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Use of a fluoride channel as a new selection marker for fission yeast plasmids and application to fast genome editing with CRISPR/Cas9

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Abstract

Fission yeast is a powerful model organism that has provided insights into important cellular processes thanks to the ease of its genome editing by homologous recombination. However, creation of strains with a large number of targeted mutations or containing plasmids has been challenging because only a very small number of selection markers is available in *Schizosaccharomyces pombe*. In this paper, we identify two fission yeast fluoride exporter channels (Fex1p and Fex2p) and describe the development of a new strategy using Fex1p as a selection marker for transformants in rich media supplemented with fluoride. To our knowledge this is the first positive selection marker identified in *S. pombe* that does not use auxotrophy or drug resistance and that can be used for plasmids transformation or genomic integration in rich media. We illustrate the application of our new marker by significantly accelerating the protocol for genome edition using CRISPR/Cas9 in *S. pombe*.

Keywords

Schizosaccharomyces pombe; fluoride channel; plasmid; CRISPR/Cas9

Introduction

Schizosaccharomyces pombe is a powerful model system since it is a simple eukaryote that is easy to manipulate. Creation and identification of mutants are facilitated by the existence of selectable markers (Bahler *et al.*, 1998; Grimm *et al.*, 1988; Hentges *et al.*, 2005; Sato *et al.*, 2005) and has allowed fission yeast to be used to make fundamental discoveries in key cellular processes such as cell cycle regulation (Fantes and Nurse, 1977; Nasmyth *et al.*, 1979; Nurse, 1975), cytokinesis (Mulholland *et al.*, 1994; Satterwhite and Pollard, 1992), epigenetics (Nakayama *et al.*, 2001; Volpe *et al.*, 2002) and endocytosis (Arasada and Pollard, 2011; Basu and Chang, 2011; Berro *et al.*, 2010; Sirotkin *et al.*, 2010). Two different

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classes of positive markers are used in *S. pombe*: auxotrophic markers and drug resistance markers. The disadvantage of auxotrophic markers over drug selection marker is that they cannot be selected on rich media, typically yeast extract (YE), and require plating on minimal media, typically Edinburgh minimal medium (EMM), which allows appearance of new colonies only very slowly (after about a week). In addition, this strategy does not allow for selection of mutants that show limited or no growth on minimal media (Luo *et al.*, 2009; van Slegtenhorst *et al.*, 2004).

A recent advance in *S. pombe* genome edition has been the implementation of the CRISPR/Cas9 system (Jacobs *et al.*, 2014), which does not require the introduction of any marker in the genome since selection of the plasmid containing Cas9 and the guide RNA is sufficient to ensure high integration efficiency. Yet the protocol developed remains slow since the plasmid contains the auxotrophic selection marker *ura4⁺*, which requires selection on minimal media lacking uracil, therefore increasing the time to recover transformants to about 1 week. This process could be accelerated if one could use a plasmid marker that can be selected on rich media.

Recent studies have shown that a large number of bacteria, archaea and fungi express fluoride export channels protecting them from fluoride toxicity (Baker *et al.*, 2012; Li *et al.*, 2013; Stockbridge *et al.*, 2013). In *Saccharomyces cerevisiae*, strains lacking both genes encoding these channels (FEX1 and FEX2) are highly sensitive to fluoride, at concentrations as low as 100 μ M (Smith *et al.*, 2015). In addition, expression of the FEX1 channel from a low copy plasmid is able to fully complement the absence of both fluoride channels and allows budding yeast cells lacking both channels to grow in rich media at high fluoride concentration (up to 5 mM), indistinguishably from wild-type strains. These results suggest that fluoride and fluoride channels might be used as a selection marker for plasmid transformation in strains lacking both fluoride channels in *S. cerevisiae* and *S. pombe* grown in rich media.

In this paper, we identify the *S. pombe* homologues of the *S. cerevisiae* fluoride export channels, which we name Fex1p and Fex2p, and show that they are required to protect cells from fluoride toxicity. We then show that growth of fission yeast strains lacking *fex1* and *fex2* genes in media containing fluoride can be rescued by expressing Fex1p from a plasmid or at a different genomic locus, and use these properties to repurpose Fex1p as a selection marker for plasmid or genomic integration in rich media supplemented with fluoride. Lastly, we use this new marker to improve the throughput of *S. pombe* strain construction using the CRISPR/Cas9 system.

Material and methods

Strains, media and growth curves

The fission yeast strains FY527 (h⁻, *his3-D1 leu1-32 ura4-D18 ade6-M216*) and FY528 (h⁺, *his3-D1 leu1-32 ura4-D18 ade6-M216*) were used as the master strains for most transformations. The strain JBSp224 (h⁺, *his3-D1 leu1-32 ura4-D18 ade6-M216fex1 ::natMX6fex2 ::kanMX6*) was created by two successive transformations of FY528 using the standard lithium acetate procedure (Moreno *et al.*, 1991). The strain

JBSp289 was created by transformation of JBSp224 using the lithium acetate procedure to tag the gene *pil1* with mEGFP and using Fex1p as a selection marker. All the strains created and used in this study are listed in Table 1.

Yeast cells were grown in rich medium (YE5S; 0.5% yeast extract and 3% dextrose) or Edinburgh minimal medium (EMM5S), both supplemented with adenine, leucine, lysine, histidine and uracil (0.225 g/l each). Media preparation and basic manipulation methods of *S. pombe* were carried out as described previously (Moreno *et al.*, 1991).

For spot assays, cells were grown overnight and diluted to 0.1 optical density at 595 nm (OD₅₉₅) and serially diluted in sterile ddH₂O by 10× increments. 10 µl of cell suspension was spotted onto YE5S plates with varying concentrations of fluoride (NaF) (Sigma, St Louis, MO, USA) and grown at 32 °C for 3 days prior to imaging. For liquid growth assays, cultures were diluted to OD₅₉₅ = 0.1 in 24-well flat-bottom plates (Corning Inc., Corning, NY, USA) containing either YE5S and varying concentrations of fluoride, or EMM5S or YNB5S in a final volume of 1 ml. One experiment was performed for each strain and in each condition. The OD₅₉₅ was measured every 5 min until growth plateaued using a BioTek Synergy 4 plate reader set at 25 or 32 °C with continual shaking.

Escherichia coli TOP10 cells were used for subcloning polymerase chain reaction (PCR) products and were grown in LB medium.

Cloning and vector design

Plasmids used in this study are listed in Table 2. The plasmid pMZ374 (Jacobs *et al.*, 2014) was modified by Gibson assembly (Gibson *et al.*, 2009) in order to replace the coding sequence (CDS) of *ura4⁺* by the CDS of *fexI⁺*, which was amplified from the gDNA of the *S. pombe* strain FY527.

All the guide RNA (sgRNA) vectors used in this study were created by Gibson assembly. The vectors pMZ374 or pJB166 were amplified with JB'330 (GTTCATCCACGACCTTAACG), JB'331 (CGTTAAGGTCGTGGATGAAC) and two oligonucleotides designed on the vector around the sgRNA site (TTCTTCGGTACAGGTTATG and GTTTTAGAGCTAGAAATAGCAAG) and also containing a tail for the target sequence to insert.

Genome integration using the pFA6a plasmid and fluoride selection

The vector pFA6a-mEGFP-kanMX6 was amplified using the primers JB'801 (GGTTATA CTGAAAAGTATTAGTCAGTACTGACAATAAAAAG) and JB'802 (GTAAGACTGTGT TAATAGCATGGTTGTTTATGTTTCGGATGTG). In parallel, the CDS of the *fexI⁺* gene was amplified from pJB166 using the primers JB'799 (CACATCCGAACATAAACAACCATGCTATTAACACAGTCTTAC) and JB'800 (CTTTTTATTGTCAGTACTGACTAATACTTTTCAGTATAACC). The two PCR products were annealed by Gibson cloning to produce pJB193 (pFA6a-mEGFP-*fexI⁺*). The vector pJB193 was amplified as described (Bahler *et al.*, 1998) so that it contains annealing ends for the gene *pil1*. The PCR product created was used to tag *pil1* with mEGFP in the strain JBSp224 using Fex1p as a selection marker.

Genome editing using the CRISPR/Cas9 plasmid and fluoride selection

For genome edition with CRISPR/Cas9, we used strains where both fluoride channels were deleted. Our master strain (JBSp224) was created from FY527 by deleting *fex1⁺* and *fex2⁺*. It was transformed using the lithium acetate method (Moreno *et al.*, 1991) with 250 ng of plasmid expressing Fex1p, Cas9 and the sgRNA specific to the region to target (e.g. pJB172) and with 1 µg of the PCR fragment used for homology-directed repair, which contains a sequence with the intended editions flanked with sequences homologous to the genomic region around the Cas9 cut. The transformation product was plated on YE5S+1 mM NaF and incubated at 32°C for 2–3 days. Twelve colonies were restreaked on YE5S+1 mM NaF for a further 2–3 days. Colonies appearing after the restreak can be directly screened using colony PCR.

Imaging

All pictures were taken using a spinning disc confocal microscope based on a TiE inverted (Nikon) instrument equipped with a 100×/1.45 NA objective, a Yokogawa CSU-W1 confocal spinning unit and a Andor iXon Ultra888 EMCCD camera.

Results

Identification of genes coding for the fluoride export channels Fex1p and Fex2p in *S. Pombe*

Saccharomyces cerevisiae fluoride export channels FEX1 and FEX2 contain 375 amino acids and are 99.2% identical to each other, with only three different but similar amino acids: V210I, I301V and A351V (supporting information, Figure S1A). We found two homologues of *S. cerevisiae* FEX1 and FEX2 in *S. pombe*: SPAC977.11 and SPBPB8B6.06c. Similarly to *S. cerevisiae*, the proteins encoded by SPAC977.11 and SPBPB8B6.06c are 100% identical (supporting information, Figure S1B). However, conservation of the protein sequences between *S. cerevisiae* and *S. pombe* FEX channels is poor (36%), but several domains contain stretches of amino acid that are conserved or with similar biophysical properties (Figure 1). The high intraspecies and poor interspecies conservation make it difficult to annotate the genes SPAC977.11 and SPBPB8B6.06c. SPAC977.11, being located on chromosome I and SPBPB8B6.06c on chromosome II, we respectively annotated them as *fex1* and *fex2* in *S. pombe*.

Schizosaccharomyces pombe strain lacking *fex1⁺* and *fex2⁺* is highly sensitive to fluoride

To confirm that Fex1p and Fex2p are indeed fluoride export channels and to test whether the export of fluoride is as essential for survival in *S. pombe* as it is in *S. cerevisiae*, we created an *S. pombe* strain where both *fex1* and *fex2* genes were deleted (JBSp224). Before further analysis, we showed that this newly created strain does not present any obvious morphological phenotype (supporting information, Figure S2). We also showed that it has the same behaviour as a wild-type strain in rich and poor media (Figure 3A, B, blue line; supporting information, Figure S3) and calculated that both strains have a doubling time of about 180 min in YE5S. This finding suggests that the deletion of both *fex1⁺* and *fex2⁺* genes does not have an impact on the physiology of the cell. However, growth of the *fex1*

fex2 strain was impaired in the presence of as low as 10 μ M fluoride on solid or liquid rich media (YE5S), whereas strains expressing Fex1p and/or Fex2p showed normal growth in the presence of up to 5 mM fluoride (Figures 2 and 3A, B). Therefore, fluoride is toxic for *S. pombe* even at low concentrations, and specific channels that export fluoride ions are required for normal growth.

Fluoride sensitivity of *fex1* *fex2* can be rescued by expression of *S. Pombe* Fex1p from a plasmid

We tested whether fluoride sensitivity of the *fex1* *fex2* strain can be rescued by expression of *S. pombe* Fex1p from a plasmid. We modified the plasmid pMZ374, originally designed by Jacobs *et al.* (2014) to perform CRISPR/Cas9 in *S. pombe*, by replacing the *ura4⁺* auxotrophic allele with *S. pombe fex1⁺* gene, expressed under the *ura4* promoter (pJB166; supporting information, Figure S4). We transformed our *fex1* *fex2* strain with either our new plasmid expressing Fex1p (pJB166) or a control plasmid expressing the *ura4⁺* allele (pMZ374), and plated transformed cells on YE5S containing 1 mM fluoride, a concentration that prevents growth of cells lacking both fluoride channels (Figures 2 and 3B). Absence of colonies in our control and the presence of a large number of colonies in our transformation with the plasmid containing *fex1⁺* (pJB166) indicate that Fex1p can be expressed from a plasmid and is able to complement the inability of the *fex1* *fex2* strain to export fluoride (supporting information, Figure S5). This result shows that Fex1p expression can be used as a selection marker for transformations into the *fex1* *fex2* strain grown on a rich medium containing fluoride. To our knowledge, Fex1p is the first marker identified in *S. pombe* that does not use auxotrophy or drug resistance and that can be used to select for plasmid in rich media.

Effect of Cas9 on cell growth

In our initial complementation experiment, we observed that cells transformed with the plasmid expressing Fex1p and Cas9 grew more slowly (Figure 3C) than wild-type cells (Figure 3A) in the presence or absence of fluoride. Since Cas9 is under the control of the strong pTEF promoter in this plasmid, we hypothesized that slower growth could be attributed to the abundant expression of Cas9, as suggested in Jacobs *et al.* (2014). We engineered a new vector where the Cas9 gene was removed (pJB186; supporting information, Figure S4) and observed that the growth rate of the *fex1* *fex2* strain transformed with this vector was similar to that observed for wild-type cells (Figure 3D). This result confirms that expression of a large amount of Cas9 has some toxic effects for fission yeast growth. A surprising observation is that in the absence of fluoride we do not observe a delay of growth when the cells are transformed with the plasmid expressing Cas9. That result could probably be explained by the fact that in the absence of fluoride there is no selection pressure for cells to keep the plasmid. Cells that have lost the plasmid would then not express Cas9 and grow faster than cells expressing Cas9. Therefore, they would likely have a competitive advantage and rapidly outnumber cells containing the plasmid, which would explain the apparently normal growth curve when no fluoride is present.

Use of our new fluoride marker (Fex1p) to accelerate genome edition with CRISPR/Cas9

The search for a non-auxotrophic marker that can be expressed on a plasmid was motivated in the first place by the urge to improve the CRISPR/Cas9 protocol developed by Jacobs *et al.* (2014) in *S. pombe*. Using a plasmid that expresses Fex1p rather than the *ura4⁺* allele makes it possible to recover transformants after 2–3 days on rich media (YE5S) containing 1 mM fluoride, whereas using the original plasmid requires growth on minimal media depleted of uracil (EMM5S-Ura), and colonies appear after 6–7 days. To compare the efficiency of both methods, we used in parallel the original plasmid and our newly developed plasmid to delete the gene coding for the eisosome protein Pil1p. In addition to their selection markers, both plasmids expressed the Cas9 protein and a guide RNA with homology with a sequence in the *pil1* gene and containing an NGG PAM sequence (GGACGGCGGTTTGCTGAGGA). In both transformations, we also added a donor DNA created by PCR as the fusion of the 5' UTR (primers used: TCGCCCTTTGGTAAAGTGTT and CAAATA GAAAAAAGTAAAGAGTTTTTCTTGCAGA AATAGAAATAGATGT) and of the 3' UTR of the *pil1* gene (primers used: ACATCTATTTCTAT TTCTGCAAGAAAACTCTTTACTTTTTTTC TATTTG and CGAAGCAATTCAATCATAC ACC), which cells could use to repair the double-stranded break induced by Cas9, leading to the deletion of the entire *pil1* gene. As a control, we transformed a wild-type strain (expressing Fex1p and Fex2p) with the plasmid with the *ura4⁺* marker (pJB109) and selected on EMM5S-Ura plates. In parallel, we transformed the *fex1⁺ flex2⁺* strain with the plasmid containing the *fex1⁺* marker and plated on rich medium (YE5S) supplemented with 1 mM fluoride. After appearance of colonies, we restreaked 12 of them on the appropriate selective medium and screened them to identify *pil1⁻* strains. In both transformations, four out of 12 colonies were positive, showing that both methods used to edit strains with CRISPR/Cas9 have comparable efficiency (~33%). The major advantage of using *fex1⁺* as a selection marker is that it allowed us to identify edited strains in about 5 days, whereas it took more than 11 days to recover strains using the auxotrophic marker *ura4⁺* (Figure 4).

Fex1p can also be used as a selection marker in traditional integration protocols

We tested whether Fex1p and fluoride can also be used as a marker for traditional genomic integration. As a proof of principle, we modified the pFA6a vector used for C-terminal tagging with mEGFP (Bahler *et al.*, 1998) by replacing the *kanMX6* drug resistance gene with the *fex1⁺* gene. We used this plasmid (pJB193) to C-terminally tag with mEGFP the eisosome gene *pil1* using the protocol developed by Bahler *et al.* (1998). Plating the transformants on rich media supplemented with fluoride produced a large number of colonies whereas the control transformation produced no colonies (supporting information, Figure S6). We confirmed proper integration of the mEGFP and *fex1⁺* cassette by PCR and by imaging the cells with a spinning disc confocal microscope (supporting information, Figure S7). These results show that Fex1p and fluoride can also be used as a selection marker for genomic integration.

Discussion

In this study, we overcame the limitations of selection markers currently available in *S. pombe* by repurposing fluoride toxicity and fluoride export channels into a new strategy for selecting transformants. Our new strategy is of particular interest for plasmid transformation since most plasmid markers are auxotrophic and require selection on minimal media, making plasmid transformation slow and poorly efficient in mutants with growth defects. Another advantage of our new strategy is that fluoride is significantly cheaper than classic antibiotics used in *S. pombe* (e.g. 100 times less than Geneticin and 1000 times less than Clonat). An immediate application of this new selection marker has been the dramatic shortening of the protocol of genome edition using the CRISPR/Cas9 system to timeframes comparable to traditional integration methods with antibiotic selection. We expect our new method to have an impact beyond the *S. pombe* community and will be adaptable to other species since genes encoding for fluoride export channels are expressed in most eukaryotes and prokaryotes. The only minor constraint of our new method is that strains that can be edited require all genes coding for fluoride export channels to be removed. We do not expect any issue with these strains since, as far as we have observed, they do not behave any differently than wild-type strains in media containing trace fluoride levels found in double-distilled water and commercially available reagents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Sc_FEX1: MIFNPVISNHNKLSHYIHVFCTFTTF--CILGTETRQAITALSTYTPAFVTAPTVLWSNC
Fex1p: -----MLLTQSYFCIMSMGLTLARLGLTALNTYPGAP--FSGLLWVQF
      : : * : * : * : * : * : * : * : * : * :
Sc_FEX1: SSCMLMGIMQSLNAYTW-MKDHQVLFGLGVTGTCGALSSFSMMLLEMFEHSTNLTNGNIA
Fex1p: VGCVMGFCQTESVFFPRPKHNATFLLAITTGFCGSLTTFSSWMLQMFTGMANLDP----
      . : * : * : * : * : * : * : * : * : * :
Sc_FEX1: NHTKLPNRAYGIMEFLSVLLVHLMVSMGSLIFGRQLGKEVIV-AYGSSSFSPKPYTPPSDT
Fex1p: --FERRGRGYFSLVVSDFMVTMCIAMSSLIWGKQIGHTTGQWRIGKVAFAWP-----
      : * : * : * : * : * : * : * : * : * :
Sc_FEX1: VKENAGDVTQEMEKNILEFKFTAPAPFFKKFFDIVDKLAYALAFPLIILFVVLCAYYEN
Fex1p: -----IPAHTHIV--V---RVLLLLLSICFFVGAIFYTA
      * : * : * : * : * : * : * : * : * :
Sc_FEX1: Y----SRGKWTLPCLFGIFAGFLRYWLAEMFNKTNKKFPLGTFLANVFATLLIGIFTMVQ
Fex1p: YTTNVTHRGIGFSLIFSPFAALTRYLARFLNSPQYFIPYGTLCANVFATLLLSIMYMIP
      * : : : : * : * : * : * : * : * : * :
Sc_FEX1: RGKKHFSTDVPIVNSLNSCHIVSALISGFCGTLSTISTFINEGYKLSFINMLIYYTVSIA
Fex1p: QITHCT----P----VSRVSMYGIQNGFCVAVLSTLSTFSNELHTMPIKRAYIYCIISVA
      : : * : * : * : * : * : * : * : * :
Sc_FEX1: ISYCLLVITLGSYAWTRGLTNPIC
Fex1p: ISFSICVIVDGATAWGHGYTEKY
      * : * : * : * : * :

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Figure 1.

The fluoride channels from *S. pombe* (Fex1p and Fex2p) and *S. cerevisiae* (FEX1 and FEX2) share only 36% identical amino acids but have several conserved regions that contain amino acids with similar properties (boxes). See also Figure S1. The alignment was performed with Clustal Omega. Symbols: asterisk (*), fully conserved residues; colon (:), residues with strong similar properties; point (.), residues with weak similar properties

Figure 2.

Spot assays showing that growth of wild-type strains on solid media is insensitive to fluoride up to 5 mM, and growth of the *fex1* *fex2* double mutant is impaired starting at 10 μM but can be rescued by expression of Fex1p from a plasmid (also expressing Cas9)

Figure 3.

Growth of *S. pombe* in the presence of different concentrations of fluoride. (A) Growth of the wild-type strain in liquid media is insensitive to fluoride concentrations up to 5 mM. (B) Growth of the *fex1* *fex2* strain is similar to wild-type in the absence of fluoride but its growth is impaired for fluoride concentration higher than 10 μM. (C) Growth of the *fex1* *fex2* strain on fluoride can be rescued by the expression of Fex1p on a plasmid. Note that the time-scale for this figure is longer than for other figures because the expression of Cas9 from the plasmid delays the growth of *S. pombe*. (D) The absence of expression of Cas9 allows the growth of *S. pombe* to be rescued with growth curves similar to wild-type

Figure 4.

Comparison of timelines using auxotrophic or fluoride channel markers, as observed in genome editing using CRISPR/Cas9. (Top) Using a Fex1p-expressing plasmid and selection on rich media with fluoride allows the screening for positive colonies 5 days after the initial transformation. (Bottom) Using a *ura4⁺*-expressing plasmid and minimal media lacking uracil allows the screening for positive colonies after at least 11 days

Table 1
Strains used in this study

Name	Genotype	Reference
FY527	<i>ade6-M216 his3-D1 leu1-32 ura4-D18</i>	S. Forsburg
JBSp217	<i>pil1 ade6-M216 his3-D1 leu1-32 ura4-D18</i>	This study
JBSp224	<i>fex1 ::natMX6 fex2 ::kanMX6 ade6-M216 his3-D1 leu1-32 ura4-D18</i>	This study
JBSp289	<i>pil1-mEGFP- fex1⁺ fex1 ::natMX6 fex2 ::kanMX6 ade6-M216 his3-D1 leu1-32 ura4-D18</i>	This study

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Table 2

Plasmids used in this study

Name	Description
pJB109	pMZ374 with a gRNA to target <i>pil1</i>
pJB166	pMZ374 with <i>ura4⁺</i> replaced by <i>fexI⁺</i>
pJB172	pJB166 with a gRNA to target <i>pil1</i>
pJB186	pJB166 with Cas9 deleted
pJB193	pFA6a-mEGFP- <i>fexI⁺</i>