ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2016. Випуск 73. С. 154–159
Visnyk of the Lviv University. Series Biology. 2016. Issue 73. P. 154–159

УДК 582.282.23+575.113

GENERATION OF MUTATIONS IN *ADE2* GENE USING A CRISPR-CAS9 SYSTEM IN THE FLAVINOGENIC YEAST *CANDIDA FAMATA*

O. Lyzak[1], K. Dmytruk[1], A. Sibirny[1, 2]

[1]Department of Molecular Genetics and Biotechnology
Institute of Cell Biology, NAS of Ukraine
14/16, Drahomanov St., Lviv 7900, Ukraine
[2]Department of Biotechnology and Microbiology, University of Rzeszow
ul. Zelwerowicza, 4, Rzeszow 35-601, Poland
e-mail: sibirny@cellbiol.lviv.ua

The CRISPR-Cas9 system is one of the defense mechanisms against foreign DNA such as plasmids and phages in some bacteria and most archaea. CRISPR-Cas9 system inhibits phage infection, conjugation, and natural transformation by degrading foreign nucleic acids that enter the cell. The system was optimized and adapted for laboratory application as versatile genome editing tool. Currently, it is widely used for all types of organisms like bacteria, plants, animal or human cell lines and fungi. The method is relatively easy and fast compared to *other techniques*. Riboflavin (vitamin B_2) plays a key role in metabolism of all living organisms. Currently, the main way to obtain riboflavin is biotechnological synthesis. Yeast *Candida famata* has huge industrial potential as producer of riboflavin and its deviates. Some mutants of this species are the most flavinogenic organisms known. However, alternative genetic code, low frequency of homological recombination and restricted number of selective markers are main drawbacks for application of *basic* molecular techniques for this yeast species. In this study, CRISPR-Cas9 system adapted for yeast *Candida albicans* with alternative genetic code was successfully applied for flavinogenic yeast *C. famata* resulted in generation of mutations in *ADE2* gene.

Keywords: ADE2, CRISPR, *Candida famata*

The yeast *Candida famata* (*C. flareri*) is a cryotolerant, marine yeast, which can tolerate high salinity levels [4, 9]. Furthermore, it also can be found in various types of food such as cheese, meat, etc. [7]. It also belongs to the group of so called “flavinogenic yeasts” which are able to overproduce riboflavin under conditions of iron limitation. Riboflavin (vitamin B_2) is one of the most important vitamins required for human and animals as it is the precursor of flavin nucleotides, FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), which are coenzymes for a wide range of enzymes. Currently, this chemical is produced in large quantities for agriculture and medicine, thus yeast *C. famata* has great biotechnological potential and can be used in large-scale industry [3].

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins form prokaryotic “immune” system against viruses and plasmids [6]. Laboratory CRISPR-Cas9 system is based on II type and consist of only two components: RNA-guided-DNA endonuclease Cas9 and guide RNA [8]. Variations of CRISPR-Cas9 have been developed for numerous groups of organisms [2, 5, 11, 13, 16].

This work is focused on application of new widespread method – CRISPR-Cas9 system for genome editing in the yeast *C. famata*. We applied this method to introduce mutations in *ADE2* gene to evaluate efficiency of the system in this yeast species. In addition, *ADE2* gene was used as new selective marker for complementation of acquired adenine deficiency mutation in *C. famata* mutant.

© Лизак О., Дмитрук К., Сибірний А., 2016

Materials and methods

The *Escherichia coli* strain DH5α [Φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r^-_K m^+_K), *supE*44, *relA*1, *deoR*, Δ(*lacZYA*-*argF*)U169] was used in experiments that required a bacterial host. DH5α was grown at 37°C in rich (LB) medium as described [10]. Transformed *E. coli* cells were maintained in rich medium containing 100 mg l^{-1} of ampicillin.

The *C. famata* L20105 (*leu2*) strain was grown on rich (YPD) medium consisting of peptone (1%), yeast extract (0.5%) and glucose (2%) or mineral (YNB) medium consisting of yeast nitrogen base (1.7 g/l), ammonium sulfate (0.5%), glucose (2%). Amino acids and nucleotides were added to YNB in final concentration 40 mg/l. For selection of yeast transformants 4 mg/l nourseothricin was added to YPD.

Standard cloning techniques were used [10]. Plasmid pV1093 was kindly provided by Gerald R. Fink [15]. Restriction endonucleases and DNA ligase (Thermo Fisher Scientific) were used according to the manufacturer's instructions. Plasmid isolation from *E. coli* was carried out as described in elsewhere [10]. PCRs were performed using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). The list of used primers is presented in Table 1.

Table 1

List of primers used in this work

OL94	ATATGGATGGAAAGACTATAGGTATCTTAGGTGGTTAAGCTTTGATTAGGTCGTATG-ATTGTTGAAGCAG
OL96	TAGTTTTAATATTTAATCTATGTGCTGCTTCAACAATCATACGACCTAAT
OL97	ATTTGCAACAATCATACGACCTAATG
OL98	AAAACATTAGGTCGTATGATTGTTGC
OL104	CTTAATCCAAGAGATGGACTTATATATTG
OL105	CCTTACATGGGCTTCAAATTG
OL139	GGGGATATCAAGCTTTAGTGCTAAAACTGAAAATTAAATCAG
OL140	AAAGTCGACAAAAGACAAGAAGAAATGAAGCAG
SEQ	CGTTGGCGTCAACATGTTC

Variable part of guide DNA was a result of hybridization of two single-strand primers: top primer for Watson strand (5'→3') and bottom primer for Crick strand (3'←5'). Following mix: top primer (100 µM) – 1.5 µl, bottom primer (100 µM) – 1.5 µl, Buffer 2 (NEB) – 5 µl, H_2O – 92 µl was prepared for annealing, then it was heated at 95˚C for 10 minutes and then slowly cooled to room temperature. Obtained product was represented with double-strand oligonucleotide with sticky ends and was able to be ligated with plasmid pV1093, linearized with restriction endonuclease BsmBI.

Repair template was generated as described [15].

Transformation of *C. famata* was performed as described [14].

Results and discussion

For validation of CRISPR-Cas9 system, it was decided to disrupt *ADE2* gene. Disruption of the gene leads to accumulation of red pigment to confer easily detectible phenotype of the *ade2* mutants [12]. The efficiency of this approach was demonstrated for *C. albicans* and *Saccharomyces cerevisiae* [1, 15]. Plasmid pV1093, harboring *cas9* gene under control of *ENO1* promoter of *C. albicans*, *SAT1* gene providing resistance to nourseotricin under control of *ACT1* promoter of

C. albicans and cassette for expression of gRNA under control of *SNR52* promoter as a starting plasmid. The 20-nt guide targeting 38-58nt region of *ADE2* gene obtained with pair of primers OL97/OL98 was cloned into plasmid pV1093 as describer in Materials and Methods. Constructed plasmid designated as pcrADE (Fig. 1,A) was linearized with SacI and KpnI endonucleases and used for *C. famata* transformation. Repair template, obtained from PCR amplification with pair of primers OL94/OL96 was added into transformation mix with linearized pcrADE plasmid and was expected to introduce HindIII site into the target region trough homological recombination. However, this plasmid resulted in a low frequency of transformation yielding less than 5 colonies per 1 mg of DNA and no red colonies were detected.

To increase transformation frequency *LEU2* gene was used as selective marker in part with ARS element.

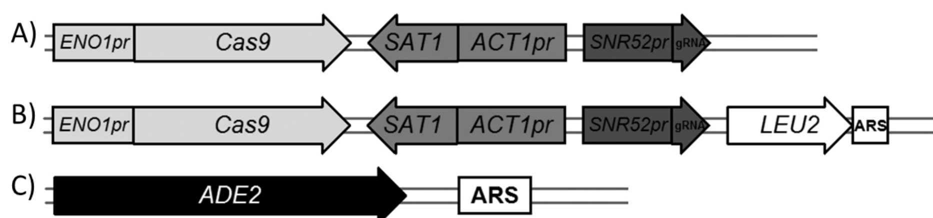

Fig. 1. Linear schemes of plasmids: A – pcrADE (14064 bp), B – pcrADE/LEU2/ARS (15024 bp), C – pCfARS16/ADE2 (5784 bp). Double line indicates the bacterial vector backbone

Plasmids pcrADE and pCfARS16 [14] harboring *LEU2* gene and ARS element of *C. famata* were combined via ligation of both SacI-linearized vectors. Then, plasmid backbone of pCfARS16 was removed using restriction endonucleases AatII and SacII with subsequent self-ligation. Constructed plasmid was designated as pcrADE/LEU2/ARS (Fig. 1,B). This plasmid resulted in a significantly higher transformation frequency reaching more than 200 transformants per 1 mg of DNA. Several red colonies were picked up among selected transformants. Red colonies (*ade* strains) were unable to grow on medium lacking adenine. However, HindIII site, presenting in repair template, did not arise in target location in frame of *ADE2* gene, revealing repair with non-homologous end joining (NHEJ). To disclose mutations, the 974 bp fragment of *ADE2* gene was isolated from four *ade* strains using primers OL104/OL105 and sequenced with primer SEQ. Results of sequencing is presented in Table 2.

The *ade* strains were cultivated in non-selective rich medium to remove plasmid pcrADE/LEU2/ARS from cells. Then, their ability to grow on mineral media lacking adenine or/and leucine was tested to verify *leu* and *ade* auxotrophy. It was demonstrated that none of *ade* strains were able to grow on mineral medium without leucine and adenine (Fig. 2). Strain #4 (*C. famata leu2ade2*) was chosen for next work.

Table 2

Sequence of target region in wild-type (WT) and CRISPR-Cas9 induced mutants.

WT	ATTAGGTCGTATGATTGTTG
#2	ATTA-GTCGTATGATTGTTG
#3	ATT**G**-GTCGTATGATTGTTG
#4	ATT**G**-GTCGTATGATTGTTG
#5	ATTA**C**GGTCGTATGATTGTTG

Notes: Bold underlined nucleotides or hyphen indicate mutations.

Selected *leu2ade2* strain gave an opportunity to use *ADE2* gene as a new selective marker for *C. famata*. Genome fragment harboring *ADE2* ORF with 673 bp upstream start codon as promoter and 518 bp downstream stop codon as terminator was isolated with pair of primers OL139/OL140 and cloned into HindIII/SalI sites of pCfARS16 instead of *LEU2* gene. Constructed plasmid, pCfARS16/ADE2 (Fig. 1,C), was tested on its ability to transform *C. famata* #4 strain solely or in combination with pCfARS16. Thus, the transformation frequency for pCfARS16/ADE2 alone was equal to pCfARS16, reaching ~200 colonies transformants per 1 mg DNA. In case of co-transformation with both pCfARS16/ADE2 and pCfARS16, transformation frequency dropped in 4-5 fold.

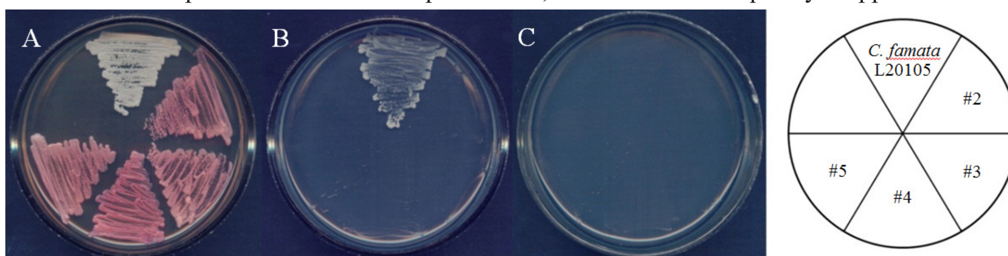

Fig. 2. Growth test of parental (*C.famata* L20105, *leu2*) and selected *ade2* strains on YPD (A), on YNB with leucine (B) and on YNB without leucine (C)

To conclude, CRISPR-Cas9 system is promising tool for genome engineering of *C. famata*. Set of *ade2* strains was constructed with CRISPR-Cas9. All selected *ade2* strains revealed NHEJ type of reparation of double-strand break caused by Cas9. DNA fragment harboring *ADE2* gene form *C. famata* was isolated and successfully used as a new selective marker.

Acknowledgement

We thank Valmik K. Vyas, M. Inmaculada Barrasa, and Gerald R. Fink for provided plasmid pV1093 and Anastasia Sybirna for helpful discussions. The work was partially supported by NAS of Ukraine (grant № 36–16).

REFERENCES

1. *Bao Z., Xiao H., Liang J.,* et al. Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae* // ACS Synth Biol. 2015. Vol. 4. № 5. P. 585-594.
2. *DiCarlo J., Norville J., Mali P.,* et al. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems // Nucleic Acids Res. 2013. Vol. 41. № 7. P. 4336-4343.
3. *Dmytruk K., Lyzak O., Yatsyshyn V.,* et al. Construction and fed-batch cultivation of *Candida famata* with enhanced riboflavin production // J. Biotechnol. 2014. № 172. P. 11-17.
4. *Dmytruk K., Sibirny A. Candida famata* (*Candida flareri*) // Yeast. 2012. Vol. 29. № 11. P. 453-458.
5. *Gilbert L., Larson M., Morsut L.,* et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes // Cell. 2013. Vol. 154. № 2. P. 442-451.
6. *Horvath P., Barrangou R.* CRISPR/Cas, the immune system of bacteria and archaea // Science. 2010. Vol. 327. № 5962. P. 167-170.
7. *Lépingle A., Casaregola S., Neuveglise C.,* et al. Genomic exploration of the hemiascomycetous yeasts: 14. *Debaryomyces hansenii* var. *hansenii* // FEBS Lett. 2000. Vol. 487. № 1. P. 82–86.
8. *Makarova K., Wolf Y., Alkhnbashi O.,* et al. An updated evolutionary classification of CRISPR-Cas systems // Nature Reviews Microbiology. 2015. Vol. 13. P. 722-736.

9. *Norkrans B.* Studies on marine occurring yeasts: growth related to pH, NaCl concentration and temperature // Archives of Microbiology. 1966. Vol. 54. № 4. P. 374-392.
10. *Sambrook J., Fritsch E., Maniatis T.* Molecular cloning: Cold spring harbor laboratory press. New York. 1989.Vol. 2.
11. *Sander J., Joung J.* CRISPR-Cas systems for editing, regulating and targeting genomes // Nat. Biotechnol. 2014. Vol. 32. № 4. P. 347-355.
12. *Stotz A., Linder P.* The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors // Gene. 1990. Vol. 95. № 1. P. 91-98.
13. *Stovicek V., Borodina I., Forster J.* CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains // Metabolic Engineering Communications. 2015. № 2. P. 13-22.
14. *Voronovsky A., Abbas C., Fayura L.,* et al. Development of a transformation system for the flavinogenic yeast *Candida famata* // FEMS Yeast Res. 2002. Vol. 2. № 3. P. 381-388.
15. *Vyas V., Barrasa I., Fink G.* A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families // Sci. Adv. 2015. Vol. 1. № 3. e1500248.
16. *Xie K., Yang Y.* RNA-guided genome editing in plants using a CRISPR-Cas system // Mol. Plant. 2013. Vol. 6. № 6. P. 1975-1983.

Стаття: надійшла до редакції 19.07.16
доопрацьована 2.09.16
прийнята до друку 5.09.16

ОДЕРЖАННЯ МУТАЦІЙ У *ADE2* ГЕНІ З ВИКОРИСТАННЯМ CRISPR-CAS9 СИСТЕМИ У ФЛАВІНОГЕННИХ ДРІЖДІВ *CANDIDA FAMATA*

О. Лизак[1], К. Дмитрук[1], А. Сибірний[1 2,*]

[1]Відділ Молекулярної генетики та біотехнології
Інститут біології клітини НАН України
вул. Драгоманова, 14/16, Львів 79005, Україна
[2]Відділ біотехнології і мікробіології, Жешувський університет
вул. Zelwerowicza, 4, Жешув 35-601, Польща
e-mail: sibirny@cellbiol.lviv.ua

Система CRISPR-Cas9 є одним із механізмів захисту деяких бактерій і більшості архей від чужорідної ДНК, наприклад, від плазмід і фагів. CRISPR-Cas9 система пригнічує інфікування фагами, трансформацію та кон'югацію шляхом руйнування чужорідної ДНК, яка потрапила в клітину. Цю систему було оптимізовано й адаптовано для лабораторного застосування як універсальний інструмент редагування геному. На сьогодні CRISPR-Cas9 система широко використовується у генетичній інженерії бактерій, грибів, рослин, тварин чи людських клітинних ліній. Порівняно з іншими, цей метод є відносно легким і швидким. Рибофлавін (вітамін B_2) відіграє ключову роль в обміні речовин усіх живих організмів. У даний час біотехнологічний синтез є основним способом отримання рибофлавіну. Дріжджі *C. famata* мають великий промисловий потенціал як продуценти рибофлавіну та його похідних. Деякі мутанти цього виду дріжджів є найбільш флавіногенними серед усіх відомих організмів. Проте альтернативний генетичний код, низька частота гомологічної рекомбінації та невелика кількість селективних маркерів обмежують можливості генетичної інженерії цього виду дріжджів. У цій роботі CRISPR-Cas9 система, адаптована для дріжджів

Candida albicans з альтернативним генетичним кодом, була успішно застосована для отримання мутацій у гені *ADE2* флавіногенних дріжджів *C. famata*.

Ключові слова: ADE2, CRISPR, *Candida famata.*

ПОЛУЧЕНИЕ МУТАЦИЙ В *ADE2* ГЕНЕ С ИСПОЛЬЗОВАНИЕМ CRISPR-CAS9 СИСТЕМЫ В ФЛАВИНОГЕННЫХ ДРОЖЖАХ *CANDIDA FAMATA*

А. Лизак[1], К. Дмитрук[1], А. Сибирный[1 2,*]

[1]Отдел молекулярной генетики и биотехнологии
Институт биологии клетки НАН Украины
ул. Драгоманова, 14/16, Львов 79005, Украина
[2]Отдел биотехнологии, Жешувский университет
ул. Zelwerowicza, 4, Жешув 35-601, Польша
e-mail: sibirny@cellbiol.lviv.ua

Система CRISPR-Cas9 является одним из механизмов защиты некоторых бактерий и большинства архей от чужеродной ДНК, такой как плазмиды и фаги. CRISPR-Cas9 система подавляет инфицирование фагами, трансформацию и конъюгации путем разрушения чужеродной ДНК, которая попала в клетку. Эта система была оптимизирована и адаптирована для лабораторного применения как универсальный инструмент редактирования генома. На сегодня CRISPR-Cas9 система широко используется в генетической инженерии бактерий, грибов, растений, животных или человеческих клеточных линий. По сравнению с другими, этот метод является относительно легким и быстрым. Рибофлавин (витамин В2) играет ключевую роль в обмене веществ всех живых организмов. В настоящее время биотехнологический синтез является основным способом получения рибофлавина. Дрожжи *C. famata* обладают большим промышленным потенциалом в качестве продуцента рибофлавина и его производных. Некоторые мутанты этого вида дрожжей являются наиболее флавиногенными среди всех известных организмов. Однако альтернативный генетический код, низкая частота гомологической рекомбинации и небольшой набор селективных маркеров ограничивают возможности генетической инженерии этого вида дрожжей. В этой работе CRISPR-Cas9 система, адаптированная для дрожжей *Candida albicans* с альтернативным генетическим кодом, была успешно использована для получения мутаций в гене *ADE2* флавиногенных дрожжей *C. famata*.

Ключевые слова: ADE2, CRISPR, *Candida famata.*