Changing the Substrate Specificity of PDE7B by Mutation and Selection in *Schizosaccharomyces pombe*

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Abstract

Phosphodiesterases (PDEs) hydrolyze important cell-singaling molecules: cAMP, cGMP, or both, thereby terminating the signal. The molecular basis for substrate specificity of PDEs remains poorly understood, although there are two hypotheses: glutamine switch and multiple elements. In a genetic screen for yeast strains that express change-in-substrate mutations in the human PDE7B, which is cAMP-specific, we identified eleven candidates that display an enhanced resistance to the toxic effect of cGMP as they enter stationary phase. We developed a protocol by which we can enrich for mutants of interest. Sequence comparison of one candidate with wild type PDE7B revealed an A to C mutation (transversion) that caused an amino acid change (E352A).

Summary

Phosphodiesterases (PDEs) are enzymes that break down two related compounds which play a key role in cell signaling pathways: cAMP, cGMP, or both, in this way ending the signal. What decides which compound can be hydrolyzed is not fully understood, but there are two hypotheses: one amino acid is important, or several of them. We developed a procedure to enrich for and identify yeast strains expressing a cAMP-specific PDE that had been mutated so as to recognize both cAMP and cGMP. We identified eleven such candidates for PDE7B, an enzyme which belongs to the PDE family. The change in the action of the enzyme was caused, in one candidate, by the mutation that resulted in the change of glutamic acid to alanine in position 352.

1 Introduction

1.1 Cyclic Nucleotide Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that catalyze the hydrolysis of cyclic adenosine monophosphate (cAMP) to adenosine monophosphate (AMP), or cyclic guanosine monophosphate (cGMP) to guanosine monophosphate (GMP) (Figure 1). As such, PDEs help to regulate the levels of the second messengers cAMP and cGMP in the cell by controlling their rates of degradation [1]. Second messengers are molecules that transmit signals from cell surface receptors to target molecules inside the cell. Both cAMP and cGMP are important because they mediate the response of cells to many hormones and neurotransmitters [2]. They are also involved in cardiac and smooth muscle contraction, inflammation, memory, and circadian regulation [2]. Several important drugs are PDE inhibitors, including Viagra, which is used to treat erectile dysfunction and pulmonary arterial hypertension. Viagra inhibits cGMP-specific phosphodiesterase type 5 (PDE5) [1].

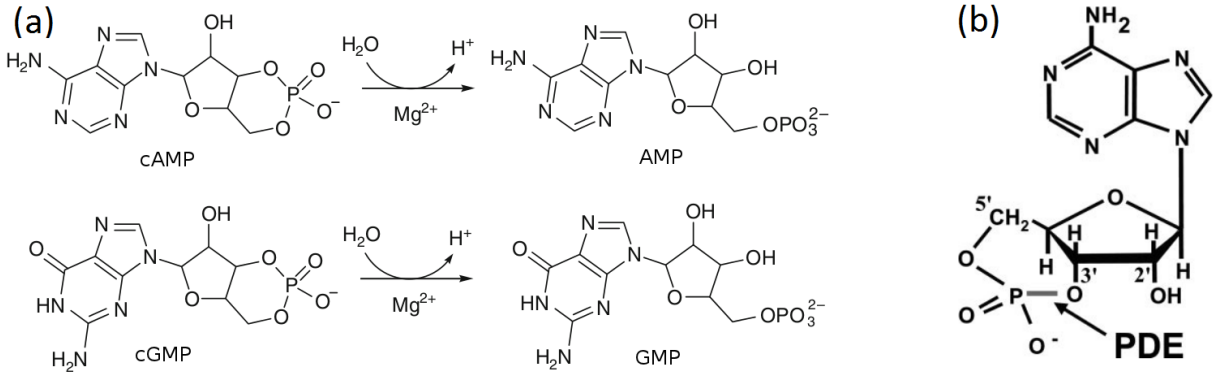

Figure 1: (a) Hydrolysis of cAMP and cGMP to 5'-AMP and 5'-GMP by PDEs [2]. (b) PDEs hydrolyze the 3' cyclic phosphate bond [1].

1.2 PDE Families and PDE7B

The PDE superfamily in mammals consists of 11 PDE families that are encoded by 21 genes, each of which has different isoforms and splice variants [1]. PDEs differ in substrate specificity. PDE4, PDE7, and PDE8 are cAMP-specific, whereas PDE5, PDE6, and PDE9 are cGMP-specific, and PDE1, PDE2, PDE3, PDE10, and PDE11 have dual specificity [1]. PDEs also differ in three-dimensional structure, kinetic properties, cellular expression, inhibitor sensitivities, and modulated regulatory pathways [1]. PDE7B specifically hydrolyzes cAMP with high affinity, showing little to no activity against cGMP [3]. It is expressed in pancreas, brain, heart, thyroid, skeletal muscle, eye, ovary, submaxillary gland, epididymus, and liver [3].

1.3 Determination of Substrate Specificity: Hypotheses

Substrate specificity is determined by amino acids in the active site of the enzyme. Zhang *et al.* proposed a glutamine switch mechanism for nucleotide selectivity of PDEs [4]. According to their hypothesis, a particular glutamine residue in the active site may have different orientations when hydrogen-bonded to cAMP or cGMP. In contrast, Ke *et al.* described a mechanism in which the substrate specificity is determined by multiple elements [2]. The argument against the glutamine switch hypothesis is that the conserved glutamine in some dual-specific PDEs is not free to rotate, and binding of the products does not simulate binding of the substrates. Furthermore, the crystal structures of protein-cAMP complexes of PDE4D2 and PDE10A2 determined by Wang *et al.* reveal that this invariant glutamine (Gln369) forms only one hydrogen bond with the adenine of cAMP [5]. Each PDE family may have a different mechanism for substrate specificity, as structural comparison between PDE4D2-cAMP and PDE10A2-cAMP showed an *anti* configuration of cAMP in PDE4D2 but a *syn* configuration in PDE10A2, and different contact patterns of cAMP in both structures [5].

1.4 Yeast as a Model Organism to Study Mammalian PDEs

Saccharomyces cerevisiae (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) are both popular model organisms. Yeast transformation was a model system for the study of recombination [6]. As a eukaryote, yeast has been used extensively to study yeast proteins and to express and study mammalian proteins, including PDEs. Molecular genetic research in these organisms has been facilitated by working with cloned genes carried on autonomous plasmids. For example, Colicelli *et al.* cloned a mammalian gene encoding a high-affinity cAMP phosphodiesterase from a rat brain cDNA library in *S. cerevisiae* [7]. Yeast expressing PDEs has been also important in screening for drugs and drug-resistant mutants. For instance, Pillai *et al.* performed a screen to isolate drug-resistant mutant of mammalian PDE4B [8]. In their experiment, yeast with no PDE were heat-shock sensitive, whereas yeast with PDE were heat-shock resistant, and addition of inhibitor made yeast expressing PDE heat-shock sensitive [8]. Mutations of PDEs help to discover key factors determining substrate specificity. Sequence comparison shows that cAMP-specific PDE4D3 has a conserved aspartic acid at position 333, and cGMP-specific enzymes have a conserved asparagine at this position [9]. Mutants with alanine at this position can hydrolyze cGMP, which suggests that aspartic acid inhibits cGMP binding rather that asparagine facilitates it [9].

1.5 Studies of Fission Yeast

Hoffman and Winston discovered that in *S. pombe* glucose detection leads to a cAMP signal that regulates *fbp1* transcription [10] (Figure 2). Glucose is sensed by the Git3 G-protein coupled receptor, which activates the heterotrimeric G-protein (Gpa2, Git5, Git11). The α subunit of the G-protein (Gpa2) activates adenylate cyclase (Git2/Cyr1), which produces a cAMP signal to activate protein kinase A (Pka1). In turn, Pka1 represses *fbp1* transcription

(*fbp1* encodes fructose-1,6-bisphosphatase) [11]. Activity of mammalian PDEs in *S. pombe* can be assayed using an *fbp1-ura4* reporter to allow for small molecule inhibitor screening. Cells expressing *ura4* can be selected in media lacking uracil, or cells not expressing *ura4* in media containing 5FOA [11]. In addition, DeVoti *et al.* found that loss of PDE inhibits mating and reduces viability in stationary phase [12].

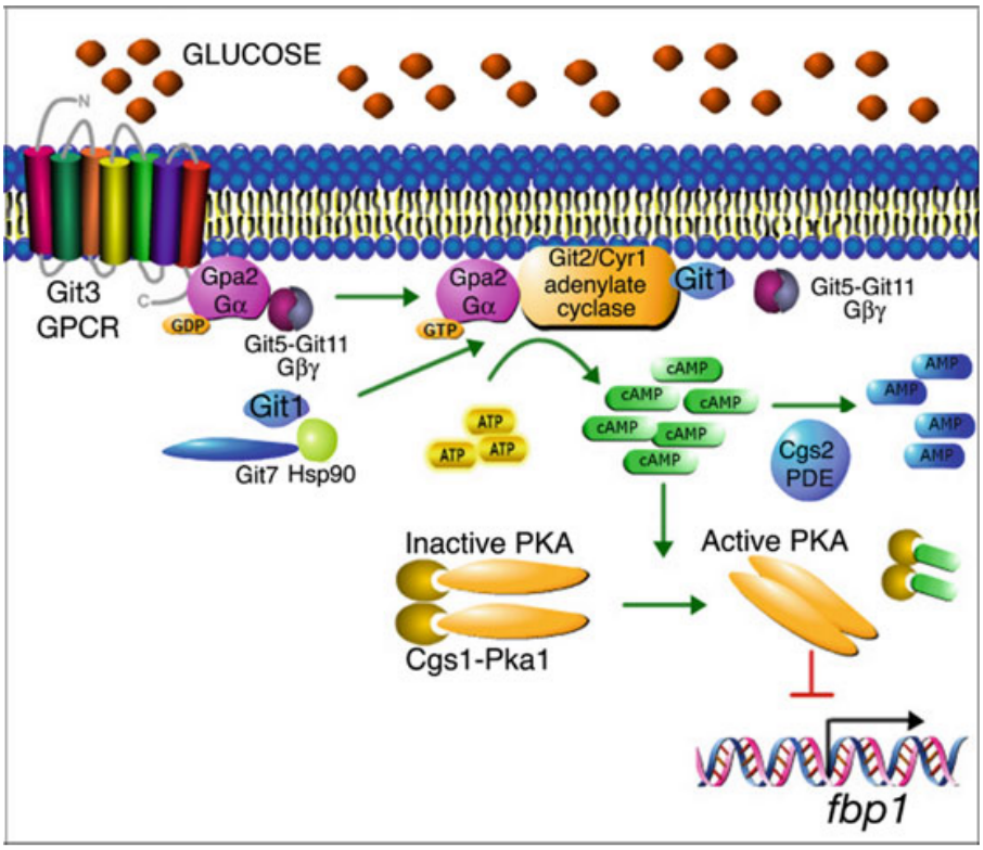

Figure 2: The *S. pombe* glucose/cAMP signaling pathway that regulates *fbp1* transcription. The Git3 G-protein coupled receptor senses glucose, and activates the heterotrimeric G-protein (Gpa2, Git5, Git11). The α subunit of the G-protein (Gpa2) then activates adenylate cyclase (Git2/Cyr1), leading to a cAMP signal which activates protein kinase A (Pka1). In turn, Pka1 represses *fbp1* transcription (*fbp1* encodes fructose-1,6-bisphosphatase) [11].

1.6 Objectives

The goal of this project was to use *S. pombe* to screen for mutant forms of human PDE7B that have the ability to hydrolyze cGMP (Figure 3). We amplified the PDE7B gene by PCR that introduced mutations. We did not use error-prone PCR because it might introduce too many mutations at one time. The PCR product and the linearized plasmid were used to co-transform *S. pombe* to create a library of mutant alleles, as described previously by Kostrub *et al.* [13]. Homologous recombination between the insert and the vector produced a collection of yeast transformants with a possibly mutated copy of the PDE7B gene [13]. These transformants were enriched for cells that could survive in the presence of cGMP and screened for mutants possessing cGMP PDE activity. In the absence of a PDE that could hydrolyze cGMP, exogenous cGMP prevented stationary phase entry, leading to cell death. In this way, the population was enriched with cells expressing PDE7B that acquired the ability to hydrolyze cGMP. After two to three rounds of enrichment, single colonies were examined for enhanced survival in cGMP solution compared to the control. Successful candidates were sequenced to determine which mutations caused the change in PDE7B substrate specificity. The amino acid sequence was analyzed in conjunction with structural data.

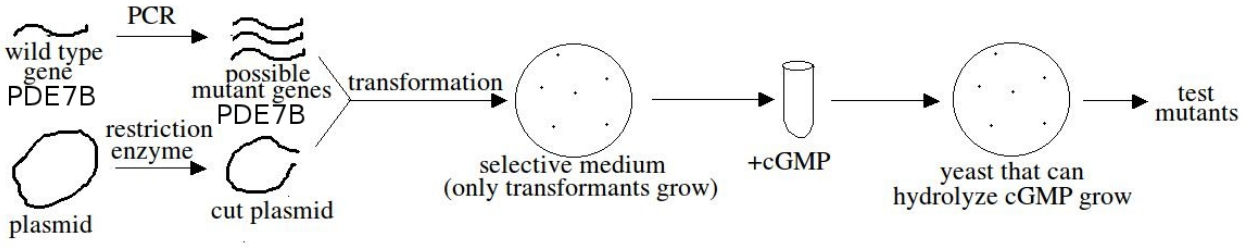

Figure 3: Outline of the project. A collection of mutant PDE7B alleles was created by the transformation of *S. pombe*. After several rounds of enrichment with cells expressing PDE7B that acquired the ability to hydrolyze cGMP, single colonies were examined for enhanced survival in cGMP solution. Sequencing of successful candidates led to determining which mutations were responsible for the change in PDE7B substrate specificity.

2 Materials and Methods

2.1 *S. pombe* strains

Yeast strains used are listed in Table 1. CHP1265 and CHP1611 strains that possess a plasmid pKG3-dropout (without PDE gene) or pKG3-PDE7B (with wild type PDE7B gene) were control strains (developed previously by Hoffman lab).

Strain	Genotype	Phenotype
CHP1265	*gpa2* mutant *cgs2-2* mutant	very low cAMP production no PDE activity
CHP1611	*git3* mutant *cgs2-2* mutant	moderately low cAMP production no PDE activity

Table 1: Yeast strains used in the study.

2.2 Growth Media

Yeast extract medium (YES – solid, YEL – liquid) and Edinburgh Minimal Medium (EMM) were used as growth media. YES/YEL are rich media, supplemented with 225mg/l histidine, leucine, lysine, uracil and adenine. EMM (US Biological) is a selective medium, containing appropriate supplements. EMM-leu is EMM medium lacking leucine, which enables selection for cells able to synthesize leucine (in our experiments, transformed yeast carrying the plasmid).

2.3 PCR Amplification of PDE7B Gene

The gene encoding human PDE7B was amplified using a polymerase chain reaction (PCR). Reactions were prepared using four different FailSafe buffers (Epicentre). Each PCR mix contained 10 μl sterile water, 12.5 μl 2$\times$ buffer (B, C, J, or L), 0.5 μl forward primer (cgs2-PCRmut-for, 50 μM, TCATAGCATACTTCTTCACCAAGC), 0.5 μl reverse primer (cgs2-

PCRmut-rev, 50 μM, AAAGTGTCCGATGAGAAAAGCGTG), 1.0 μl template plasmid (1:1000 dilution), and 0.5 μl enzyme mix. The PCR program used was: (1) 2 min at 95 °C (denaturation of plasmid DNA), (2) 25 cycles of 30 s at 95 °C (denaturation of DNA), 30 s at 58 °C (annealing of primers to template DNA), 2.5 min at 72 °C (polymerization), (3) 10 min at 72 °C, (4) hold at 4 °C. After PCR, 5 μl of the reaction was removed, 2 μl STOP solution was added and gel electrophoresis was performed. The expected PCR product had the length of 1.75 kb.

2.4 Transformation of *S. pombe*

S. pombe strains CHP1265 and CHP1611 were transformed with linearized plasmid pKG3-dropout and PCR products of gene PDE7B (Figure 4), separately for the PCR product from each buffer. Yeast was cultured overnight in 5 ml EMM to a concentration of 10^7 cells/ml the next morning. Cells were diluted to 5×10^6 and set back to 10^7 cells/ml. Cells were pelleted and washed with equal volume sterile water. Cells were pelleted, brought up in 1 ml water and transferred to Eppendorf tube. Cells were pelleted and washed with 1× LiOAc/TE (buffer composition: 2 ml 10× LiOAc (1 M, pH = 7.5), 2 ml 10× TE, 16 ml sterile water). Cells were pelleted again and brought to 2×10^9 cells/ml in 1× LiOAc/TE. 1 μl linearized plasmid pKG3-dropout cut with StuI, 2 μl PCR product (one of B ,C, J, and L for each of the four transformations) and 50 μl cells were incubated 10 min at room temperature. Then 130 μl 40% PEG/LiOAc/TE (buffer composition: 2 ml 10× LiOAc, 2 ml 10× TE, 8 g PEG (3350), 9.75 ml sterile water, boiled 6–10 min) was added and incubated 2–4 h at 30 °C. 21.5 μl DMSO was added. Cells were heat shocked for 5 min at 42 °C. Transformant cells were plated on three EMM-leu plates for each transformation, using 10 μl, 95 μl and 95 μl for each plate. After seven days, colonies from plates with 10 μl were counted and colonies from all plates were collected.

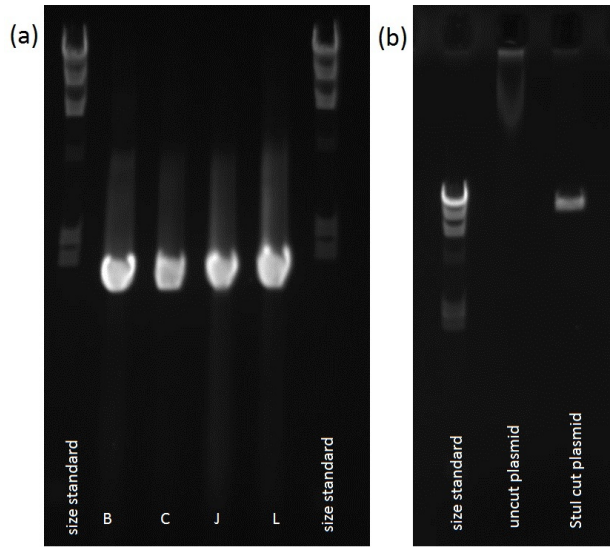

Figure 4: Insert and vector creation. (a) PCR of PDE7B. From left: size standard (bacteriophage lambda DNA cut with HindIII), PCR products from buffers B, C, J, and L, size standard. (b) Restriction digest of plasmid pKG3-dropout with StuI. From left: size standard, uncut plasmid pKG3-dropout, StuI cut plasmid pKG3-dropout.

2.5 Stationary Phase Entry Test

5×10^5 CHP1611-DO and CHP1265-DO control transformants were transferred into 100 μl YEL medium containing 0, 100, 200 and 500 μM cGMP and incubated for three days at 30 °C. 5×10^5 CHP1611-7B and CHP1265-7B control transformants were transferred into 100 μl YES medium containing 0, 100 and 200 μM cGMP and incubated for three days at 30 °C. 5×10^5 CHP1611-7B and CHP1265-7B transformants (B, C, J, and L) were transferred into 100 μl YEL medium containing 0, 100 and 200 μM cGMP and incubated for three days at 30 °C.

2.6 Viability Spot Test

In the experiments described in Section 2.5, the viability of cells was assessed by microscopy and/or a viability spot test for colony-forming units. In the viability spot test, hundred-fold diluted cultures and their dilutions (each of the next four dilutions is a five-fold dilution of

the previous) were spotted onto YES plate and allowed to grow for three days. The dilutions help to compare the number of colony-forming units. Viability was scored by counting the colonies that formed.

2.7 Enrichment for Cells Expressing PDE with cGMP Hydrolysis Activity

The first round of enrichment occured when yeast grew during the viability test. The second round of enrichment was initiated by transferring 10 μl from the first round to 90 μl of YEL with the same concentration of cGMP after three days. Similarly, the third round of enrichment was initiated by transferring 10 μl from the second round to 90 μl of YEL with the same concentration of cGMP after three days. Concentration of cGMP for enrichment was 200 μM for both CHP1265-7B and CHP1611-7B transformants (B, C, J, and L).

2.8 Generation of Single Colonies

5 μl of CHP1265-7B transformants after the second and third round of enrichment and CHP1611-7B transformants after the second round of enrichment (B, C, J, and L) were plated on EMM-leu plates to select for transformants, in a way that allows formation of single colonies. The single colony is a colony in which all cells have identical DNA because they descend from a single cell.

2.9 Screening

Twenty single colonies were collected from each of the four (B, C, J, and L) plates with CHP1265-7B transformants after the second round of enrichment, placed into 50 μl YEL with [cGMP] = 200 μM in 384-well microtiter dish and incubated for three days at 30 °C. There were also control CHP1265-7B transformants placed into one well. Each of the candidates

was examined by microscopy for enhanced viability (more live cells than in the control). Two chosen candidates were photographed and used to perform whole cell PCR. 5 μl was plated on EMM-leu plate to regrow and the rest was frozen. Ten single colonies were collected from each of the four (B, C, J, and L) plates with CHP1265-7B transformants after the third round of enrichment and CHP1611-7B transformants after the second round of enrichment, placed into 50 μl YEL with [cGMP] = 200 μM in 384-well microtiter dish and incubated for three days at 30 °C. Each of the CHP1265-7B candidates was examined by microscopy for enhanced viability. Nine candidates were chosen and plated on EMM-leu plates.

2.10 Whole cell PCR

Whole cell PCR was used to amplify genes of interest directly from yeast cells. Three reactions were performed on CHP1265-7B control transformants using three different FailSafe buffers (Epicentre) to optimize conditions prior to identifying mutants of interest. Each PCR mix contained 10 μl sterile water, 12.5 μl 2× buffer (A, F, or G), 0.5 μl forward primer (7Bsh-F-, ATGTCTTGTTTAATGGTTGAGAGG), 0.5 μl reverse primer (cgs2-PCRmut-rev, AAAGTGTCCGATGAGAAAAGCGTG) and a small amount of yeast cells. The PCR program used was: (1) 10 min at 98 °C (high temperature causes cell lysis and release of DNA), (2) 35 cycles of 30 s at 94 °C (denaturation of DNA), 30 s at 58 °C (annealing of primers to template DNA), 3 min at 72 °C (polymerization), (3) 10 min at 72 °C, (4) hold at 4 °C. After PCR, 5 μl of the reaction was removed, 2 μl STOP solution was added, and gel electrophoresis was performed. As the machine was cooling to 58 °C during the first cycle of amplification, PCR was paused, 0.5 μl enzyme was added to each tube, and PCR was restarted. The whole cell PCR was performed using two candidates from CHP1265-7B transformants (B20 and L8) and buffer A, which was chosen after the pilot experiment. Before the PCR, the cell wall was digested by the enzyme zymolase, and the cells were opened by osmosis (sorbitol solution and then water) and by heating for 10 min at 98 °C.

2.11 Sequencing

The PCR products of candidates chosen among CHP1265-7B transformants after the second round of enrichment (B20 and L8) were purified using the spin column kit (QIAquick, Qiagen). The concentration of the purified DNA product was measured using NanoDrop spectrophotometer. 8 μl purified DNA at the concentration in range of 30-60 ng/μl and 2 μl of 2 mM sequencing oligonucleotide were mixed. The PCR product was sent out for sequencing to Eurofins. Three different oligonucteotides were used for each candidate: 7Bsh-F- (ATGTCTTGTTTAATGGTTGAGAGG), 7B-627F (TGCAGCAGCACACGATGTGG) and 7B-1345R (TGTCGCCTTCCTGCTCCTC). The oligonucleotides for sequencing were different from the oligonucleotides in order not to sequence possible contaminations.

2.12 Analysis of Sequence Data

Nucleotide and protein sequences of mutant PDE7B were compared with wild type PDE7B using Basic Local Alignment Search Tool (BLAST). The 3D structure of PDE7B is not available, so the amino acid sequences of PDE7B and PDE4D were aligned. The position of the amino acid which was altered in PDE7B was determined in PDE4D, for which there is available a structure of the PDE4D-cAMP complex. While PDE7B is more closely related to PDE7A than to PDE4D, there is a crystal structure of a mutant PDE4D enzyme bound to cAMP (MMDB ID: 60177, PDB ID: 2PW3), whereas the crystals of PDE7A are of enzymes bound to inhibitors that do not reveal the interactions that are relevant to this study (MMDB ID: 33884, PDB ID: 1ZKL, and MMDB ID: 71413, PDB ID: 3G3N).

2.13 Plasmid DNA Preparation for the Transformation of *E. coli.*

The plasmids pKG3, each having a copy of PDE7B gene, were isolated from CHP1265-7B yeast transformants after two or three rounds of enrichment and used to transform *E. coli*

to amplify and purify them, as described by Hoffman and Winston [14]. Yeast transformants were patched onto EMM-leu plates and collected into a tube. 0.2 ml of Smash and Grab buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris pH = 8.0, 1 mM EDTA, sterile water), 0.2 ml phenol-chloroform and 0.3 g acid-washed glass beads were added. The mix was vortexed 5 min, pelleted 5 min, and 50 μl was transferred to a new tube. 50 μl isopropanol was added. The mix was put on ice for 10 min, pelleted 10 min and resuspended in 5 μl sterile water. *E. coli* was transformed with 0.5 μl of the concentrated DNA by electroporation.

3 Results

3.1 Generation of Mutants of Interest

Transformation of the CHP1265 strain with the amplified PDE7B gene resulted in the following number of colonies on plates receiving 10 μl cells (for respective PCR buffers): B – 128, C – 321, J – 149, L – 669. Transformation of the CHP1611 strain with the amplified PDE7B gene resulted in the following number of colonies on plates receiving 10 μl cells (for respective PCR buffers): B – 164, C – 58, J – 107, L – 157. The stationary phase entry test and the subsequent viability spot test of CHP1265-DO, CHP1265-7B, CHP1611-DO and CHP1611-7B (Figure 5) optimized the concentration of cGMP for enrichment. Enrichment is the process in which the percentage of cells able to hydrolyze cGMP is increased because cells unable to do so fail to enter stationary phase and die. Too low concentration of cGMP may not lead to cell death, whereas too high concentration of cGMP may kill some mutants that have the ability to hydrolyze cGMP. The optimal concentration of cGMP is the smallest concentration that leads to cell death, and the [cGMP] = 200 μm was chosen for enrichment. On plates receiving 10 μl cells, 1267 colonies of CHP1265-7B transformants after the second round of enrichment were obtained. In total (from all 200 μl cells), there were approximately

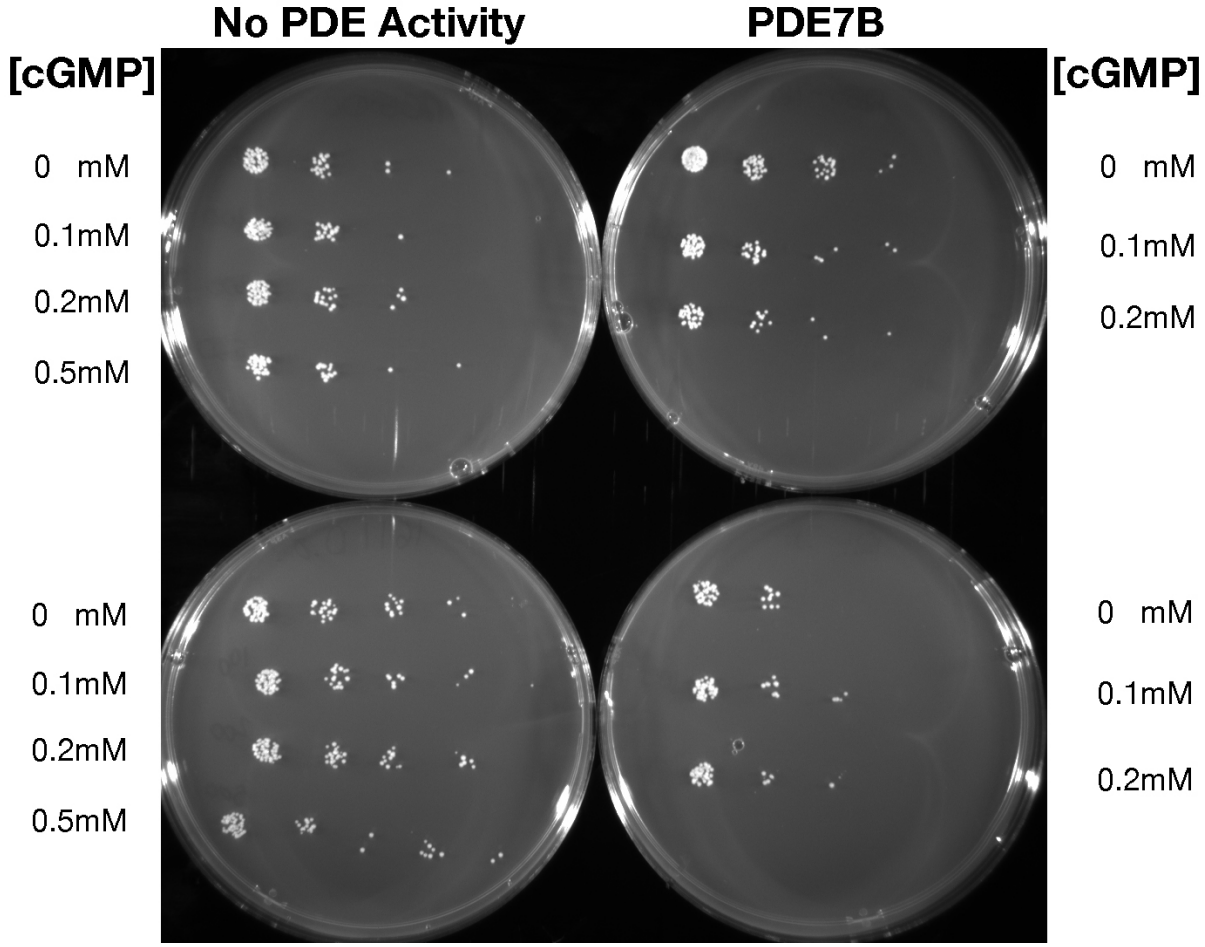

Figure 5: Viability spot test. Top: strain CHP1265. Bottom: strain CHP1611. From left (in each row): 100-fold, 500-fold, 2500-fold, 12500-fold, and 62500-fold dilutions.

25,000 colonies. Viability spot test showed that the enrichment was approximately eight-fold. The dilutions helped to estimate that the enrichment was more than five-fold and less than ten-fold. During the screening of eighty single CHP1265-7B colonies after the second round of enrichment, two colonies with enhanced viability (higher percentage of alive cells) were found (Figure 7). This means that after the second round of enrichment there was 2.5% of mutants of interest, which is equal to 1/40 (1 mutant out of 40 transformants). Assuming that the rate of enrichment was eight-fold, the initial frequency of mutant alleles of PDE7B that acquired the ability to hydrolyze cGMP was 1/2560 (Figure 6).

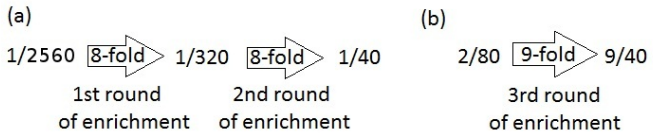

Figure 6: (a) Calculations of the frequency of mutants of interest after the first round of enrichment and before enrichment. (b) Observed frequencies after the second and third rounds of enrichment.

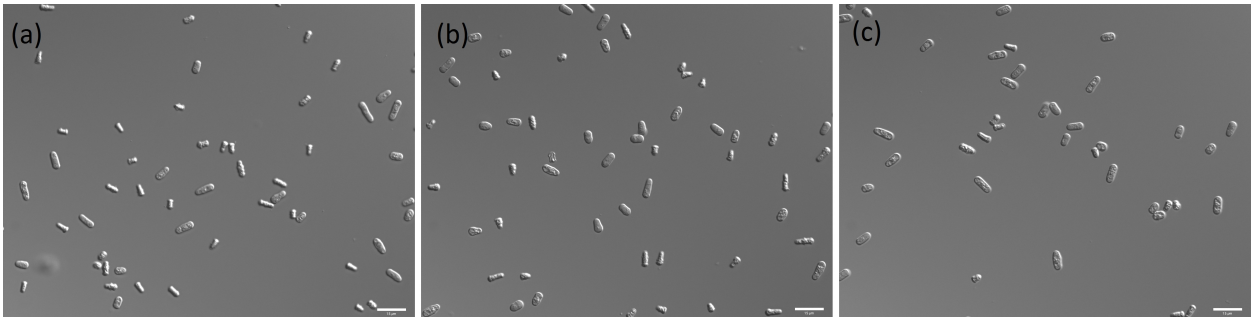

Figure 7: Representative candidates from CHP1265-7B after the second round of enrichment. (a) B10 - low viability. (b) B20 - enhanced viability. (c) L8 - enhanced viability. Scale bar – 15 μm. Dead cells are narrow, alive cells are more round.

3.2 Sequence data

PCR successfully amplified the PDE7B gene (Figure 8). The sequence of B20 candidate obtained after screening the cells after the second round of enrichment (from the 7B-627F primer) indicated that an adenine was mutated to cytosine (Figure 9). This transversion (purine to pyrimidine) mutation caused the change in the PDE7B protein sequence: glutamic acid at position 352 was changed to alanine (Figure 10).

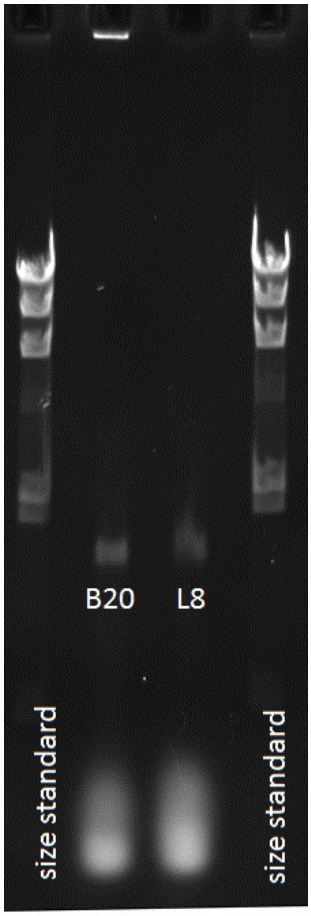

Figure 8: Whole cell PCR of CHP1265-7B-2-B20 and CHP1265-7B-2-L8.

4 Discussion

4.1 Analysis of the Results of Viability Spot Test

The results of the viability spot test showed that in the CHP1265 strain the expression of PDE7B increased viability. This occurred because PDE7B hydrolyzed excess cAMP that otherwise would have been lethal. In CHP1265 cells lacking PDE, the increase of the cGMP concentration did not lead to decreased viability because the elevated level of cAMP had already caused cell death. cAMP did not affect CHP1265 cells expressing PDE7B, although they were killed by the elevated level of cGMP. In CHP1265-7B cells, the increase of the

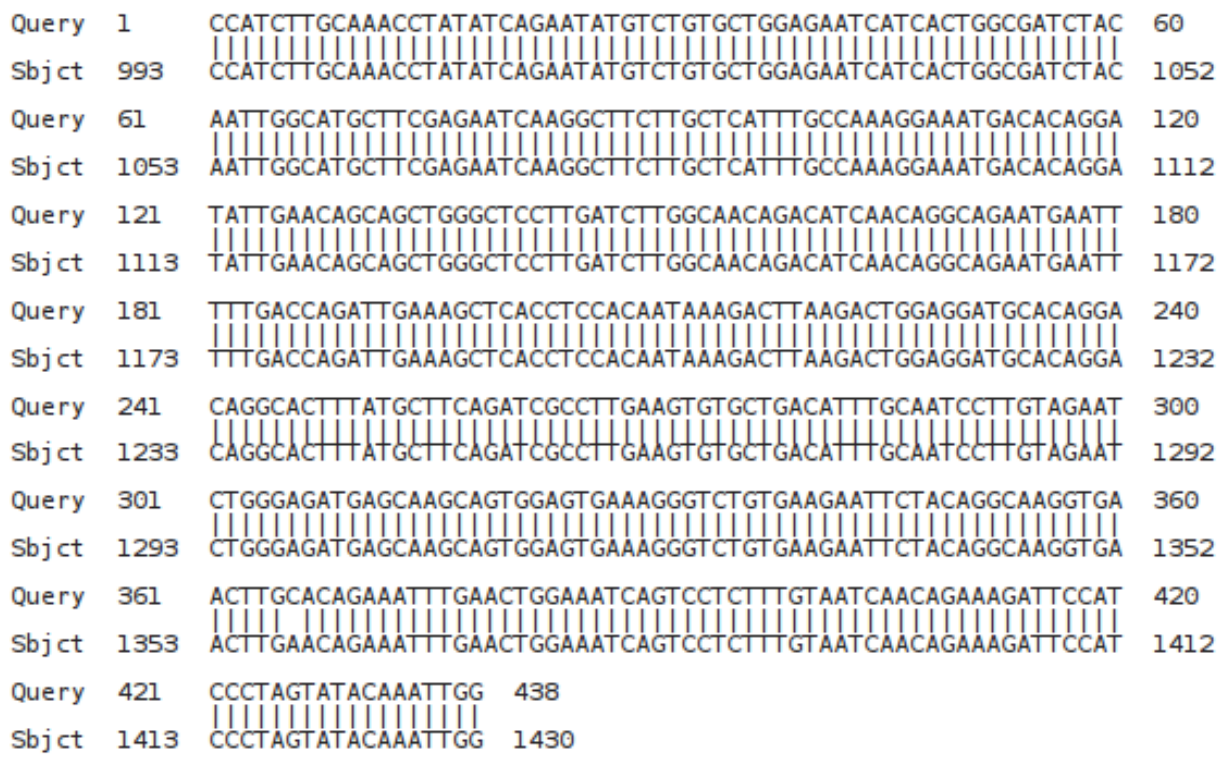

Figure 9: Sequence alignment of obtained mutant DNA with human PDE7B gene using Standard Nucleotide BLAST. A to C mutation occured.

cGMP concentration led to decreased viability because PDE7B hydrolyzed cAMP but could not hydrolyze cGMP. This result supported that CHP1265-7B cells with enhanced viability in high cGMP concentrations were the cells that had acquired a mutation in PDE7B that allowed them to hydrolyze both cAMP and cGMP. The strains used purposefully had a lower level of cAMP production than wild type (Table 1 in Section 2.1) because a mutation in PDE7B that changes the substrate specificity may decrease the affinity for cAMP. In contrast, the CHP1611 strain did not benefit from expression of PDE7B. The viability of CHP1611-7B was even less than that of CHP1611 without PDE. The experiments with the CHP1611 strain were discontinued because any mutation enhancing the viability would not be in PDE7B.

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Query  2    HLANLYQNMSVLENHHWRSTIGMLRESRLLAHLPKEMTQDIEQQLGSLILATDINRQNEF  181
            HLANLYQNMSVLENHHWRSTIGMLRESRLLAHLPKEMTQDIEQQLGSLILATDINRQNEF
Sbjct  231  HLANLYQNMSVLENHHWRSTIGMLRESRLLAHLPKEMTQDIEQQLGSLILATDINRQNEF  290

Query  182  LTRLKAHLHNKDLRLEDAQDRHFMLQIALKCADICNPCRIWEMSKQWSERVCEEFYRQGE  361
            LTRLKAHLHNKDLRLEDAQDRHFMLQIALKCADICNPCRIWEMSKQWSERVCEEFYRQGE
Sbjct  291  LTRLKAHLHNKDLRLEDAQDRHFMLQIALKCADICNPCRIWEMSKQWSERVCEEFYRQGE  350

Query  362  LAQKFELEISPLCNQQKDSIPSIQI  436
            L QKFELEISPLCNQQKDSIPSIQI
Sbjct  351  LEQKFELEISPLCNQQKDSIPSIQI  375
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Figure 10: Sequence alignment of obtained mutant protein with human PDE7B using Translated BLAST: blastx. E352A mutation occured.

4.2 Enrichment

In previous efforts, the percent of loss-of-function mutants in the *S. pombe ura5* gene was from 3% to 13% [15]. It is much more common for the random mutation to cause loss of function than a specific change in function (in this case, in the substrate specificity). Therefore, the frequency of 1/2560 is reasonable. A frequency = 1/2560 and a number of colonies = 25,000 gives the expected number of mutants = ten. Nine mutants of interest were found after the third round of enrichment after screening forty colonies. This indicates that nine-fold enrichment occurred between the second and third rounds, which is consistent with the results of the viability test. These mutants may be the same as the mutants found among cells after the second round of enrichment. However, there were at least four independent mutations, because it is improbable that the same two mutations might occur independently in two separate PCRs. If two identical mutations were discovered, it would suggest that there is a limiting number of mutations that could produce this phenotype. Three rounds of enrichment were sufficient to identify a rare mutant. If a frequency of a given mutation was 1/N, the x-fold enrichment that we should seek should be $x < N$. In addition, we wanted to find a mutation in PDE7B gene, not a rare but more effective mutation in other gene or diploidization that could cause yeast to have enhanced viability.

4.3 Analysis of the Mutant PDE7B Sequence

The change of glutamic acid at position 352 to alanine could alter the conformation of the helices surrounding the substrate. The position of this residue is shown in yellow in the image of PDE4D bound to cAMP. (Figure 11). The side chain of glutamic acid is negatively charged, whereas the side chain of alanine is hydrophobic. Therefore, the mutation of glutamic acid to alanine could change the pattern of electrostatic interactions. Moreover, the side chain of alanine is smaller than the side chain of glutamic acid. This could alter the relative position of amino acids in space. In a similar study performed by Friedberg and Hoffman, a mutation of glutamic acid at position 365 to glutamine (E365Q) caused that the cAMP-specific PDE7A

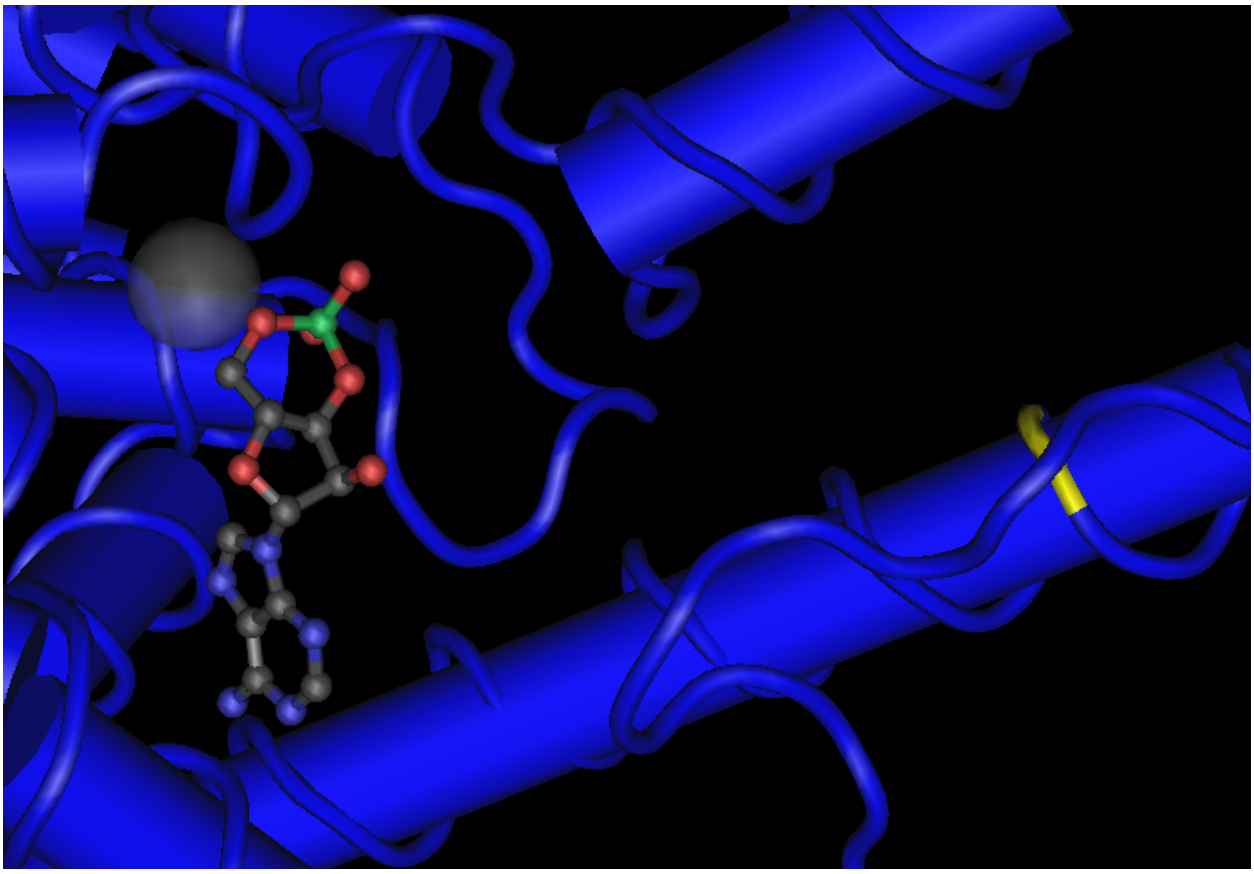

Figure 11: Position of the residue mutated in PDE7B shown in PDE4D bound to cAMP. Structure from Molecular Modeling Database (MMDB).

acquired the ability to hydrolyze cGMP [16]. The amino acid sequence alignment revealed that this was the same glutamic acid residue, which emphasizes its importance in determining the substrate specificity [16].

5 Conclusion

We obtained yeast cells having a plasmid with a copy of PDE7B gene that has a mutation which allows the cAMP-specific phospodiesterase to hydrolyze cGMP. We developed an effective method by which to enrich for and identify mutants of interest. Future work will involve determining mutations and amino acid changes that altered the substrate specificity in our other transformants.

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A Screening

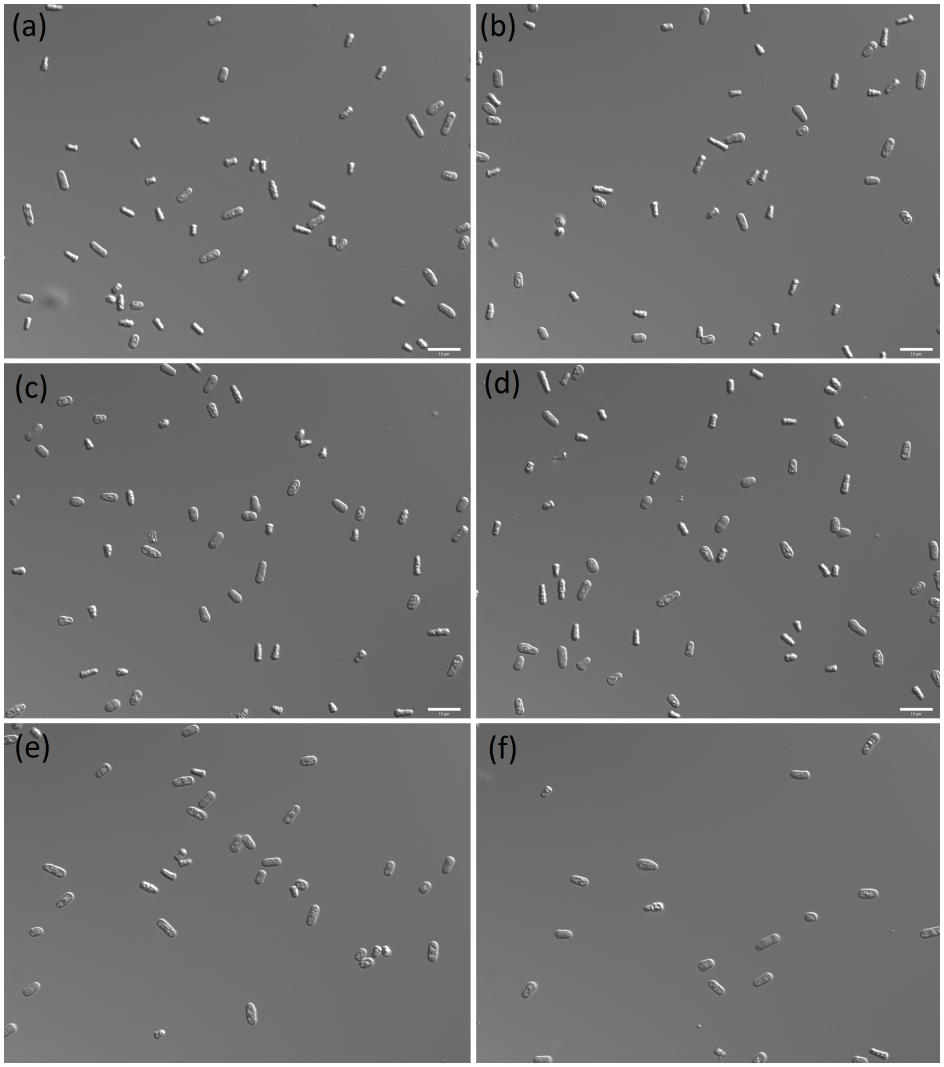

Figure 12: Representative candidates from CHP1265-7B after the second round of enrichment. These are enlarged photos from Figure 7 and duplicate photos of these cells, shown here to more easily see the change in morphology an viability. (a–b) B10 - low viability. (c–d) B20 - enhanced viability. (e–f) L8 - enhanced viability. Scale bar – 15 μm.