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Identification of amino acid residues of mammalian mitochondrial phosphate carrier important for its functional expression in yeast cells, as achieved by PCR-mediated random mutation and gap-repair cloning

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Footnotes:
Abbreviations used: PiC, mitochondrial phosphate carrier; hPiC, rPiC, and yPiC, PiC of human, rat, and yeast, respectively; ΔNPiC, PiC lacking its presequence

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Abstract

The mitochondrial phosphate carrier (PiC) of mammals, but not the yeast one, is synthesized with a presequence. The deletion of this presequence of the mammalian PiC was reported to facilitate the import of the carrier into yeast mitochondria, but the question as to whether or not mammalian PiC could be functionally expressed in yeast mitochondria was not addressed. In the present study, we first examined whether the defective growth on a glycerol plate of yeast cells lacking the yeast PiC gene could be reversed by the introduction of expression vectors of rat PiCs. The introduction of expression vectors encoding full-length rat PiC (rPiC) or rPiC lacking the presequence (ΔNrPiC) was ineffective in restoring growth on the glycerol plates. When we examined the expression levels of individual rPiCs in yeast mitochondria, ΔNrPiC was expressed at a level similar to that of yeast PiC, but that of rPiC was very low. These results indicated that ΔNrPiC expressed in yeast mitochondria is inert. Next, we sought to isolate "revertants" viable on the glycerol plate by expressing randomly mutated ΔNrPiC, and obtained two clones. These clones carried either of two mutations, F267S or F282S; and these mutations restored the transport function of ΔNrPiC in yeast mitochondria. These two Phe residues were conserved in human carrier (hPiC), and the transport function of ΔNhPiC expressed in yeast mitochondria was also markedly improved by their substitutions. Thus, substitution of F267S or F282S was concluded to be important for functional expression of mammalian PiCs in yeast mitochondria.

1. Introduction

Mitochondrial ATP synthesis is achieved in the matrix space owing to the electrochemical gradient of H^+ across the mitochondrial inner membrane acting as a driving force. To enable efficient ATP synthesis, the mitochondrial inner membrane shows high resistance against the permeation of molecules. However, numbers of molecular species involved in biochemical reactions such as ATP synthesis, β-oxidation or TCA cycle must be transported into the matrix space of mitochondria. The transport of individual molecule species is catalyzed by their specific transport proteins. These transporters show structural similarities with each other and are thought to have been formed from a common ancestral gene; and, hence, they are referred to as members of the mitochondrial solute carrier family, SLC25a (For reviews, see refs. 1-5).

Most carrier proteins have been conserved in both yeast and mammals. As manipulation of the yeast genome is much easier than that of the mammalian one, yeast would seem to be very useful for structure/function studies on mammalian mitochondrial solute carriers. Actually, numbers of yeast mitochondrial carriers such as Sam5p, Flx1p, Oac1p, Leu5p, Rim2p, Ort1p, and Crc1p have been successfully complemented by the human orthologs SLC25A26 [6], SLC25A32 [7], SLC25A34 [8], SLC25A42 [9], SLC25A33 and SLC25A36 [10], SLC25A15 [11, 12], and SLC25A20 [13]. However, complementation of yeast mitochondrial carriers by their mammalian ortholog is not always successfully achieved. In the case of the mitochondrial ADP/ATP carrier, introduction of the expression vector of the native bovine carrier into yeast cells lacking a functional ADP/ATP carrier gene was not effective in rescuing them from their defect in ATP synthesis, indicating that the native bovine carrier cannot be functionally expressed in yeast mitochondria [14]. As the yeast carrier has a longer N-terminal sequence than the bovine one, we examined earlier whether it would be possible to express a chimeric bovine carrier whose N-terminal sequence had been substituted with the corresponding region of the yeast carrier and found that this was the case [14]. Not only the bovine carrier but also the human one could be functionally expressed in yeast cells as chimeric proteins [15].

In the case of the mitochondrial phosphate carrier (PiC), the mammalian one, but not the yeast one, is synthesized with a presequence, which is cleaved at the mitochondria [16-18]. Furthermore, an earlier study clearly demonstrated that deletion of the presequence of the mammalian mitochondrial phosphate carrier facilitates the import of the carrier into yeast mitochondria [19]. However, the more intriguing question of whether or not the mammalian phosphate carrier can be expressed in a functional form in yeast mitochondria had not been addressed. Therefore, in the present study we

examined whether the mammalian mitochondrial phosphate carrier could be functionally expressed in yeast cells.

2. Materials and Methods

2.1. Materials

The haploid strain of *Saccharomyces cerevisiae* W303-1B (MATα *ade2-1 leu2-3, 112 his3-22,15 trp1-1 ura3-1 can1-100*) was used as the wild type [14]. The single- and multi-copy type expression vectors in yeast cells were prepared by introducing the promoter region of the yeast type 2 ADP/ATP carrier gene into pRS314 and pYO326, respectively, as described previously [14]. Antibody against the FLAG tag (code F7425-2MG) was purchased from Sigma-Aldrich.

2.2. Preparation of a yeast strain lacking its MIR1 gene (mir1Δ)

Disruption of the *MIR1* gene encoding the mitochondrial phosphate carrier in the W303-1B strain was achieved by homologous recombination (*mir1::HIS3*). The structure of the targeting construct used for disruption of the *MIR1* gene is shown in **Supplementary Fig. S1a**. This construct was prepared by PCR. Briefly, the first PCR was carried out by using GE2857 and GE2859 as primers, and pRS313 as a template, as shown in **Supplementary Fig. S1b**. The nucleotide sequences of one-third of the 3’ side of these two primers corresponded to those at the 5' and 3' regions of the *HIS3* gene, respectively, in the pRS313; and the nucleotide sequences of their remaining regions corresponded to those at the 5’ and 3’ regions of the *MIR1* gene of the yeast genome. The second PCR was carried out by using GE2858 and 2860 as primers, and the PCR product of the first reaction as a template. Actual nucleotide sequences of individual primers are shown in **Supplementary Fig. S1c**. The nucleotide sequences of GE2857 and GE2859, shown in green, are those that annealed to the *HIS3* gene; and the boxed nucleotide sequences represent overlapped sequences between primers GE2857 and GE2858, and those between primers GE2859 and GE2860. The resultant PCR product was gel purified and used for transformation.

2.3. Preparation of cDNA and expression vectors of yeast, rat, and human mitochondrial phosphate carriers (abbreviated as yPiC, rPiC, and hPiC, respectively), and their mutants

The cDNA fragments encoding yPiC, rPiC, and hPiC were prepared by RT-PCR or PCR.

Strategies for PCR, template DNA, and nucleotide primers used for preparation of these cDNA fragments are summarized in **Supplementary Table SI**. These cDNA fragments were subcloned into single copy-type (pRS314) or multi copy-type (pYO326) expression vectors having the promoter region of the yeast type 2 ADP/ATP carrier gene (referred to as pRS314/yA2P and pYO326/yA2P, respectively). The methods used for preparation of expression vectors of PiCs having a C-terminal FLAG tag are summarized in **Supplementary Fig. S2**.

2.4. Observation of cell growth

To examine the growth-defect phenotype, we streaked a yeast cell suspension on agar plates containing 1% yeast extract, 2% bactopeptone, and 2% agar supplemented with either 2% glucose (YPD) or 3% glycerol (YPGly) as a carbon source. After incubation at 30° C for 2 or 5 days, the cell growth was assessed photographically.

2.5 Preparation of mitochondrial fraction from cultured yeast cells and Western blotting

For preparation of mitochondria, yeast cells were cultured in a liquid medium containing 1% yeast extract, 2% bactopeptone, and 2% galactose at 30° C. The mitochondria fraction was prepared as described previously [20].

Western blotting was performed as described earlier [20,21]. Samples of 20 μg (for detection of FLAG-tagged PiC) or 5 μg (for Por1p) aliquots of mitochondrial fractions were subjected to SDS-PAGE and subsequent Western blotting, and PiCs having the FLAG tag were detected by use of anti-FLAG antibody. Yeast Por1p (mitochondrial porin, also referred to as voltage-dependent anion channel) was also detected as a control by using anti-Por1p antibody, which was raised in a rabbit immunized with a synthetic peptide with the amino acid sequence of IVGGAEFGYDISAGSISRYC (amino acids 147 – 165 of yeast Por1p (NM_001182894) plus a C-terminal Cys residue for conjugation with hemocyanin) as the immunogen.

To ascertain the proper preparation of the mitochondrial fraction from yeast cells, we subjected a whole cell lysate and mitochondrial fraction to Western blotting by using antibodies against FLAG tag, Por1p, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Results obtained from *mir1Δ* and that expressing yPiC are shown in **Supplementary Fig. S3.**

2.6. Identification of amino acids important for functional expression of rPiC by PCR-based random mutation and gap-repair cloning

The outline of the PCR-based random mutation and gap-repair cloning procedure is

depicted in **Supplementary Fig. S4**.

The randomly mutated cDNA fragments corresponding to the open reading frame of rat PiC lacking its N-terminal presequence (ΔNrPiC-A) were obtained by PCR using *Taq* DNA polymerase, because it is well known that mutation of DNA will occur at a certain frequency during ordinary PCR using this polymerase [22]. The amplification conditions used (25 μl) consisted of 0.2 ng template DNA (pYO326/yA2p/ΔNrPiC-A), 25 pmol each of primers (GE2046, 5'-GTAATACGACTCACTATAG and GE2047, 5'-TACAAGTCAAAGGAGCCCC), 1 × HY buffer, 200 μM dNTP, and 1.25 units of *Taq* DNA polymerase (Greiner, code 986007). The mixture was heated at 95° C for 5 min and then subjected to a chain reaction of 35 cycles of heating at 95° C for 30 sec, 56° C for 30 sec, and 72° C for 1 min, with a final extension at 72° C for 15 min.

The resulting amplification mixture was mixed with 10 μg aliquots of the expression vector of ΔNrPiC (rPiC lacking its N-terminal presequence) digested with *Bam*HI and *Bgl*II. Then, the yeast cells lacking their *MIR1* gene were transformed with the above mentioned mixture, and spread on an agarose plate containing minimum medium with glycerol as a carbon source but lacking uracil and histidine. After 10 days' incubation at 30° C, two viable colonies on the plate were obtained.

2.7. Measurement of phosphate transport function of PiCs expressed in yeast mitochondria

The phosphate transport function of the expressed protein was evaluated either by measuring the turbidity change in the mitochondrial suspension or by measuring the uptake of [^{32}P]phosphate [23]. As for the former, mitochondria (0.25 mg protein) prepared from individual transformant yeast cells were suspended in 1.5 ml of medium containing 120 mM ammonium chloride or 120 mM ammonium phosphate, 20 mM Tris (pH 7.4), 1 mM EDTA, and 5 mM rotenone; and time-dependent absorbance changes in the suspension at 546 nm were recorded by use of a Shimadzu spectrophotometer, model UV-1800. As for the latter evaluation, mitochondria (0.25 mg of protein) were incubated at 0° C in a medium consisting of 20 mM Tris-Cl, pH 6.5, 100 mM KCl, 1 mM EGTA, 1 μg/ml rotenone, and 20 mM butylmalonate. The uptake was started by adding 1.35 mM [^{32}P]phosphate, and after incubation for 20 sec, terminated by the addition of 1.5 mM mersalyl. The amount of [^{32}P]phosphate specifically taken up by mitochondria was corrected by subtracting the radioactivity observed with mitochondria from *mir1*Δ pretreated with mersalyl as background.

3. Results

3.1. Preparation for the experiments evaluating functional expression of rat PiC in yeast cells

Past studies demonstrated that disruption of the gene encoding the yeast mitochondrial phosphate carrier causes a growth-defect phenotype of yeast grown on plates containing glycerol as a sole carbon source [24, 25] and that this growth defect can be reversed by introduction of an expression vector encoding the yeast phosphate carrier [25]. We also observed the growth-defect phenotype of a yeast strain lacking a functional phosphate carrier gene, *MIR1* (*mir1*Δ), on a glycerol plate, and its rescue by introduction of an expression vector for the yeast mitochondrial phosphate carrier (see **Supplementary Fig. S1d**). Thus, this experimental system was successfully confirmed.

We next prepared the expression vectors for the rat mitochondrial phosphate carrier for introduction into yeast cells. The reason for choosing rat as an animal species is because functional studies on mitochondria have been mainly carried out by using mitochondria isolated from rat liver. In this manuscript, PiCs of yeast and rat are referred to as yPiC and rPiC, respectively.

As for the mammalian mitochondrial phosphate carrier, two splice variants, referred to as PiC-A and PiC-B, respectively, generated by alternative use of exons 3A and 3B, are known to be expressed (see **Fig. 1a** and ref. [18]). Thus, the entire cDNAs encoding the full-length form of these two variants of rat PiCs were prepared by RT-PCR. Because PiC-A is reported to be expressed in the heart and skeletal muscle, and PiC-B is ubiquitously expressed [26,27], first-strand cDNAs prepared from total RNA of rat heart were used as templates for preparation of rPiC-A; and those from rat brain, for preparation of rPiC-B. After confirmation of the proper nucleotide sequence of individual cDNAs, they were subcloned into the expression vector pYO326/yA2P [14].

We also prepared expression vectors of rPiC-A and rPiC-B lacking their presequence (designated as ΔNrPiC-A and ΔNrPiC-B, respectively; see also **Figs. 1b and 1c**), because a past study clearly demonstrated that the deletion of the presequence of the mammalian PiC facilitates the import of the carrier into yeast mitochondria [19]. Totally, 4 expression vectors of A and B isoforms of rPiCs (rPiC-A and rPiC-B) and the precursor/mature forms containing/lacking their presequence (rPiC and ΔNrPiC) were prepared.

3.2. The growth of the mir1Δ cells on the glycerol plate was not recovered by

introduction of expression vectors of rat PiCs

When we transformed *mir1*Δ cells with the expression vector for the full-length open reading frame of either rPiC-A or rPiC-B, the yeast with the disrupted phosphate carrier were not rescued from their growth defect on the glycerol plate (**Fig. 2a**), indicating the failure of the functional expression of rPiC in yeast mitochondria by using these vectors. Unexpectedly, *mir1*Δ cells transformed with the expression vector for either ΔNrPiC-A or ΔNrPiC-B were also unable to grow on the glycerol plate (**Fig. 2a**).

3.3. Evaluation of the expression levels of the rPiC in yeast mitochondria

In the above experiments, if the growth defect of the *mir1*Δ cells on the glycerol plate had been successfully rescued by the introduction of an expression vector, this result would have reflected the functional expression of the rPiC encoded by the introduced expression vector. However, as rescue was not achieved by this means, this result might be attributed to i) failure in proper expression of the rPiC in yeast mitochondria or ii) lack of the transport function of the expressed rPiC. To ascertain which of these two possibilities was the case, we next examined the expression levels of rPiC in yeast mitochondria.

For this purpose, we prepared expression vectors of PiC/FLAG, each having a flanking sequence of the FLAG tag at its C-terminal end (4 rPiCs and yPiC, totally 5 PiCs). When we transformed the *mir1*Δ cells with any of these expression vectors, the transformants showed phenotypes similar to those observed for the cells transformed with the expression vector not having the FLAG tag (**Supplementary Fig. S5**), suggesting that the addition of the FLAG tag at their C-terminal end did not affect the function of the individual rPiC.

When we prepared the mitochondrial fraction from the transformed yeast cells and subjected them to SDS gel electrophoresis and subsequent Western analysis using anti-FLAG antibody, we observed strong immunoreactive protein bands for mitochondrial fractions obtained from the cells transformed with the expression vector encoding yPiC/FLAG or type A or type B isoforms of ΔNrPiC/FLAG, as shown in **Fig. 2b**. These results clearly demonstrated that type A or type B isoforms of ΔNrPiC were well expressed at the mitochondrial membrane, as was yPiC.

Interestingly, moreover, mitochondrial fractions from yeast cells transformed with the expression vector encoding type A or type B isoforms of rPiC/FLAG also showed a faint immunoreactive protein band having slower migration than that of yPiC/FLAG or type A or type B isoforms of ΔNrPiC/FLAG (**Fig. 2b**). This result might indicate that a small amount of full-length rat PiC with its presequence could also be expressed at the

mitochondrial membrane.

These data clearly demonstrated that rPiC lacking its presequence, i.e., ΔNrPiC, could be more effectively expressed than its full-length form (rPiC), in yeast mitochondria, in accordance with the results reported earlier [19]; and this property was consistently observed with rPiCs regardless of differences in isoform type (type A or B).

More importantly, it was evident that presequence-depleted forms of rPiC, i.e., ΔNrPiC-A or ΔNrPiC-B, were well expressed in yeast mitochondria; although *mir1*Δ cells transformed with expression vectors for these proteins failed to grow on a glycerol plate, indicating the lack of transport activity of ΔNrPiC expressed in yeast mitochondria.

3.4. Isolation of revertants viable on the glycerol plate by PCR-mediated random mutation and gap-repair cloning

Then the question arose as to why ΔNrPiC was inactive in yeast mitochondria. To answer to this question, we sought to isolate "revertants" viable on glycerol plates. As the above results clearly indicated that two splice variants of rPiC-A and rPiC-B showed essentially the same results, the following experiments were achieved using rPiC-A variants. Also, hereafter, rPiC-A and ΔNrPiC-A, will be simply referred to as rPiC and ΔNrPiC, respectively.

For screening of yeast cells in which randomly mutated ΔNrPiC was expressed, we employed the techniques of "PCR-mediated random mutation" and "gap-repair cloning" (for details, see Methods, **Supplementary Fig. S4** and ref. 28). When yeast cells lacking the *MIR1* gene (*mir1*Δ) were incubated with a mixture of randomly mutated DNA fragments encoding ΔNrPiC and an expression vector digested with *Bgl*II and *Bam*HI, and spread onto a selective agarose plate (agarose plate with minimum medium containing glycerol as a carbon source but lacking uracil and histidine), only two viable colonies were obtained (i.e., the total number of clones obtained with the above mentioned strategy was 2). When plasmid vectors in these colonies were subjected to sequence analysis, these expression vectors in both colonies were found to carry a distinct mutation of F267S or F282S in their rPiC (these two Phe residues are highlighted in blue in **Fig. 1c**). To confirm whether one of these mutations had enabled the growth of the *mir1*Δ cells on the glycerol plate, yeast cells lacking the *MIR1* gene were transformed with each expression vector of ΔNrPiC having one of these mutations; and their growth on the glycerol plate was then examined. As a result, either mutation effectively enabled the growth of the *mir1*Δ cells on the glycerol plate (**Fig. 3a**). These two colonies carrying the F267S or F282S mutation, obtained by

PCR-mediated random mutation and gap-repair cloning from the pre-sequence-depleted background (i.e., ΔNrPiC), will hereafter be referred to as ΔNrPiC (F267S) and ΔNrPiC (F282S), respectively.

3.5. Characterization of ΔNrPiC (F267S) and ΔNrPiC (F282S) mutants

To characterize these two mutants, ΔNrPiC (F267S) and ΔNrPiC (F282S), we further conducted two experiments. First, we roughly examined the differences in the growth rates of the yeast cells expressing these mutants with those of *mir1*Δ cells and *mir1*Δ cells expressing yPiC, rPiC or ΔNrPiC. For this examination, culture suspensions of individual yeast cells were serially diluted and spotted on YPGly plates; and after incubation at 30° C for 3 days, the growth of individual yeast cells was compared. As a result, as shown in **Fig. 3b**, the degree of cell growth was in the order of yeast cells expressing yPiC > ΔNrPiC (F282S) > ΔNrPiC (F267S); and the growth of *mir1*Δ cells or *mir1*Δ cells transformed with expression vectors of rPiC or ΔNrPiC was not observed. However, it was difficult to ascertain whether the differences in the observed growth were due to differences in their expression levels or due to differences in the functionality of the expressed proteins. Thus, we conducted a second experiment to evaluate the expression levels of the individual proteins. For this purpose, we constructed expression vectors of individual mutants having a C-terminal FLAG tag, as described above (**Fig. 2b**). As shown in **Fig. 3c**, the signal intensities of the immunoreactive protein band observed with mitochondria fractions prepared from yeast cells expressing ΔNrPiC (F267S)/FLAG or ΔNrPiC (F282S)/FLAG were not markedly different from that intensity observed with ΔNrPiC/FLAG. Thus, the effects of these mutations on the growth of the *mir1*Δ on the glycerol plate were concluded to reflect the improved functionality of the expressed proteins. It should be noted that the migration of ΔNrPiC (F267S)/FLAG or ΔNrPiC (F282S)/FLAG in the polyacrylamide gel was slightly slower than that of ΔNrPiC/FLAG. The exact reason causing changes in the degree of migration in the polyacrylamide gel by this point mutation is uncertain, but it seems not to be such a rare case; because we have observed a similar phenomenon with the mitochondrial voltage-dependent anion channel (VDAC) [29].

3.6. Measurement of the phosphate transport function of the mutant rPiCs expressed in yeast mitochondria

To confirm the effects of a mutation in ΔNrPiC on its transport function, we further assessed the transport activity of the PiC expressed in yeast mitochondria. Toward this end, we prepared mitochondria from individual transformants and subjected them to

swelling in either an NH_4Cl or NH_4Pi solution [23]. As shown in **Fig. 4a**, mitochondria prepared from *mir1*Δ cells showed no remarkable difference in the swelling in these two solutions; whereas those prepared from yeast cells expressing yPiC swelled more significantly in the NH_4Pi solution than in the NH_4Cl solution, reflecting functional phosphate transport in the latter mitochondria. Mitochondria prepared from yeast cells expressing ΔNrPiC also showed no remarkable difference in the swelling in either solution, but those from cells expressing ΔNrPiC (F267S) or ΔNrPiC (F282S) showed stronger swelling in the NH_4Pi solution than in the NH_4Cl one. These results clearly validated our conclusion that ΔNrPiC (F267S) or ΔNrPiC (F282S) but not ΔNrPiC showed the phosphate transport activity.

We also evaluated the transport function of individual carrier proteins by direct measurement of the phosphate uptake by using [^{32}P]phosphate as a tracer, and obtained essentially the same results, as shown in **Fig. 4b**, as those observed with the swelling experiments.

Ideally, it would be possible to demonstrate a difference in sensitivities of mammalian and yeast mitochondrial phosphate carriers to *N*-ethylmaleimide (NEM) [30,31] by using mitochondria prepared from yeast cells expressing yPiC and ΔNrPiC (F267S) or ΔNrPiC (F282S). We succeeded in observing the inhibition of both mammalian and yeast mitochondrial phosphate carriers by exposing the mitochondria to higher concentrations of NEM (data not shown), but trials to demonstrate a difference in sensitivities of mammalian and yeast mitochondrial phosphate carriers to NEM were not successful, due to the difficulties in adjusting the proper experimental conditions to demonstrate such a difference, because sufficient amounts of mitochondria with high purity would be required.

3.7. Depletion of presequence and amino acid substitution were also effective in the functional expression of human PiC in yeast mitochondria

The above results clearly indicated that depletion of the presequence was essential for expression of rat PiC in yeast mitochondria, but ΔNrPiC thus expressed was inactive, and that further substitution of F267 or F282 with Ser was necessary for restoring the transport function of the expressed ΔNrPiC in yeast mitochondria. We next tested whether depletion of the presequence and amino acid substitution were also effective in the functional expression of human PiC in yeast mitochondria.

As two splice variants of hPiC, i.e., hPiC-A and hPiC-B, showed essentially the same properties (data not shown) as observed with rPiCs (Fig. 2), we conducted the following studies using the hPiC-A variant, and hPiC-A and ΔNhPiC-A will hereafter be simply

referred to as hPiC and ΔNhPiC, respectively.

When we compared the growth of yeast cells expressing full-length human PiC (hPiC), its presequence-depleted form (ΔNhPiC), and yPiC on the glycerol plate, cells expressing hPiC failed to grow. The yeast cells expressing ΔNhPiC showed slight growth, but its level was almost negligible in comparison with that of cells expressing yPiC (**Fig. 5**). Substitution of either F267 or F282 in ΔNhPiC with Ser caused remarkable acceleration of the growth rate of the host cells, to a level of growth comparable to that of the yPiC transformant.

Thus, mutations of these Phe residues to Ser ones were concluded be effective for functional expression of not only rat PiC but also human PiC. The introduction of the mutated (F267S or F282S) hPiC expression vector harboring its presequence into the *mir1*Δ cells also slightly accelerated the growth of these cells on the glycerol plate, but this effect was negligible. Thus, in addition to the depletion of the presequence, the substitution of F267 or F282 with Ser was concluded to be effective for functional expression of not only rat PiC but also human PiC in yeast cells.

3.8. Further substitution studies on the amino acids of yPiC and mammalian PiC

To explore a possible reason why substitution of F267S or F282S of mammalian PiC was effective in restoration of their function in yeast cells, we further conducted two experiments. The first experiment involved reverse substitution of P274 or G289 of yPiC, corresponding to F267 or F282 of ΔNrPiC or ΔNhPiC, respectively, with Phe. As shown in **Fig. 6a**, yeast cells expressing yPiC (P274F) or yPiC (G289F) grew well on the glycerol plate, indicating that a Phe residue at these positions was not inhibitory for the function of yPiC. In the second experiment we examined the effect of substitution of the Phe residue of the mammalian PiC with amino acids other than Ser. This experiment was conducted with the F282 residue of ΔNrPiC. As shown in **Fig. 6b**, yeast cells expressing ΔNhPiC of which F282 was substituted with a basic (F282K) or acidic (F282E) or hydrophobic (F282L) residue failed to grow, but those substituted with Thr or Gly grew slightly.

4. Discussion

An earlier study clearly demonstrated that deletion of the presequence of the mammalian mitochondrial phosphate carrier facilitates the import of the carrier into yeast mitochondria [19]. In the present study, we addressed the more intriguing question of whether the mammalian phosphate carrier could be expressed in a functional

form in yeast mitochondria. For this purpose, we first examined whether the growth-defect phenotype of a yeast strain lacking a functional phosphate carrier gene, *MIR1* (*mir1*Δ), on a glycerol plate could be rescued by introduction of an expression vector of rPiC. The introduction of expression vectors encoding neither full-length rat PiC (rPiC) nor rat PiC lacking its presequence (ΔNrPiC) was effective in rescuing *mir1*Δ cells from their growth defect. To understand the reason for this ineffectiveness, we further examined the expression levels of individual proteins. As a result, the presequence-depleted form (ΔNrPiC), but not the full-length form (rPiC), was well expressed at the mitochondrial membrane, as was yPiC (**Fig. 2**). Thus, the presequence-depletion effects of the mammalian PiC on the effective sorting of PiC into yeast mitochondria [19] were clearly reconfirmed in the present study. In addition, these results clearly indicated that ΔNrPiC expressed in yeast mitochondria was inert.

Thus, we next considered the reason as to why it was inert. To address this issue, by expressing mutated ΔNrPiC in *mir1*Δ cells, we sought to isolate "revertants" that would be viable on the glycerol plate. For such a purpose, mutagenesis by ethyl methanesulfonate has been employed [32,33]. However, this procedure induces non-selective mutations in the genome; and hence, higher efforts are required for identification of the mutation responsible for the functional expression of the target carrier. In the present study, we employed the strategy combining the "PCR-mediated random mutation" and "gap-repair cloning". The use of this strategy seems to have the great advantage of avoiding the side effects, i.e., "off target effects". Actually, in the present study, we obtained only two colonies; and both carried effective mutations responsible for the functional expression of ΔNrPiC in yeast mitochondria. Thus, the use of this strategy combining the "PCR-mediated random mutation" and "gap-repair cloning" procedures was proven to be quite effective for identification of the amino acid residues responsible for the functional expression of the mitochondrial phosphate carrier.

With these experiments, we succeeded in clearly demonstrating the restorative effect of the mutation F267S or F282S in ΔNrPiC on its transport function. Interestingly, the amino acid residues F267 and F282 were conserved in hPiC. Thus, we next conducted an experiment to determine whether these mutations would also be effective in the functional expression of the hPiC. The *mir1*Δ cells transformed with expression vector encoding hPiC failed to grow on a glycerol plate, as in the case of those transformed with expression vectors of rPiCs; whereas those transformed with the ΔNhPiC expression vector grew slightly (**Fig. 5**). Mutation of F267S or F282S in ΔNhPiC significantly improved the growth of the transformant, and this result clearly indicated

that either mutation was also effective in restoring the transport activity of ΔNhPiC expressed in yeast mitochondria. The reason why *mir1*Δ cells transformed with the ΔNhPiC expression vector could grow slightly on a glycerol plate, distinct from cells transformed with the expression vector encoding ΔNrPiC, is uncertain. The 16 amino acid residues not conserved between ΔNrPiC and ΔNhPiC could be responsible for the observed difference, but this difference was too slight and seemed not intriguing enough to warrant further investigation.

Then, the question arose as to why replacement of Phe residues at positions of 267 or 282 to Ser was effective in restoring the transport function of ΔNrPiC and ΔNhPiC expressed in yeast cells. At this moment, we do not have any clear evidence that could explain such an elevation of this transport function. The inevitable roles of a Ser residue at these positions for transport function of the PiC could be excluded, because the amino acids in yPiC corresponding to the Phe residues at positions of 267 and 282 in mammalian PiC are not Ser ones (**Fig. 1c**). Furthermore, reverse substitution of P274 or G289 of yPiC, corresponding to F267 or F282 of ΔNrPiC or ΔNhPiC, respectively, with Phe did not affect the growth of the host cells (**Fig. 6a**). However, proper interpretation of this result would be difficult, because the amino acid sequence of PiC is not highly conserved between yeast and mammals. The difference in phospholipid composition between mammalian and yeast mitochondria [34,35] might be a possible reason, because it is well known that certain phospholipids interact with mitochondrial solute carriers or are essential for their function [36,37]. This interpretation would be reasonable, because these two Phe residues in PiC identified in the present study have been estimated to be located at the putative 6th transmembrane region and to face the phospholipid phase rather than the internal cavity of the carrier [38]. In addition, the results of our substitution study on F282 in ΔNrPiC with several amino acids (**Fig. 6b**) also accord with this interpretation. However, to ascertain the validity of this interpretation, further studies such as reconstitution experiments would be necessary.

In the present study, we did not examine whether the expressed protein was properly localized at the mitochondrial inner membrane, but just dealt with it as “expressed well in the mitochondria”. However, in an exact sense, we should also examine the sub mitochondrial localization of the expressed protein, as well as the effects of a point mutation (F/S) on it.

In summary, we explored the factors required for functional expression of the mammalian mitochondrial phosphate carrier in yeast mitochondria. The depletion of the presequence was effective for expression of mammalian PiC proteins in yeast

mitochondria, but further substitution of F267 or F282 with Ser was necessary for their functionality. The results obtained in this study are important for further investigation of the mitochondrial phosphate carrier in mammals.

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Figure Legends

Fig. 1. Structural features of the gene encoding mammalian PiC and its mRNA, N-terminal amino acid sequences of proteins for expression in yeast mitochondria examined in the present study, and comparison of the amino acid sequences of rat and human PiCs.

Panel a, Organization of the gene encoding mammalian PiC. The gene consists of 8 exons, and two splice variants (PiC-A and -B) are formed by alternative use of one of these exons, 3A or 3B. **Panel b,** Amino acid sequences of the N-terminal region of proteins used for expression in yeast mitochondria examined in the present study. **Panel c,** Comparison of the amino acid sequences of rat and human PiCs. The amino acid sequences of the two splice variants of rat PiC (rPiC-A and rPiC-B) and of human PiC (hPiC-A and hPiC-B) are cited from NM_001270788.1 (rPiC-A), NM_139100.2 (rPiC-B), NM_005888.3 (hPiC-A), and NM_002635.3 (hPiC-B), respectively; and their homology alignment was achieved by using the GENETYX program (GENETYX Corporation, Tokyo). Numbers in the left and right margins represent the amino acid numbers counted with the N-terminal Ala residue in the mature protein taken as +1 [18]. The amino acid residues perfectly conserved among the 4 sequences are indicated by asterisks. The two broken perpendicular lines indicate the borders of amino acid sequences encoded by the alternative third exons, and the N-terminal presequences are highlighted by the box. The two Phe (F) residues found to be critical for lack of functional expression of rPiC-A in yeast mitochondria, and corresponding human residues, are highlighted in blue. The amino acid sequence of the C-terminal region of yPiC (NM_001181735) is given on the bottom line to show that these two Phe residues are not conserved in yeast.

Fig. 2. Effects of introduction of expression vectors into yeast cells lacking functional *MIR1* gene (*mir1*Δ) on their growth on a glycerol plate, and expression analysis of various PiCs in yeast mitochondria

Panel a, Culture suspensions of yeast cells lacking a functional *MIR1* gene (*mir1*Δ) and transformants of the *mir1*Δ strain with a multi-copy type (pYO326) expression vector for yPiC, rPiC-A, ΔNrPiC-A, rPiC-B or ΔNrPiC-B were streaked onto a YPD or YPGly plate. After incubation at 30° C for 2 or 3 days, cell growth on the plates was evaluated from photographs. **Panel b**, Mitochondria fractions were prepared from the *mir1*Δ strain transformed with the indicated individual expression vectors. Samples of 20-μg (for detection of FLAG-tagged PiC) or 5-μg (for Por1p) aliquots of mitochondrial

proteins were subjected to SDS-PAGE and subsequent Western analysis with anti-FLAG (for detection of PiC having FLAG tag) or Por1p (control experiment) antibodies.

Fig. 3. Growth properties of yeast cells expressing rPiC and its mutants, and evaluation of their expression levels

Panel a, Observation of the growth of yeast cells transformed with various expression vectors. Culture suspensions of yeast cells lacking a functional *MIR1* gene (*mir1*Δ) and transformants of the *mir1*Δ strain produced with the multi-copy type (pYO326) expression vector for yPiC, rPiC-A, ΔNrPiC-A, F267S or F282S were streaked onto a YPD or YPGly plate. After incubation at 30° C for 2 (YPD plate) or 5 days (YPGly plate), cell growth on the plates was evaluated from photographs. **Panel b**, For evaluation of the growth rates of individual yeast cells, culture suspensions of yeast cells were diluted with sufficient amounts of water to obtain an optical density at 600 nm of 1, 0.1 or 0.01, spotted onto a YPD or YPGly plate, and incubated for 2 or 3 days. **Panel c**, For evaluation of the expression levels of rPiC-A, ΔNrPiC-A, ΔNrPiC (F267S), and ΔNrPiC (F282S), expression vectors encoding FLAG-tagged proteins were prepared and used for transformation. Mitochondrial fractions were prepared from individual cell lines, and samples of 20-μg (for detection of FLAG) or 5-μg (for Por1p) aliquots of proteins were subjected to SDS-PAGE and subsequent Western analysis with anti-FLAG or Por1p antibodies.

Fig. 4. Phosphate transport function of the PiCs expressed in yeast mitochondria

Panel a, Phosphate transport function of the PiCs expressed in yeast mitochondria was assessed by measuring the time-dependent turbidity change in mitochondrial suspensions containing either 120 mM NH_4Pi or 120 mM NH_4Cl [23], and the obtained results are shown by solid lines or broken lines, respectively. Mitochondrial fractions prepared from the *mir1*Δ strain or from the *mir1*Δ strain transformed with the expression vector of yPiC were used as negative and positive controls, respectively. **Panel b**, Phosphate transport function of PiCs expressed in yeast mitochondria was also conducted by measuring [^{32}P]phosphate uptake.

Fig. 5. Growth properties of yeast cells expressing hPiC and of its mutants

Experiments were performed as stated in the legend for Fig. 3b.

Fig. 6. Growth properties of yeast cells expressing mutant yPiC and ΔNrPiC

Panel a, Effects of the substitution of the amino acids in yPiC corresponding to F267 and F282 of mammalian PiC (P274 and G289, respectively) with Phe on the growth of yeast cells were evaluated. **Panel b,** Effects of the substitution of F282 of ΔNrPiC with amino acids other than Ser on the growth of yeast cells were evaluated.

Fig 1

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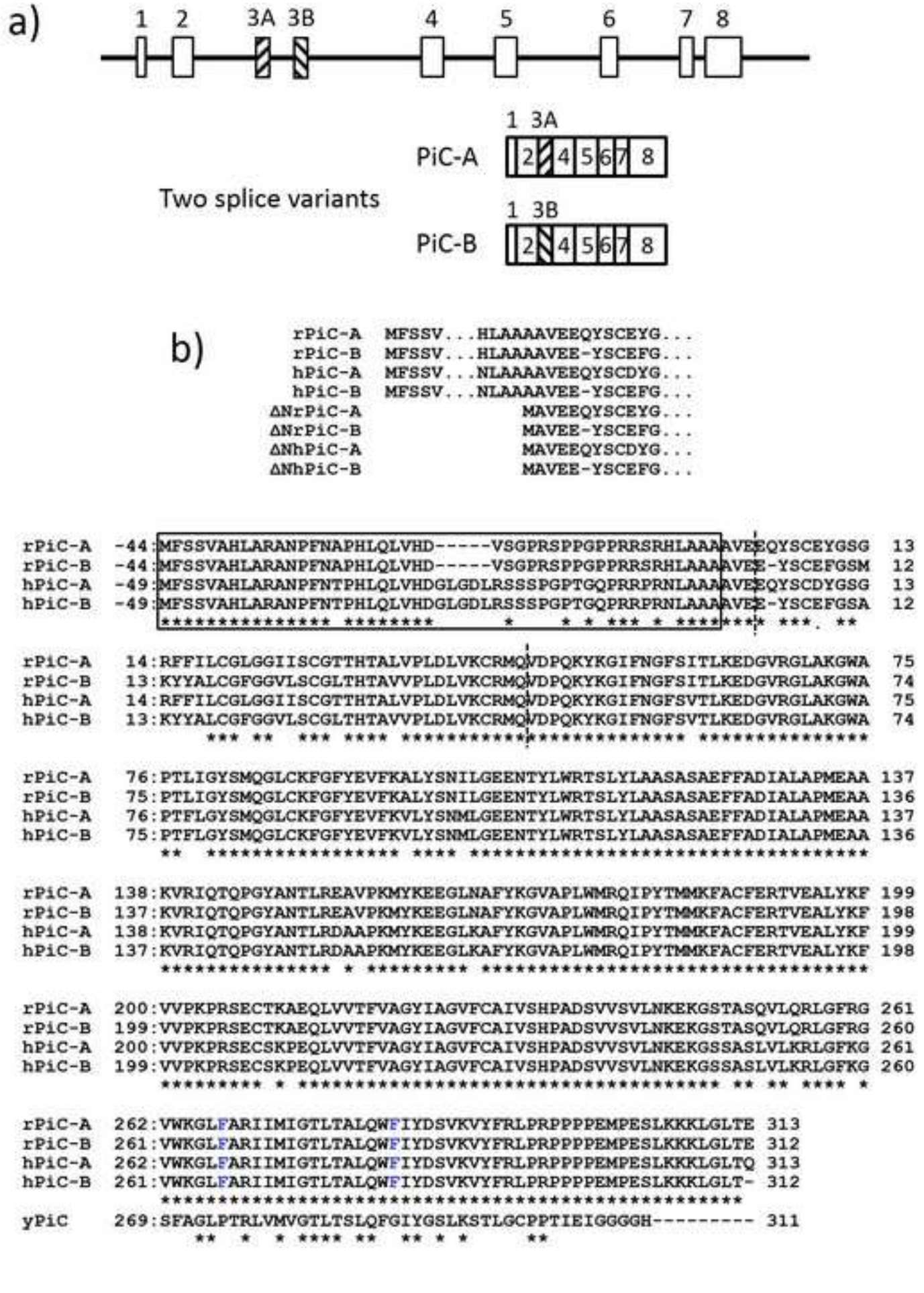

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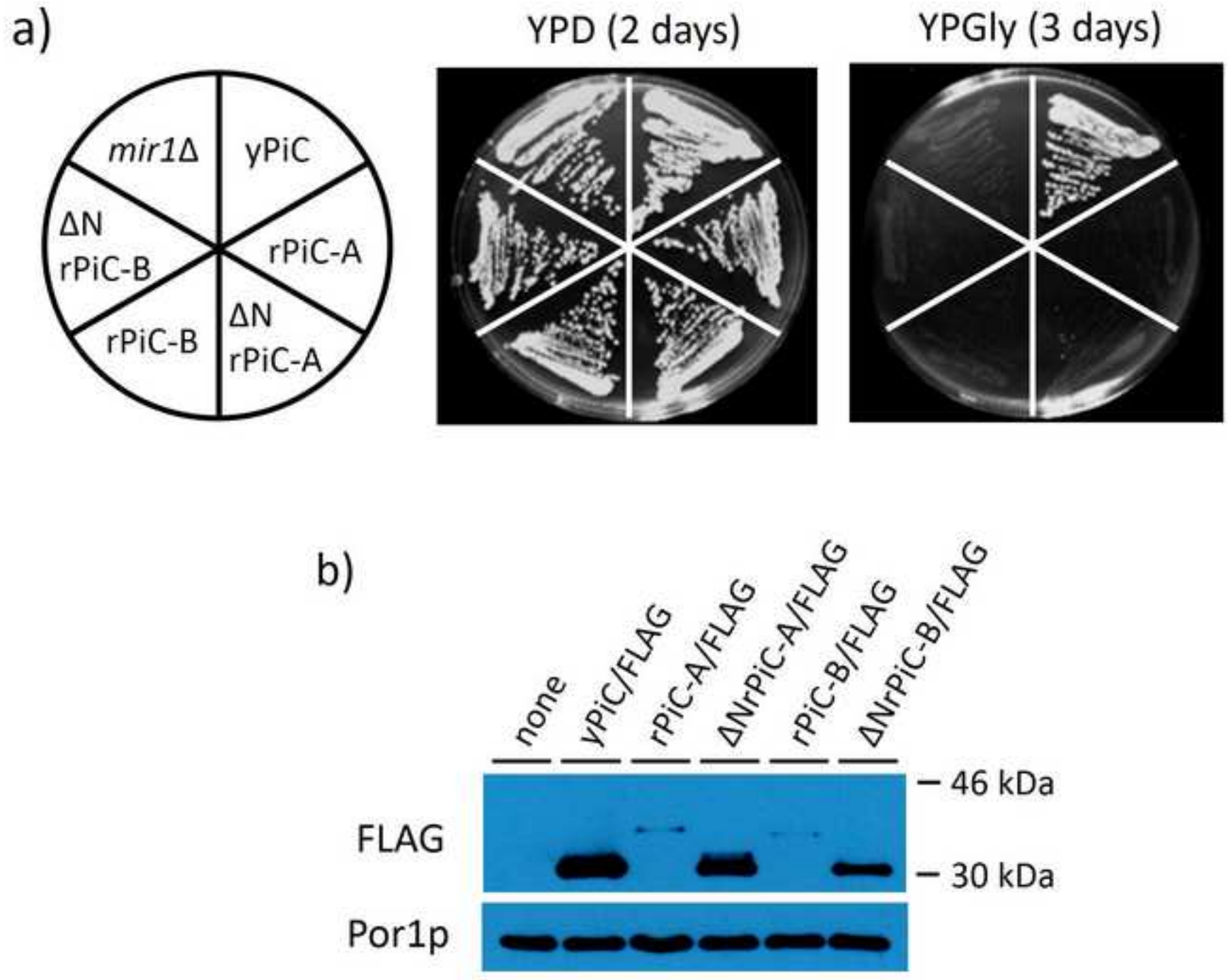

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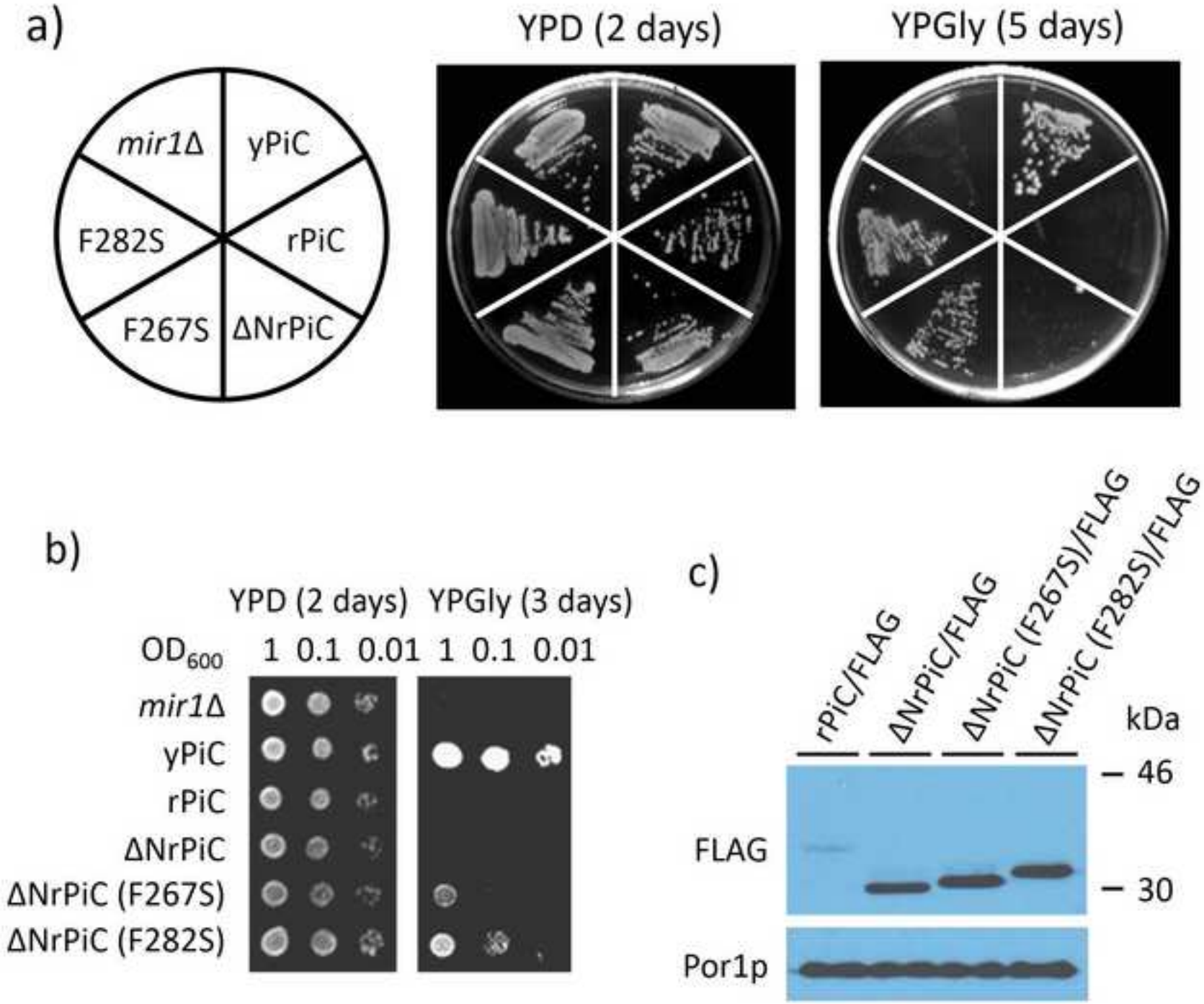

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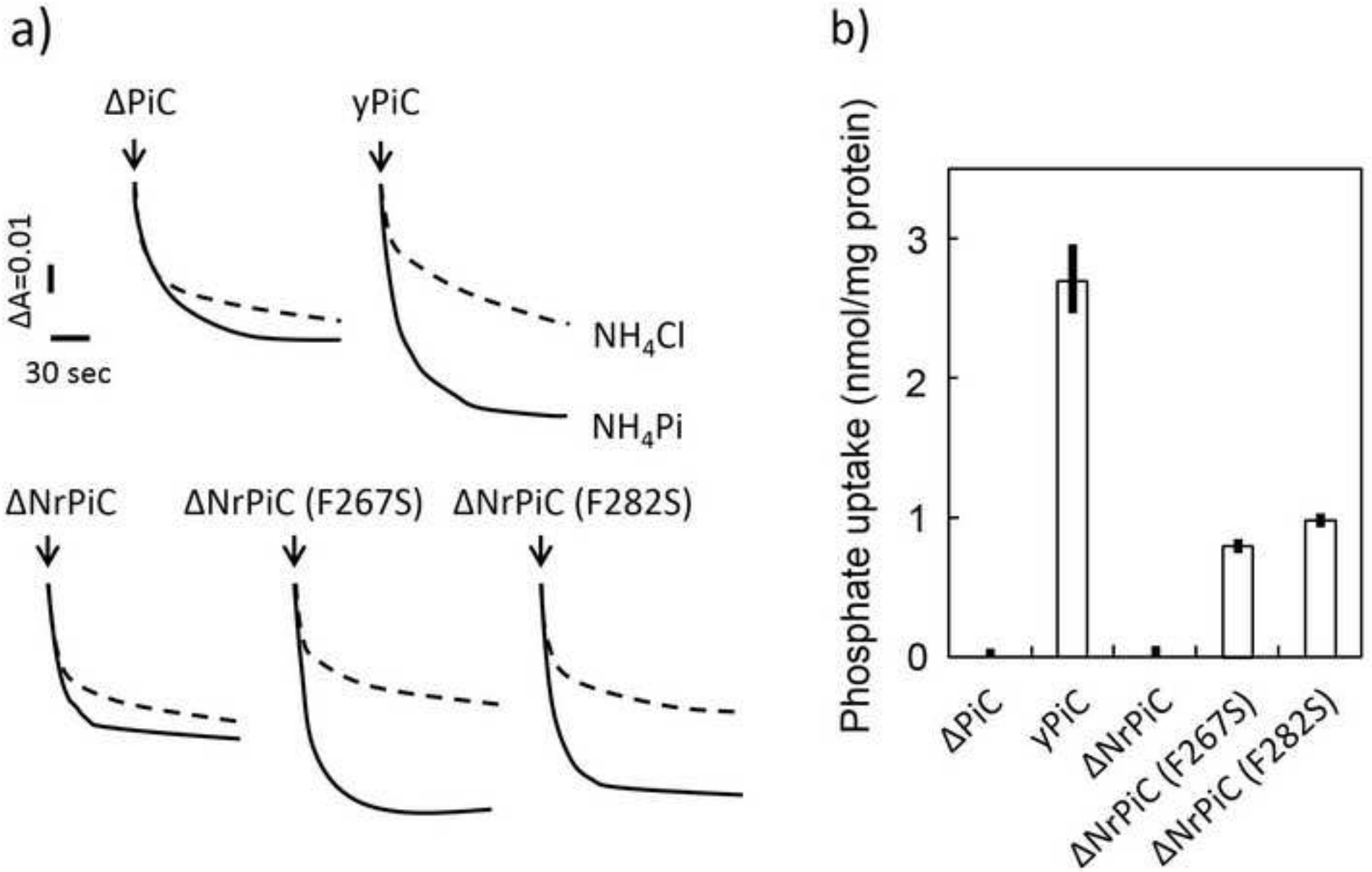

Fig 5

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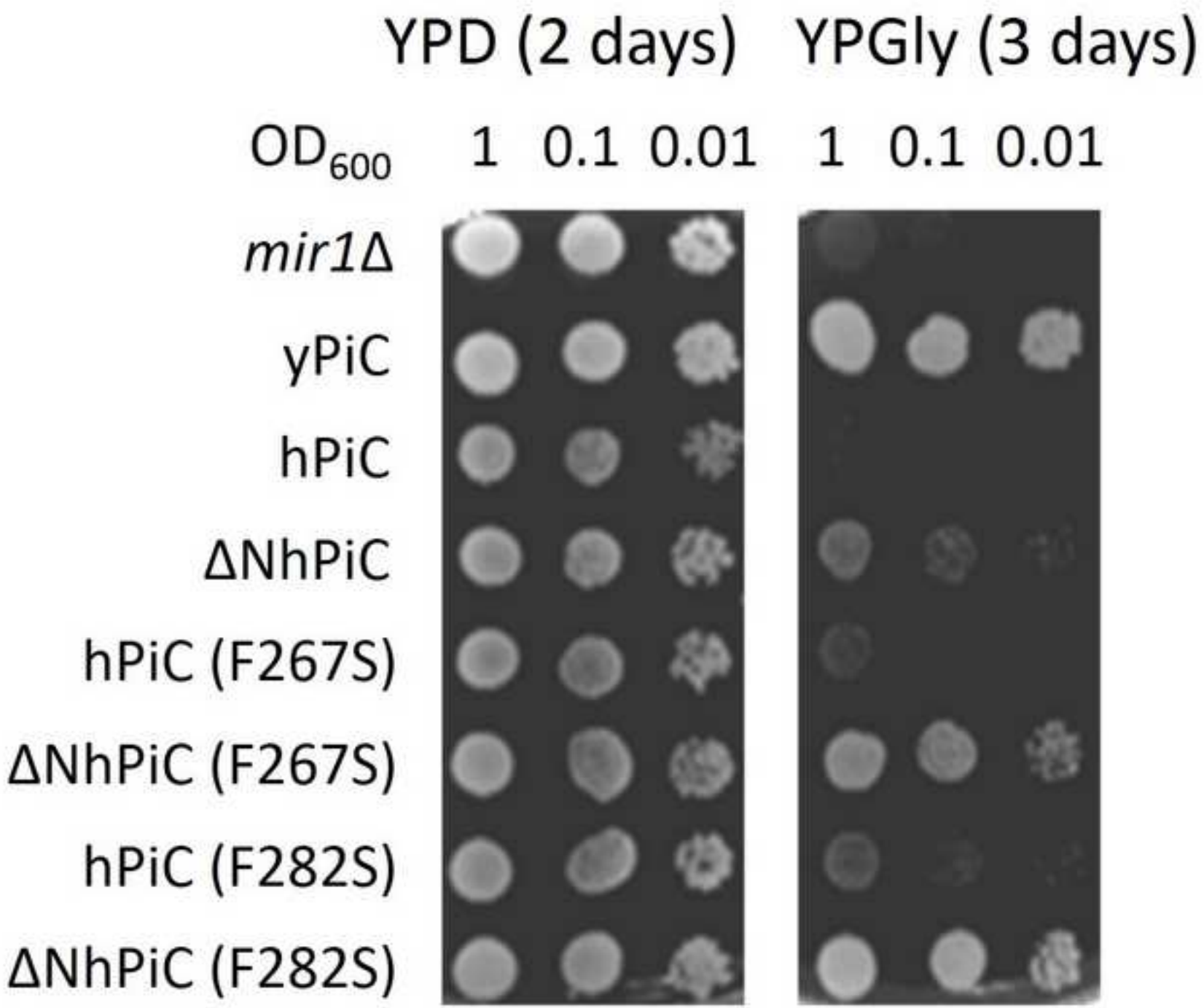

Fig 6

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