Efficacy of genetic transformation of E.coli field and reference (ATCC 8739) strains at different concentrations of $CaCl_2$ for creation of Gene libraries

Manvel Badalyan[1], *Tatevik* Aloyan[1*], *Tatev* Amirkhanyan[1], *Varya* Dilanyan[1], and *Andreas* Melikyan[1]

[1]Scientific Center of Agrobiotechnology, ANAU Armenian National Agrarian University, 74, Teryan, Yerevan, 0009, Armenia

Abstract. The importance of genetic transformation in various fields of biological research, and especially in the process of gene libraries formation, is very important. It has been proven by numerous studies that during the phase of obtaining competent cells, Ca^{2+} ions neutralize the electrostatic repulsion of membrane proteins and DNA of the bacterial cell, thereby stimulating the introduction of foreign DNA into the membrane pores. Our research aimed to elucidate the maximum efficiency of transformation in field and ATCC 8739 reference strains of *E.coli* at 0.05; 0.1 and 0.15 M concentrations of $CaCL_2$. Microbiological, molecular-biological modern methods were used in the experimental studies. From the research results, it became evident that the efficiency of genetic transformation increases with the addition of concentration of $CaCL_2$ and reaches the maximum at 0.1 M. Among the studied strains of *E.coli*, at 0.05 and 0.1 M concentrations of $CaCL_2$, the highest efficiency was recorded in the field strain. At a 0.15 M concentration of $CaCL_2$, the transformation efficiency in the field and reference strains is highly variable due to the size of the genomic DNA of the internalizing bacteria and the number of restriction sites.

1 Introduction

Genetic resources are considered the reachness of every country. Formed as a result of natural evolution and human activity, they are very important in the process of food production and environmental balance [1].

At the current stage of agricultural development, when the task is to create new varieties of crops that meet population and production requirements in a short period of time, or to improve existing ones, conservation and efficient use of genetic resources is considered as a strategic task.

Currently, the most practical method of conservation, inventory, location and acquisition of valuable gene pools is the creation of DNA banks or gene libraries. A gene

* Corresponding author: tatev.aloyan20@mail.ru

library is a group of living bacterial colonies built in a vector containing restriction sites of the genomic DNA of a single organism, which are considered a source of desired genes.

In other words, the total DNA segments of valuable species are stored in bacterial cells. It was created for the first time in 1974 by D. Khognes and employees. Practically every bacterial cell is deploying one or more genomic DNA segments that represent a hybrid of one of the many restriction segments of vector or DNA. The bacterial cell, receiving the hybrid DNA, multiplies to produce a clone. A collection of bacterial cells containing all the restriction fragments of the genomic DNA of a particular organism forms a DNA bank [2].

The whole process of creating gene libraries includes a series of operations, where the genetic transformation of bacteria is essentially the most important, because their efficiency is directly proportional to forming a complete DNA bank [3].

The phenomenon of transformation was first discovered in 1928, by F. Griffith [4]. It is a change in the hereditary information of a bacterial cell due to the introduction of DNA segments from another cell or another strain into it. In the laboratory, transformation is used to introduce recombinant plasmid DNA into bacterial cells, in which case the plasmids replicate independently of the chromosomal DNA and ensure the expression of the target genes. Transformation provides an opportunity to introduce genetic constructs that, by recombination with chromosomes, can be integrated into it and reproduced together (integrative plasmids) [5-6].

The initial material for many biological studies is the acquisition of cells containing foreign DNA, this phenomenon is considered genetic transformation. It makes it possible to obtain genetically modified organisms, combine or disable the necessary genes, determine the effect of certain proteins and finally create gene libraries or genetic banks [7].

In many cases, due to research purposes, it is necessary to accurately determine the efficiency of transformed cells, which is the ratio of the total number of transformed cells to the amount of DNA used for transformation. The efficiency of transformation is directly related to obtaining competent cells. *E.coli* cells capable of including foreign DNA into themselves are considered as a competent. One of the most common methods for obtaining competent cells is the incubation of bacteria at low temperature in a liquid medium containing Ca^{2+} ions [8-9].

The method is known as cell calcinations. The nature of the method consists in the following: calcium ions bind to the outer membrane of cells and contribute to the adsorption of foreign DNA due to ionic bonds. The maximum level of adsorption is maintained for 2 hours after incubation, after which it gradually decreases, due to which the transformation efficiency drops sharply [8].

Both the size of the vectors, the type of *E.coli* and the amounts of $CaCL_2$ used for cell calcination have some influence on the transformation efficiency. In this work, the effect of $CaCL_2$ with different molarity (0.05, 0.1 and 0.15 M) on the transformation efficiency of reference (ATCC 8739) and field strains of *E.coli* is presented. The results can be used as a test in the process of genetic transformation and especially the creation of gene libraries in various fields of biology.

2 Materials and methods

The research was carried out in 2021-2023, in the Biological Research Laboratory of the "Scientific Center of Agro-Biotechnology" branch of the Armenian National Agrarian University /ANAU/. The samples of the studied plants were taken from the *ex situ* seed collection of the National Genebank of Crops and their Wild Relatives of the Scientific Center.

Genomic DNA from crops and wild species was obtained by the SDS method [10]. The concentration of extracted DNA was determined using a NanoDrop One (Thermo Scientific, USA) spectrophotometer.

Ampicillin resistance selection of *E.coli* was carried out in order to obtain colicinogenic plasmids against ampicillin (as a selection marker) [11].

Colonies of *E.coli* were grown in 100 ml of LB medium with ampicillin (50 µg/ml) and incubated at 37°C for 18 hours. A portion of the overnight culture was transferred to fresh LB medium containing ampicillin, ensuring its 100x dilution. Growth was continued for 16-18 hours until the appropriate optical density (2-3*10^8 cells/ml) was reached.

Plasmid DNA was isolated from bacterial cells by the base digestion method [10, 12].

Restriction digestion of genomic DNA and vector was performed using EcoR I restriction enzyme [13]. The reaction mixture had the following composition: 15 9 µl water, 2 µl 10x buffer, 2 µl DNA (2 µg/ml), 1 µl restriction enzyme (5 units/µl): The mixture was incubated at 37^0C, for 16 hours.

When a single restriction enzyme is used, very often the cleaved ends of the vector stick together, forming the vector's starting forms without large amounts of internalized DNA. In order to avoid all this, dephosphorylation of plasmid DNA was carried out immediately after restriction according to the following scheme: 5 µl of 10x buffer was added to 50 µl of the reaction mixture (5 µg DNA, 50 µl deionized water, 2 µl alkaline phosphatase). The mixture was incubated at 37^0C, for 1 hours. DNA purification was carried out by phenol-chloroform method, phosphatase was removed by 96% ethanol.

Ligation of vector and genomic DNA restriction fragments was performed using T_4 DNA ligase enzyme [13-14], according to the following composition: 1 µl 10x buffer, 1,5 µl plasmid DNA (0.1 µg/ml), 3 µl (0,1 µg/ml) restriction fragments of genomic DNA, 1 µl T_4 DNA ligase (100 units), 3,5 µl water: The mixture was incubated at 16^0C, for 2 hours.

Competent cells were obtained by treatment with 0.1 M calcium chloride [15-16].

The number of competent cells was determined by the following formula [14]:

$$K= N*10^6 \quad (1)$$

Where: K - is the number of competent cells, N - total number of colonies per Petri dishes.

Genetic transformation of E.coli cells was carried out by heat shock method [17].

The transformation efficiency was determined by the following formula [14, 18]:

$$T=M/A \quad (2)$$

Where: T - is the transformation efficiency, M - total number of transformed colonies per Petri dishes, A - amount of built-in DNA (µg).

The results of DNA incorporation into transformed cells were determined by the restriction enzyme method [10].

3 Results and Discussion

In order to investigate the transformation efficiency of different strains of *E.coli* and different concentrations of calcium chloride, field and reference strains of *E.coli* were treated with different molar solutions of $CaCL_2$ to increase the competence of strains. The maximum transformation efficiency was observed in the field strain of *E.coli* when treated with 0.1 M solution of $CaCL_2$ (Table 1).

The lowest concentration efficiency was recorded when the reference strain of *E.coli* was calcinated with a 0.05 M solution of $CaCL_2$. In the case of 0.15 M calcium chloride

solution, high transformation efficiencies were reported for both field and reference strains, with a highly defined pattern depending on the genome size of the transformed plants and the number of genomic DNA restriction fragments (Table 1, Figure 1). Therefore, we hypothesize that the efficiency of transformation depends not only on the type of *E.coli*, the concentration of Ca^{2+} ions required to neutralize the negative charges of their cells and plasmid (vector), but also on the number of transformed clones.

Cells were not ampicillin resistant prior to transformation. To establish that Ca^{2+} ions are very important in the process of obtaining competent cells, non-calcified cells were transformed and grown in nutrient medium containing ampicillin. The transformation efficiency was equal to 0 in the case of both strains of *E.coli*.

Studies have shown that the presence of Ca^{2+} ions is essential for obtaining competent cells. From the analysis of the materials obtained as a result of our research, it becomes clear that during transformation, the electrostatic repulsion between the negative charges of the bacterial outer membrane and the DNA prevents the proximal association, which in his turn reduces the transformation efficiency. The presence of positively charged Ca^{2+} ions promotes transformation activity by neutralizing the negative charge associated with DNA and the cell membrane and thus reducing electrostatic repulsion.

Table 1. Transformation efficiency due to different strains of *E.coli* and different concentrations of $CaCL_2$.

No	Plant species/varieties	Genome size, kb	***E. coli* Strain and $CaCL_2$ concentration**					
			***E.coli* field strain**			***E.coli* ATCC 8739**		
			0.05 M	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
1	*Triticum urartu Tumanian ex Gandilyan*	$5*10^6$	$32*10^6$	$2.4*10^8$	$9*10^6$	$44*10^6$	$2.1*10^8$	$6*10^6$
2	*Triticum araraticum Jakubz*	$5*10^6$	$44*10^6$	$2.2*10^8$	$13*10^6$	$68*10^6$	$2*10^8$	$16*10^6$
3	*Triticum boeoticum Boiss.*	$5*10^6$	$38*10^6$	$2*10^8$	$13*10^6$	$51*10^6$	$1.6*10^8$	$10*10^6$
4	*Triticum aestivum* L., variety "Alti Aghaj"	$17*10^6$	$48*10^6$	$3*10^8$	$18*10^6$	$62*10^6$	$2.8*10^8$	$10*10^6$
5	*Triticum aestivum* L., variety "Voskehask"	$17*10^6$	$35*10^6$	$3.5*10^8$	$14*10^6$	$54*10^6$	$2.6*10^8$	$6*10^6$
6	*Triticum aestivum* L., variety "Gyulgiani"	$17*10^6$	$24*10^6$	$2.8*10^8$	$8*10^6$	$38*10^6$	$2*10^8$	$6*10^6$
7	*Triticum aestivum* L., variety "Garaseferyani"	$17*10^6$	$30*10^6$	$3.6*10^8$	$18*10^6$	$56*10^6$	$2.8*10^8$	$14*10^6$
8	*Triticum aestivum* L., variety "Qrik"	$17*10^6$	$48*10^6$	$2.4*10^8$	$6*10^6$	$68*10^6$	$1.8*10^8$	$6*10^6$
9	*Triticum aestivum* L., variety "Galgalos"	$17*10^6$	$40*10^6$	$2*10^8$	$21*10^6$	$64*10^6$	$1.3*10^8$	$11*10^6$
10	*Hordeum bulbosum* L.	$5,3*10^6$	$46*10^6$	$3.2*10^8$	$23*10^6$	$64*10^6$	$2.8*10^8$	$12*10^6$
11	*Hordeum vulgare* L., variety "Hayk 1"	$5.3*10^6$	$40*10^6$	$3.7*10^8$	$28*10^6$	$58*10^6$	$2.8*10^8$	$20*10^6$
12	*Hordeum vulgare* L., variety "Hayk 2"	$5.3*10^6$	$22*10^6$	$3.1*10^8$	$14*10^6$	$34*10^6$	$2.7*10^8$	$10*10^6$
13	*Hordeum vulgare* L., variety "Marina"	$5,3*10^6$	$46*10^6$	$3*10^8$	$11*10^6$	$64*10^6$	$2.9*10^8$	$8*10^6$
14	*Aegilops tauschii* Cosson.	$4,2*10^6$	$42*10^6$	$2.8*10^8$	$26*10^6$	$68*10^6$	$2*10^8$	$14*10^6$
15	*Aegilops umbellulata* Zhuk.	$4,2*10^6$	$37*10^6$	$2.5*10^8$	$12*10^6$	$54*10^6$	$2.2*10^8$	$6*10^6$
16	*Lactuca serriola* L.	$2,5*10^6$	$30*10^6$	$3.6*10^8$	$6*10^6$	$32*10^6$	$3.1*10^8$	$6*10^6$
17	*Spinacia tetrandra* Steven.	$989*10^3$	$42*10^6$	$3.8*10^8$	$12*10^6$	$36*10^6$	$3.2*10^8$	$4*10^6$
18	*Daucus carota* L.	$473*10^3$	$58*10^6$	$4.2*10^8$	$6*10^6$	$38*10^6$	$2.8*10^8$	$6*10^6$
19	*Beta macrorhiza* Steven.	$758*10^3$	$63*10^6$	$3.8*10^8$	$20*10^6$	$47*10^6$	$2.3*10^8$	$12*10^6$
20	*Beta lomatagona* F. et M.	$758*10^3$	$84*10^6$	$2.1*10^8$	$21*10^6$	$60*10^6$	$1.8*10^8$	$12*10^6$
21	*Beta corolliflora* Zoss.et Butler	$758*10^3$	$35*10^6$	$2.8*10^8$	$18*10^6$	$27*10^6$	$2.2*10^8$	$8*10^6$
22	*Beta vulgaris* L., variety "Bordeaux 237"	$758*10^3$	$58*10^6$	$2.7*10^8$	$8*10^6$	$36*10^6$	$2.4*10^8$	$5*10^6$
23	*Physalis alkekengi* L.	$157*10^3$	$63*10^6$	$3.7*10^8$	$11*10^6$	$44*10^6$	$3.1*10^8$	$10*10^6$
24	*Coriandrum sativum* L.	$213*10^4$	$72*10^6$	$2.4*10^8$	$6*10^6$	$48*10^6$	$1.8*10^8$	$3*10^6$
25	*Phaseolus vulgaris* L., "Buzhakan local" variety-population	$587*10^3$	$75*10^6$	$3.2*10^8$	$10*10^6$	$52*10^6$	$2.8*10^8$	$10*10^6$
26	*Phaseolus vulgaris* L., "Goris local" variety-population	$587*10^3$	$56*10^6$	$2.2*10^8$	$8*10^6$	$32*10^6$	$2*10^8$	$14*10^6$
27	*Phaseolus vulgaris* L., "Armenian red" variety-population	$587*10^3$	$35*10^6$	$2.8*10^8$	$16*10^6$	$28*10^6$	$1.8*10^8$	$9*10^6$
28	*Phaseolus vulgaris* L., "Kotayk local" variety-population	$587*10^3$	$66*10^6$	$4.2*10^8$	$8*10^6$	$32*10^6$	$3.1*10^8$	$3*10^6$
29	*Cicer arietinum* L., variety "Leninakan 313"	$740*10^3$	$84*10^6$	$3.6*10^8$	$18*10^6$	$28*10^6$	$3*10^8$	$12*10^6$
30	*Cicer arietinum* L., variety "Karin"	$740*10^3$	$44*10^6$	$2.4*10^8$	$4*10^6$	$38*10^6$	$2.1*10^8$	$8*10^6$
31	*Glycine max* Merr., variety "Milena"	$115*10^3$	$66*10^6$	$2.8*10^8$	$18*10^6$	$30*10^6$	$1.9*10^8$	$11*10^6$
32	*Vitis vinifera* L. ssp. *sylvestris*	$500*10^3$	$57*10^6$	$4.4*10^8$	$24*10^6$	$38*10^6$	$3.6*10^8$	$16*10^6$
33	*Rubus* L.	$240*10^3$	$38*10^6$	$3.3*10^8$	$12*10^6$	$35*10^6$	$3*10^8$	$8*10^6$
34	*Pistacia mutica Fisch et C.A.Mey.*	$600*10^3$	$52*10^6$	$3.2*10^8$	$14*10^6$	$38*10^6$	$3*10^8$	$9*10^6$

The aim of our studies was to determine the effect of different strains of *E.coli* and different molarity of $CaCL_2$ on transformation efficiency. From the results of the studies, it is clear that at a low concentration of $CaCL_2$ (0.05 M), the highest transformation efficiency

was recorded in the field strain of *E.coli* ($4x10^6$-$28x10^6$). Under the conditions of the same concentration of calcium chloride, the efficiency of transformation of *E.coli* reference strain in all versions ranged from $3x10^6$-$20x10^6$. It is noteworthy that, when comparing the averaged data, the field strain of *E.coli* at 0.05 M $CaCL_2$ exceeds the reference strain by 28.1% in transformation efficiency.

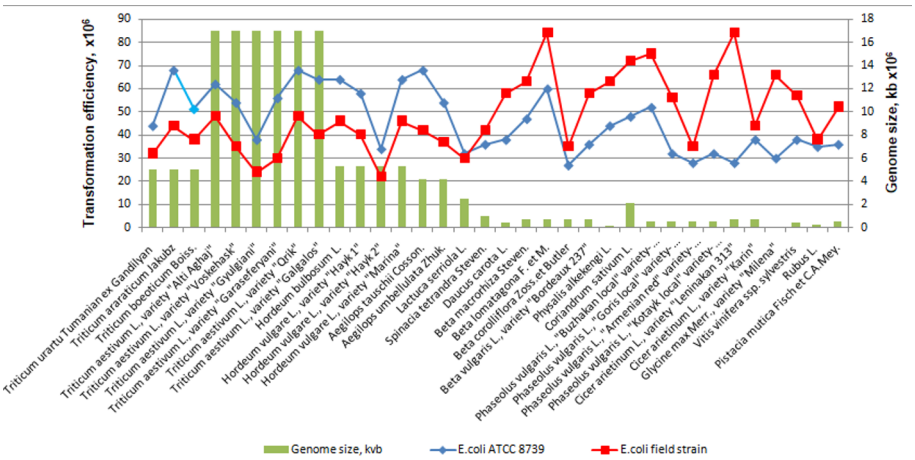

Fig. 1. Dependence of transformation efficiency on genome size for different strains of *E.coli* at 0.15 M concentration of $CaCL_2$.

Upon calcination with a 0.1 M solution of calcium chloride, the transformation efficiency is dramatically increased in both studied strains. For the field strain of *E.coli*, it ranges from $2x10^8$ to $3.6x10^8$ (average: $2.6x10^8$). In the 0.1 M version of $CaCL_2$, the field strain outperformed the reference strain by 18.75% in transformation efficiency. In fact, as the $CaCL_2$ concentration was doubled, the transformation efficiency increased dramatically.

Scientific sources prove that high concentrations of $CaCL_2$ create an environment of high osmotic stress, leading to upregulation of non-specific outer membrane proteins. Outer membrane protein C (OmpC) is a variant of a nonspecific solute, porin, whose treatment with $CaCL_2$ facilitates the uptake of foreign DNA. From this reasoning, it can be concluded that the reference strain of the *E.coli* is more resistant to osmotic stress compared to the field strain; therefore less OmpC is produced to mitigate it, which may be the reason for the relatively low transformation efficiency. However, this pattern is not maintained at 0.15 M concentration of $CaCL_2$ [19-20].

In the case of genetic transformation of restriction segments of plant species with gene sizes of $2.5x10^6$-$17x10^6$, the efficiency of transformation is high in the reference strain, it ranges from $32x10^6$-$68x10^6$ (average: $50x10^6$). In the field strain, it ranges from $24x10^6$ to $48x10^6$ (mean: $36x10^6$), falling by 28%.

The efficiency of the vector insertion process containing the restriction regions of plant species with genome sizes of $157x10^3$-$989x10^3$ is high in the field strain of *E.coli*, ranging from $35x10^6$-$84x10^6$ (average: $59.5x10^6$). In these variants, the transformation efficiency falls short of the reference strain, ranging from $27x10^6$ to $60x10^6$ (average $43.5x10^6$), falling by 27% (Table 1, Figure 1).

Regardless of the fact that the efficiency of transformation due to the size of the genome of plant species, and therefore also the number of restriction segments, is highly variable in the reference and field strains of the *E.coli*, it should be noted that, in general, the efficiency of transformation at a concentration of 0.15 M of $CaCL_2$ is very low compared with the 0.1 M. In this assay scheme, transformation efficiency in both *E.coli* strains increases dramatically from 0.05 M to 0.1 M $CaCL_2$ at the expense of increased OmpC protein

transcription, after which it decreases markedly. At a 0.15 M concentration of calcium chloride, the decrease in transformation efficiency is a consequence of high osmotic stress, which forces cells to limit the amount of uptake of foreign DNA, since the latter is not considered an essential component for cell survival.

4 Conclusion

Summarizing the results of the experiment, it can be concluded that the efficiency of genetic transformation in reference and field strains of *E.coli* is the highest at 0.1 M concentration of $CaCL_2$. Incidentally, the field strain has a significant advantage in transformation efficiency at both 0.05 and 0.1 M $CaCL_2$ concentrations. Regarding the conflicting results obtained at the 0.15 M concentration, which we believe is due to the amount of genomic DNA and the number of restriction sites of the introduced plant species, further studies are needed, and therefore we refrain from drawing conclusions.

Acknowledgement

The work was supported by the Science Committee of RA, in the frames of the research project No. 21T-4D074.

References

1. R.K. Salgotra, B.S. Chauhan, Genetic Diversity, Conservation, and Utilization of Plant Genetic Resources, Genes (Basel), **14**, 174 (2023)
2. G.G. Goncharenko, Basics of Genetic Engineering, Minsk, 183 (2005)
3. R. Etchuuya, M. Ito, S. Kitano, F. Shigi, R. Sobue, S. Maeda, Cell-to-Cell Transformation in Escherichia coli: A Novel Type of Natural Transformation Involving Cell-Derived DNA and a Putative Promoting Pheromone. PLoS One, **6**, 16355 (2011)
4. F. Griffith, The Significance of Pneumococcal Types, J. Hyg. (Lond), **27**, 113–159 (1928)
5. A.W. Downie, Pneumococcal Transformation-A Backward View Fourth Griffith Memorial Lecture, J. Gen. microbiol, **73**, 1–11 (1972)
6. M. G. Lorenz, W. Wackernagel, Bacterial gene transfer by natural genetic transformation in the environment. microbiol. Rev., **58**, 563–602 (1994)
7. R.K. Holmes, J.M. medical microbiology. In Genetics; Baron S, Ed.; Galveston (TX): University of Texas Medical Branch at Galveston
8. G. Lim, D. Lum, B. Ng, C. Sam, Differential transformation efficiencies observed for pUC19 and pBR322 in E. coli may be related to calcium chloride concentration, J. Exp. microbiol. Immunol, **20**, 1–6 (2015)
9. W.-T. Chan, C.S. Verma, D.P. Lane, Samuel Ken-En Gan, A comparison and optimization of methods and factors affecting the transformation of Escherichia coli. biosci. Rep., 33(2013)
10. V.E. Padutov, O.Yu. Baranov, E.V. Voropaev, Methods of molecular genetic analysis, ISBN 978-985-6768-12-8, Unipol, Minsk, 176 (2007)
11. E.S. Gvozdeva, Practical work on genetic engineering and molecular biology of plants, Tomsk State University, Tomsk, 96 (2012)

12. N.A. Kutlunina, A.A. Ermoshin, Molecular genetic methods in the study of plants, Educational and methodological manual, Ekaterinburg, 140 (2017)
13. A.R. Kayumov, Practical work on molecular genetics, Educational and methodological manual, KFU, Kazan, 36 (2016)
14. D.E. Zhuravleva, Z.I. Iskhakova, E. Yu. Trizna, R.G. Khamidullina, A.R. Kayumov, Practical work on heterologous expression of proteins. Educational and methodological manual, KFU, Kazan, 36 (2022)
15. A.T. Yeprintsev, V.N. Popov, D.N. Fedorin, Identification and study of gene expression, Educational and methodological manual, VSU, Ed.; Voronezh, 63 (2008)
16. A.Y. Chang, V.W.Y. Chau, J.A. Landas, Yvonne Pang, Preparation of calcium competent Escherichia coli and heat-shock transformation. undergrade, Methods Pap, **1**, 22–25 (2017)
17. N.A. Koltovaya, Guide to practical exercises in molecular biology, Dubna, 80 (2010)
18. M. Dagert, S. Ehrlich, Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells, Gene, **6**, 23–28 (1979)
19. T.X. Nguyen, E.R. Alegre, S.T. Kelley, Phylogenetic analysis of general bacterial porins: a phylogenetic case study, J Mol Microbiol Biotechnol, **11**, 291–301 (2006)
20. J-M. Pages, Role of bacterial porins in antibiotic susceptibility of Gram-negative bacteria, In Bacterial and eukaryotic porins: Structure function and mechanism.; Benz R, Ed. Wiley-VCH, Weinheim, 41–59 (2004)