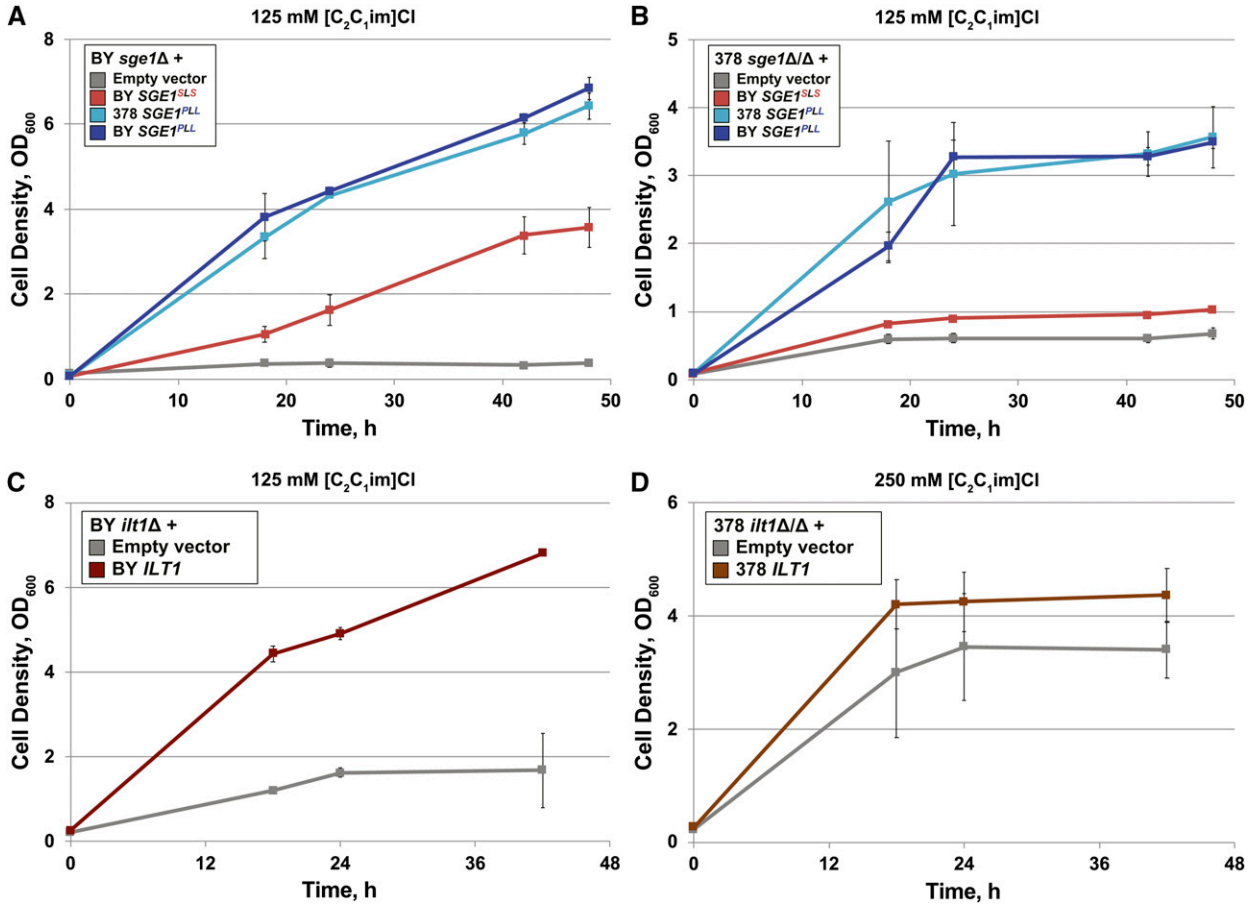

Figure 2 *SGE1* alleles from BY and 378 determine $[C_2C_1im]^+$-tolerance phenotypes. Haploid BY or diploid 378 strains harboring (A and B) *sge1*Δ or (C and D) *ilt1*Δ null mutations were transformed with plasmids containing the indicated *SGE1* or *ILT1* sequences. In (A and B), a plasmid containing site-directed mutations in the BY *SGE1* gene sequence (BY $SGE1^{PLL}$) was included. Transformed strains were then cultured aerobically in tubes containing 10 ml YPD (pH 5), 125 mM $[C_2C_1im]Cl$, and the appropriate antibiotic. Average cell densities (OD_{600}) ± SD are reported from three independent biological replicates.

dye resistance and whether the $SGE1^{PLL}$ allele conferred greater resistance to other cationic dyes than the $SGE1^{SLS}$ allele. Compared to the BY parent strain, we found that BY *ilt1*Δ mutants grew to lower cell densities in 10 and 20 μM CV (Figure 3A). In contrast, deletions of both *ILT1* copies in the 378 strain background, which retained two copies of the $SGE1^{PLL}$ allele, had insignificant effects on tolerance to CV (Figure S5A) and $[C_2C_1im]Cl$ (Figure S5B), consistent with results seen with plasmid-transformed strains (Figure 2D). Additionally, BY and 378 strains containing $SGE1^{PLL}$ grew to relatively higher cell densities than strains containing the $SGE1^{SLS}$ alleles in 10 or 20 μM CV (Figure 3B and Figure S5B) and, as expected, in 125 or 250 mM $[C_2C_1im]Cl$ (Figure S5D). As a further test of specificity, we also compared wild-type and mutant cell growth in media containing $[C_4C_1im]Cl$ (Figure 3, C and D), another IIL used in pretreatment of plant feedstocks (Binder and Raines 2010), to growth in media containing $[C_2C_1im]Cl$ (Figure 3, E and F). Similar to $[C_2C_1im]Cl$, deletion of *ILT1* significantly reduced the growth tolerance to $[C_4C_1im]Cl$, and expression of the BY $SGE1^{PLL}$ allele conferred greater resistance to $[C_4C_1im]Cl$ than the $SGE1^{SLS}$ allele. These results indicate that *ILT1* functions in tolerance to a range of cationic toxins in the BY strain background, while the $SGE1^{PLL}$ allele enables greater tolerance than the $SGE1^{SLS}$ allele across multiple cationic toxins in both BY and 378 strains.

The $Sge1^{PLL}$ H^+ antiporter maintains IIL tolerance across a wider extracellular pH range than $Sge1^{SLS}$ protein

MFS protein member Sge1 is a Dha2-like, 14-span transmembrane H^+ antiporter that couples the import of protons from the extracellular medium with the export of toxins out of the cytoplasm (Sá-Correia *et al.* 2009; Dos Santos *et al.* 2014). To test whether the efflux of IILs by the Sge1 variants is coupled with proton influx, we examined the interaction between extracellular pH and IIL tolerance for both the $SGE1^{SLS}$ and $SGE1^{PLL}$ alleles. Between pH 8 and 9, both the $SGE1^{SLS}$ and $SGE1^{PLL}$ strains failed to grow significantly in the presence of 125 mM $[C_2C_1im]Cl$ (Figure S6). From pH 5 to 7, the $SGE1^{PLL}$ strain grew to relatively higher cell densities than the $SGE1^{SLS}$ strain, whereas both strains grew to similar cell densities at pH 4. These observations indicated that the tolerant

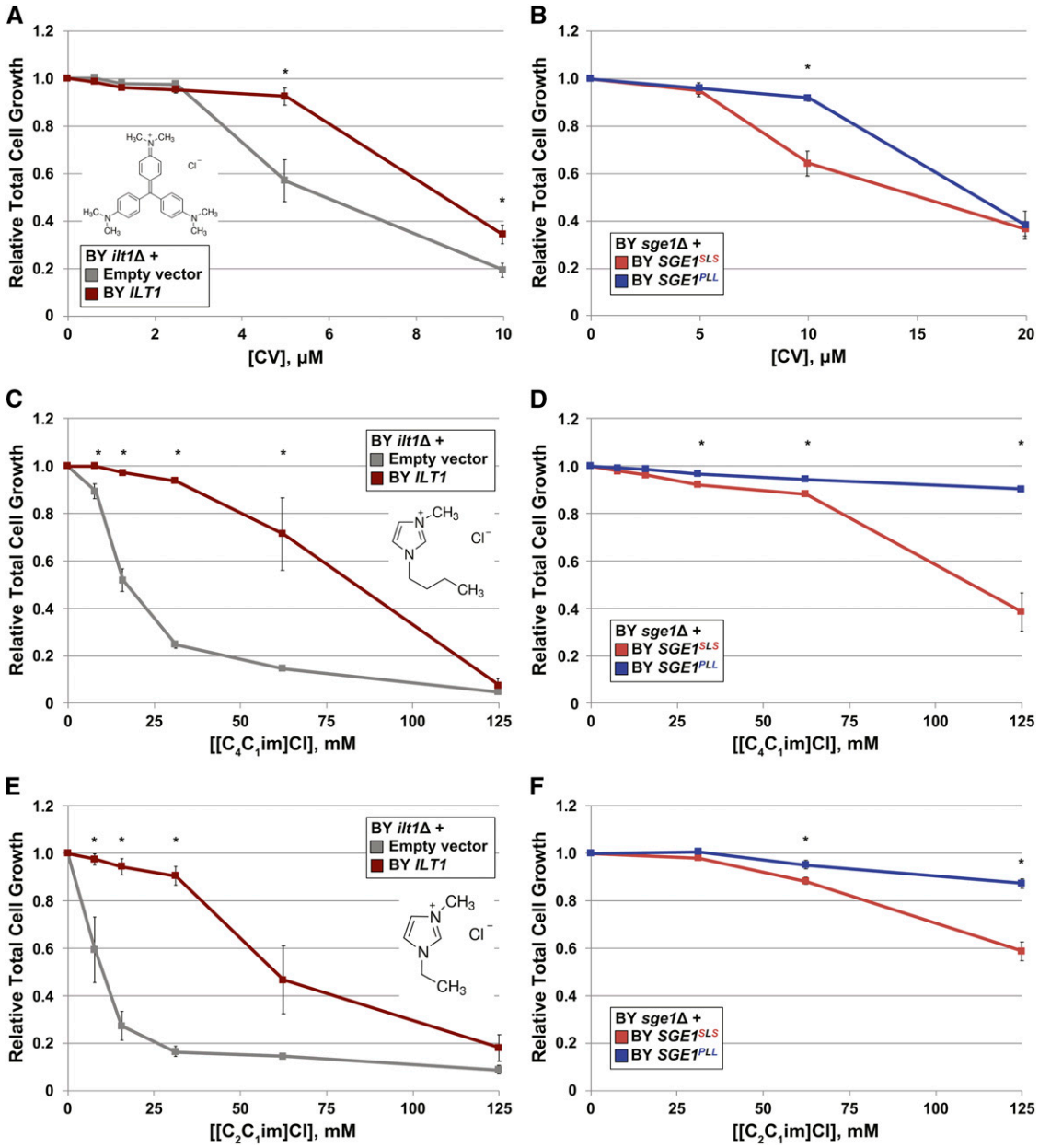

Figure 3 *SGE1* and *ILT1* function in resistance to multiple cationic inhibitors. The *sge1*Δ or *ilt1*Δ mutants were transformed with empty vector or a plasmid containing unmodified or mutant *SGE1* or *ILT1* from BY. Transformed strains were then cultured aerobically in YPD (pH 5) and the indicated concentrations of (A and B) CV, (C and D) $[C_4C_1im]Cl$, or (E and F) $[C_2C_1im]Cl$. Relative growth was determined by measuring the total cell growth after 18 hr of culturing in YPD (pH 5) containing the cationic compound normalized to total growth in YPD (pH 5) alone. Average relative total growth ± SD was plotted from three independent biological triplicates. Statistical significance determined by paired Student's *t*-test. * $P < 0.05$.

$SGE1^{PLL}$ allele enables greater IIL tolerance in a wider pH range than the sensitive $SGE1^{SLS}$ allele.

The SGE1^{PLL} allele is the ancestral sequence associated with IIL tolerance across natural S. cerevisiae strains

The discovery that two different alleles of *SGE1* conferred differential tolerance to $[C_2C_1im]^+$ prompted us to investigate whether the tolerance phenotypes of 136 haploid or homozygous diploid *S. cerevisiae* strains examined in Figure 1 correlated with the *SLS* and *PLL* genotypes. Using published genome sequences (Sardi *et al.* 2018) and targeted Sanger sequencing, we identified 37 strains containing the $SGE1^{SLS}$ allele, 16 with an $SGE1^{SLL}$ allele, 25 with $SGE1^{PLL}$, and 58 strains containing the $SGE1^{PLL}$ allele along with one or more additional polymorphisms (see "$SGE1^{PLL}$ + Additional *SGE1* SNPs" in Figure 4A, File S1, and File S2). No sequenced strains from our collection contained an $SGE1^{PLS}$ allele. In media containing 250 mM $[C_2C_1im]Cl$ (Figure 4A), the relative growth for strains containing only the $SGE1^{PLL}$ allele (average relative growth = 0.709 ± 0.159) was significantly

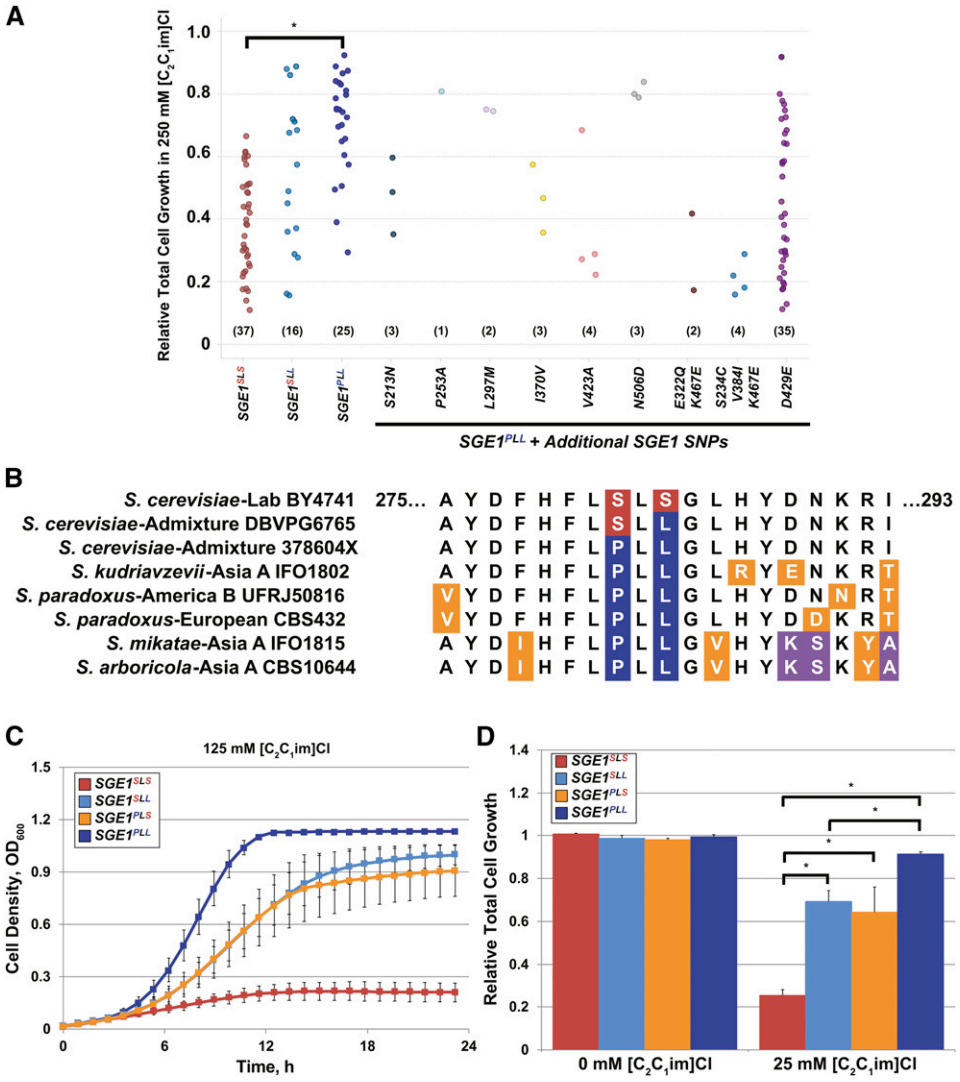

Figure 4 The *SGE1*^{PLL} allele confers greater tolerance to [C_2C_1im]Cl than the *SGE1*^{SLS} variant. (A) The individual *S. cerevisiae* strains were grouped based on their *SGE1* genotype and plotted according to their relative growth in 250 mM [C_2C_1im]Cl. For strains containing *SGE1*^{PLL} and additional *SGE1* SNPs, the amino acid changes in each strain are relative to S288c reference sequence. The numbers of strains for each genotype are listed in parentheses. Statistical significance of growth differences between strains containing the *SGE1*^{PLL} alone and *SGE1*^{SLS} alleles was determined by unpaired Student's *t*-test. * $P < 2e^{-10}$. (B) Sge1 protein sequences encompassing the region surrounding the Sge1^{SLS}/Sge1^{PLL} variants from different strains and species from the genus *Saccharomyces* were aligned to display the amino acid sequences at the corresponding SLS/PLL residues. For each species, their locations of isolation as well as common strain identifiers are listed. (C) Average ± SEM aerobic cell densities of BY *SGE1*^{SLS} cells or the indicated *SGE1* mutations introduced into the genome by CRISPR/Cas9 from three biological replicates are shown. (D) Average total cell growth + SD was normalized relative to growth for BY *SGE1*^{SLS} strain after 10 hr of culturing and plotted with SD from three independent biological replicates. Statistical significance determined by paired Student's *t*-tests. * $P < 0.05$.

higher ($P < 0.05$) than strains containing the *SGE1*^{SLS} allele (average relative growth = 0.386 ± 0.155). We did not observe any statistically significant correlations between IIL tolerance and strains containing both the *SGE1*^{PLL} sequence and additional *SGE1* SNPs, although the small number of representatives limited our statistical power. These results indicate that yeast strains containing *SGE1*^{SLS}, *SGE1*^{SLL}, and *SGE1*^{PLL} alleles persist in the natural environment and influence tolerance to cationic toxins.

The identification of the *SGE1*^{SLL} allele along with the inability to detect the *SGE1*^{PLS} allele suggested that the *SGE1*^{SLS} or *SGE1*^{PLL} allele was derived from an ancestral sequence through two successive nucleotide changes. To ascertain the order in which the derived alleles emerged, we identified the ancestral sequence by aligning analogous Sge1 protein sequences from other species within the genus *Saccharomyces* (Scannell *et al.* 2011; Bing *et al.* 2014; Yue *et al.* 2017), which diverged from *S. cerevisiae* up to 20 MYA (Figure 4B). Interestingly, *SGE1* from five different species of *Saccharomyces* contained the *SGE1*^{PLL} sequence, whereas the *SGE1*^{SLS} allele was not identified in any other sequenced non-*S. cerevisiae* species. We also found that the genome sequences of multiple *S. eubayanus*, *S. uvarum*, *S. paradoxus*, and even *S. cerevisiae* strains lacked the *SGE1* gene, and two strains of *S. cerevisiae* also appeared to contain premature stop codons (File S3). This suggests that *SGE1*^{PLL} was the tolerant ancestral allele and that the sensitive *SGE1*^{SLS} allele was derived from an initial P282S mutation followed by the secondary L284S mutation.

To understand the emergence of the derived *SGE1*^{SLS}-sensitive allele, we determined the functional effects that each individual sequence variant contributed to the IIL-tolerance phenotypes. First, CRISPR/Cas9 and homologous recombination were used to precisely edit the BY genome to generate strains containing specific *SGE1*^{SLL}, *SGE1*^{PLS}, or *SGE1*^{PLL} mutations. When cultured in 125 mM [C_2C_1im]Cl, BY strains harboring the single *SGE1*^{SLL} or *SGE1*^{PLS} mutations displayed growth rates and total cell growth phenotypes that were intermediate from the tolerant *SGE1*^{PLL} and sensitive *SGE1*^{SLS} strains (Figure 4, C and D). Furthermore, the phenotypes of

the *SGE1*^{SLL} and *SGE1*^{PLS} strains were statistically indistinguishable, indicating that both P282S and L284S changes were needed to derive the *SGE1*^{SLS} phenotype. These results further suggest the order of the P282S and the L284S mutations were interchangeable in the context of cationic toxin tolerance.

Sge1^{PLL} *protein is present at higher abundance than Sge1*^{SLS} *in cells*

One potential explanation for the increased tolerance to ionic liquids is that the Sge1^{PLL} protein is more abundant in cells than Sge1^{SLS} protein, resulting in greater export of toxic IILs from the cytoplasm. We investigated this possibility by comparing Myc-tagged Sge1^{SLS} and Sge1^{PLL} protein levels in the BY strain background by Western blotting. A Myc epitope tag was inserted in frame at the 3′ end of wild-type *SGE1*^{SLS}, mutant *SGE1*^{SLL}, *SGE1*^{PLS}, and *SGE1*^{PLL} sequences generated in the BY genome by CRISPR/Cas9. Strains containing the various Sge1-Myc fusions grew similarly to the untagged strains in the presence of 125 mM [C_2C_1im]Cl (Figure 4C and Figure S7), confirming that the fusion of Myc tags did not significantly alter Sge1p function. Equal amounts of total protein from strains expressing the variant Sge1-Myc protein fusions were electrophoresed and blotted with anti-Myc and anti-actin antibodies. The normalized Sge1^{PLL}-Myc protein signal was significantly greater than that of Sge1^{SLS}-Myc (Figure 5, A and B). Moreover, both Sge1^{PLL}-Myc and Sge1^{SLS}-Myc proteins appeared to separate into more than one band, suggesting that Sge1 protein may be post-translationally modified. These results indicate that the *SGE1*^{PLL} allele may confer greater tolerance to IILs and cationic dyes due to greater protein abundance and stability than the *SGE1*^{SLS} variant.

If *SGE1* SNPs determine IIL tolerance primarily by affecting Sge1p abundance, rather than by affecting transporter activities, we hypothesized that yeast strains expressing equivalent levels of Sge1^{SLS} and Sge1^{PLL} protein would display similar IIL tolerance. To test this model, we cultured the *sge1*Δ mutant strain transformed with a plasmid containing *SGE1-MYC* under the control of a doxycycline-inducible promoter in a range of doxycycline concentrations. Increasing doxycycline concentrations did not affect the growth of cells containing the empty control vector in the presence of 125 mM [C_2C_1im]Cl (Figure S8A) or the growth of cells containing *SGE1-MYC* in the absence of [C_2C_1im]Cl. In contrast, strains harboring the Tet-inducible *SGE1-MYC* plasmid displayed doxycycline-dependent increases in cell growth and Sge1^{SLS}-Myc (Figure S8B) and Sge1^{PLL}-Myc (Figure S8C) protein levels in the presence of [C_2C_1im]Cl. We then compared the normalized Sge1-Myc protein signal coupled to the relative total cell growth for each strain across multiple doses of doxycycline (Figure 5C). By Spearman rank analysis (not including data from $P_{Tet\text{-}On}$-Empty samples), we found that Sge1-Myc protein levels significantly correlated with relative total cell growth ($R^2 = 0.6$, $P < 3.2e^{-13}$), regardless of the Sge1 protein sequence. Together, these results support a

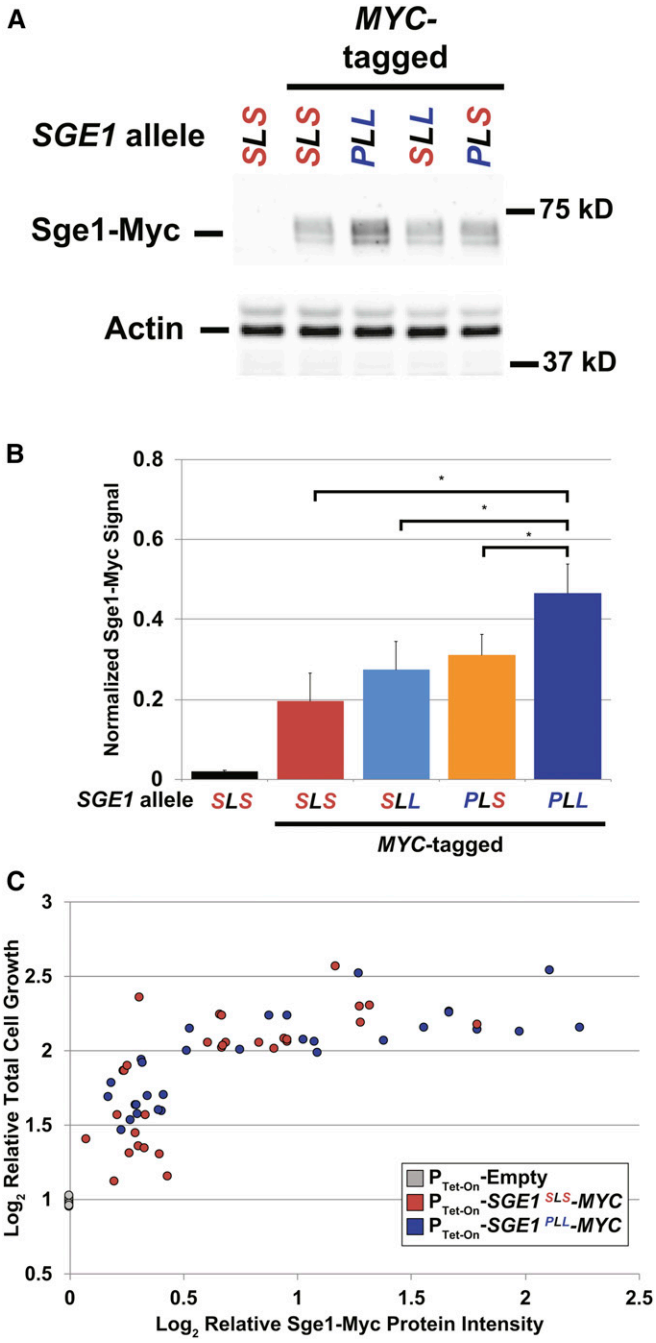

Figure 5 Increased Sge1 protein abundance correlates with increased tolerance to IILs. (A) A representative Western blot of different alleles of Myc-tagged Sge1 or actin protein from total cell lysates harvested from the indicated BY strains. Chemiluminescence signal for Sge1-Myc was normalized to actin signal from the same sample. (B) Average normalized Sge1-Myc signals + SEM were plotted from five independent biological replicates. Statistical significance was determined by paired Student's tests. * $P < 0.05$. (C) *sge1*Δ mutant cells containing a plasmid with *SGE1-MYC* alleles driven by a tetracycline-inducible promoter were cultured in YPD (pH 5) medium containing 0–625 ng/ml doxycycline and 125 mM [C_2C_1im]Cl for 24 hr. Total cell growth was recorded and cells were harvested for total cellular protein lysates after 24 hr. Sge1-Myc protein was quantified with anti-Myc antibodies and normalized for protein loading (see Figure S7 and *Materials and Methods*). Normalized Sge1-Myc signal from each strain condition was plotted against the total cell growth relative to *sge1*Δ cells transformed with $P_{Tet\text{-}On}$-Empty plasmid and grown in 0 ng/ml doxycycline and 125 mM [C_2C_1im]Cl.

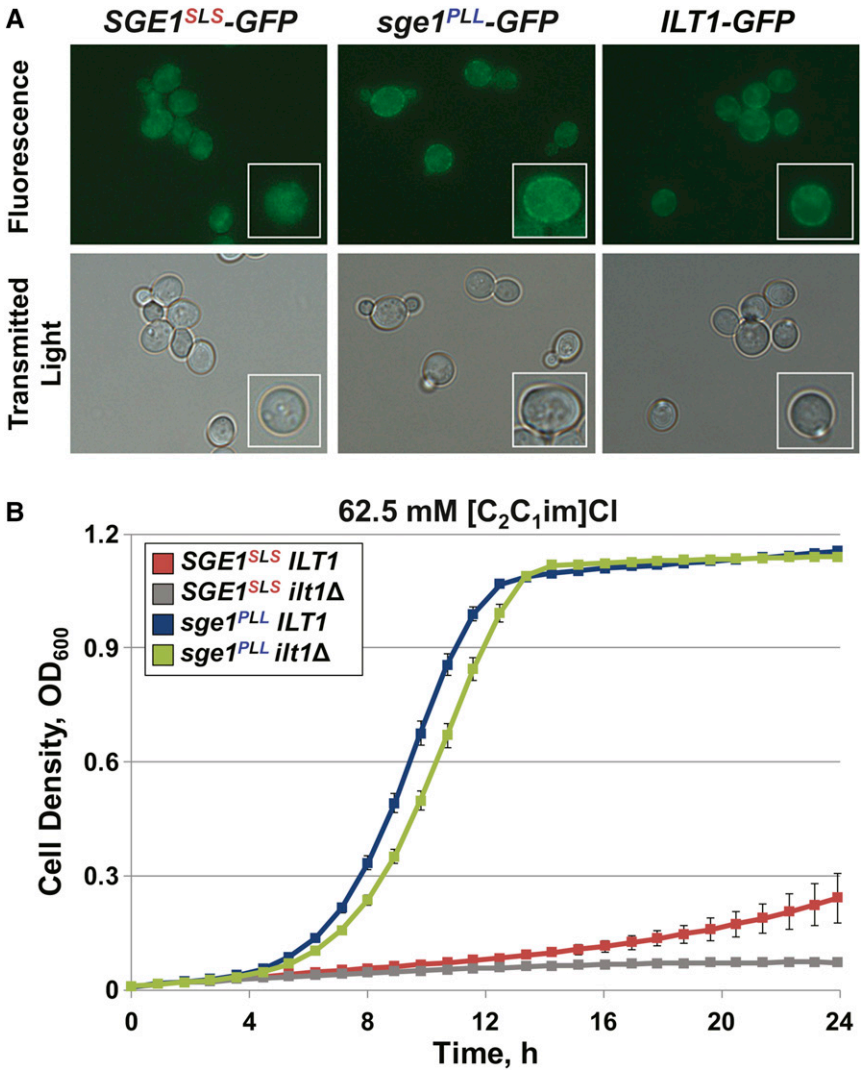

Figure 6 Plasma membrane-localized Sge1^{PLL} protein functions independently of *ILT1*. BY strains containing GFP fused to the indicated genes were cultured in YPD medium. (A) GFP fluorescence from representative cells was visualized with 100× magnification. Insets in the bottom right corners display a single representative cell with an additional 50% higher magnification. (B) *ILT1* was deleted from BY strains containing *SGE1^{SLS}* or *sge1^{PLL}* alleles. Resulting strains were cultured in YPD (pH 5) medium containing 62.5 mM [C_2C_1im]Cl. Cell growth is reported as average cell densities ± SEM from three independent biological replicates.

model in which the natural *SGE1^{SLS}* and *SGE1^{PLL}* alleles primarily affect Sge1 protein abundance, which in turn determines cellular IIL tolerance.

Sge1^{PLL} and Ilt1 proteins function independently at the plasma membrane

Sge1 protein has been proposed to function at the plasma membrane to extrude cationic toxins from the cytoplasm (Ehrenhofer-Murray *et al.* 1998). Given this, we predicted that increased abundance of Sge1^{PLL} protein could also result in greater localization to the plasma membrane than the Sge1^{SLS} variant protein. We tested this prediction by inserting GFP in frame at the carboxyl termini of chromosomal *SGE1* and *ILT1*. Strains containing these gene fusions did not grow significantly differently from strains containing the untagged genes (Figure S8). By fluorescence microscopy, both Sge1^{PLL}-GFP and Ilt1-GFP fusion proteins localized to the plasma membrane, whereas the Sge1^{SLS}-GFP fusion appeared to localize weakly to the plasma membrane and to internal organelles (Figure 6A). This suggested that, in the BY strain background, the PLL sequence promotes greater Sge1 abundance at the plasma membrane than the IIL-sensitive SLS sequence.

The localization of both Sge1^{PLL} and Ilt1 protein to the plasma membrane suggested the possibility that Ilt1 functionally interacts with Sge1 in IIL tolerance. To examine this possibility, we compared the growth of BY strains containing deletion mutations in *ILT1* in medium containing [C_2C_1im]Cl (Figure 6B). The BY *sge1^{PLL} ilt1*Δ double mutant strain was similarly tolerant to [C_2C_1im]Cl as the *sge1^{PLL}* strain containing wild-type *ILT1*. This indicated that *sge1^{PLL}* function does not require *ILT1* for IIL tolerance in the BY strain background.

Discussion

In this investigation of natural variants of *S. cerevisiae*, we discovered novel functions for *YDR090C/ILT1* and two alleles of the *SGE1* efflux pump in tolerance to IIL solvents and cationic toxins. Our genetic and biochemical analyses support a model in which Sge1 and Ilt1 function in resistance to cationic toxins (Figure 7A). The H^+ antiporter Sge1 presumably exports toxic IIL cations from the cytoplasm through the

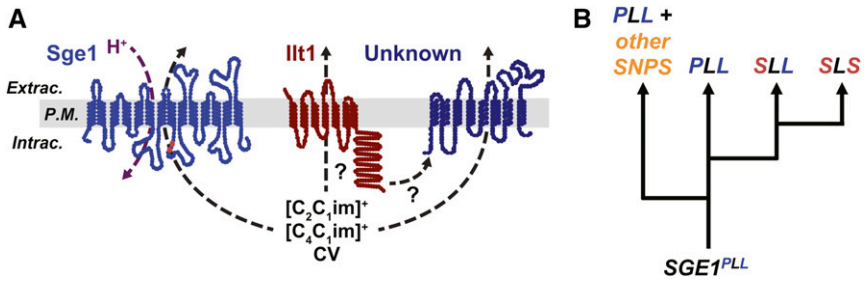

Figure 7 A model for the role of yeast transmembrane proteins in resistance to cationic toxins and the emergence of *SGE1* alleles. The model in (A) proposes the functions of *ILT1* and *SGE1* determined from this study. Sge1 functions in exporting cationic toxins, including CV and IILs, out of the cell through proton exchange. Alleles of *SGE1* determine its protein abundance and the ability to tolerate high concentrations of cationic inhibitors. *ILT1* may also directly or indirectly export cationic toxins out of the cytoplasm through the plasma membrane (P.M.). The dendogram in (B) proposes the evolutionary path for the emergence of the $SGE1^{SLS}$ sequence from the ancestral $SGE1^{PLL}$ allele. Extrac., Extracellular; Intrac., Intracellular.

plasma membrane in a similar manner as has been shown for cationic dyes (Ehrenhofer-Murray *et al.* 1994). The greater abundance of $Sge1^{PLL}$ protein at the plasma membrane likely enables greater extrusion of IILs and cationic toxins from the cell over the less abundant $Sge1^{SLS}$ protein. Ilt1 may also function at the plasma membrane and through a mechanism distinct from Sge1, but whether it directly exports ionic liquids and cationic compounds out of the cell remains unclear. Ilt1p shares homology to the paralogs Ypq1p, Ypq2p, and Rtc2p/Ypq3p, solely through a PQ-loop domain (Ponting *et al.* 2001) which is present on vacuolar/lysosomal transporters of cationic amino acids (Cherqui *et al.* 2001; Jézégou *et al.* 2012), suggesting the possibility that Ilt1 may also function in transport. Additional studies are needed to better understand the role of Ilt1p function in IIL tolerance.

The existence of multiple *SGE1* alleles encoding proteins of differing stabilities suggests that these sequence variations are tolerated in specific genetic backgrounds or environmental contexts. Since the tolerant $SGE1^{PLL}$ allele is ancestral (Figure 7B) and has been conserved across millions of years of evolution, man-made cationic toxins, such as IILs and CV, cannot be invoked as recent selective agents for adaptation at this locus. The $SGE1^{SLS}$ allele likely increased in frequency due to a bottleneck or reduced selective pressure to maintain higher levels of Sge1p expression. For example, there may have been reduced exposure to natural cationic toxins in the environment, or some genetic backgrounds may have conferred partly redundant mechanisms for coping with these toxins. Interestingly, several strains of multiple different *Saccharomyces* species lack the *SGE1* gene entirely (File S3), further suggesting the importance of its function may be conditional. Although the derived alleles are most likely conditionally neutral or slightly deleterious, the observation that many *S. cerevisiae* strains contain the sensitive $SGE1^{SLS}$ and intermediate $SGE1^{SLL}$ sequences could suggest their importance in some natural environments. The $Sge1^{SLS}$ or Sge^{SLL} proteins may have altered specificity for unknown natural toxins, causing fitness trade-offs in reduced resistance to man-made IILs and cationic dyes. Alternatively, the differences in protein abundances between natural variants suggest that Sge1 protein stability is regulated. Sge1 is ubiquitinated *in vivo* (Swaney *et al.* 2013), which could affect Sge1 stability by targeting Sge1 protein for endocytosis and delivery to the vacuole for degradation (Piper *et al.* 2014). Further molecular and genetic studies are needed to better understand the roles of each allele in Sge1 function across a broad range of ecologically and industrially relevant conditions.

To make cost-effective and sustainable biofuels and bioproducts, microbial catalysts will need to metabolize lignocellulosic sugars efficiently in the presence of inhibitory compounds such as ionic liquids, which are necessary for deconstruction of lignocellulosic feedstocks into fermentable sugars. Our results suggest that CRISPR/Cas9-based gene editing or engineered overexpression of *SGE1* sequences in industrial yeast strains may enable wider use of ionic liquid-pretreated biomass for biofuel production. Genetic modifications of other MFS transporters have been shown to improve biofuel production (Farwick *et al.* 2014; Li *et al.* 2016), further adding to the idea that exploring natural or experimental variation in MFS sequences may enable phenotypes for industrial applications. Our approach to screen and identify allelic sequences is not limited to the ionic liquid tolerance trait, but can be applied to phenotypes gathered from a large number of wild and domesticated *S. cerevisiae* strains cultured across a variety of media conditions (Fay and Benavides 2005; Liti *et al.* 2009; Schacherer *et al.* 2009; Parreiras *et al.* 2014; Sato *et al.* 2014; Wohlbach *et al.* 2014; Strope *et al.* 2015). Through this approach, distinct phenotypes can be assigned to specific genetic variants within the same species, thus providing a better understanding of how sequence differences among strains can be used to improve production of industrial biofuels and products.

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