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Molecular Evolution of a Defined DNA Sequence with Accumulation of Mutations in a Single Round by a Dual Approach to Random Chemical Mutagenesis (DuARCheM)

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Directed evolution has paved the way to a new era of protein and nucleic acid molecules with improved and enhanced properties. The utmost important component of directed evolution is random mutations in a defined DNA sequence. The utility of random chemical mutagenesis in directed evolution studies is dwindling due to the inherent flaws with whole-organism mutagenesis and the in vitro approach. Here, we report a novel Dual Approach to Random Chemical Mutagenesis (DuARCheM) to introduce random mutations in a defined DNA fragment. DuARCheM involves in vivo chemical mutagenesis and in vitro genetic manipulations. The resulting library revealed an accumulation of mutations in its members. These results imply that the parent mutation is carried in the further generations within the same library. This method might help to change random chemical mutagenesis because the combination of in vivo and in vitro approaches mimics the amplification and mutation that is performed by PCR-based mutagenesis, and at the same time the mutations are confined to the desired gene. Moreover, the mutagen pressure is greater in chemical mutagenesis than in a Taq-polymerase-based error-prone system. Concomitant amplification and mutation in the DuARCheM method leads to a better spectrum of mutants because the plasmid construct is exponentially amplified in the presence of mutagen pressure, unlike in the in vitro chemical mutagenesis system in which the template molecule does not replicate. This work is able to nullify all the disadvantages that are associated with random chemical mutagenesis, and could make random chemical mutagenesis an indispensable tool in directed evolution studies.

Introduction

With recent advances in molecular biology, one has the possibility to isolate an enzyme with a desired catalytic property by directed evolution. In theory, the properties of an enzyme can be altered by rational design; however, rational design is greatly hindered in practice by the complexity of protein function. Although there has been a continuous advancement in our understanding of protein structure and function, it is clear that there are many aspects of protein function that we cannot predict. In the last few years, the combinatorial approach toward protein engineering has gathered momentum and success, thus defining a new discipline of research that is generally referred to as directed evolution of proteins. By mimicking natural evolution, directed evolution aims to generate a protein with new activity by screening or selecting for the desired function from a large pool of protein variants.[1–5] A wide variety of methods have been developed for the construction of gene libraries. These methods include the use of physical and chemical mutagens, mutator strains and some forms of insertion and deletion mutagenesis.[6] Error-prone PCR, DNA shuffling, one-step random mutagenesis by error-prone rolling-circle amplification, stEP, NeXT, nonhomologous recombination and RAISE are some of the approaches that have been used to introduce random mutations.[7–16] In vitro random chemical mutagenesis has also been used many times to introduce random mutations within a gene, although its usage has its own drawbacks.[17–22] Gradually but steadily, the usage of random chemical mutagenesis is dwindling due to the inherent problems attached with it. Lai et al. reported the use of ethyl methane sulfonate (EMS) as a tool to introduce random mutagenesis in vitro.[21] Their study showed 60% transformation rates of the EMS treated cells. This decreased transformation rate was due to the interference of ethylated DNA with transformation efficiency. Most directed evolution experiments involve either two different methods of random mutagenesis, or they pass the best mutant from the first round to subsequent mutation rounds. The logic behind such a practice is that in addition to the useful first generation mutations, there might be other useful mutations in the second and further rounds that could act with the earlier mutations in a cooperative manner. More rounds of mutant generation necessitate more screening, and this makes the process tedious. Here, we demonstrate the accumulation of mutations in a defined gene without passing it through further rounds of mutagenesis and screening. So, the possibility of obtaining the desired mutant without passing it through further mutagenesis rounds increases. The present work uses a chemical mutagen to mutate the desired gene in vivo and is followed by in vitro genetic manipulation to ensure that only the desired gene is affected. This approach was found to be an efficient and easy method for introducing

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random mutations in the desired gene, and can be applied to any chemical mutagen and any DNA sequence of interest for directed evolution studies. This approach has been successful in alleviating the inherent flaws of in vitro random chemical mutagenesis and whole-cell mutagenesis. To the best of our knowledge, this is the first report in which a dual approach has been used to generate mutations in a defined DNA sequence with a chemical mutagen or any other method.

Results

The DuARCheM method

The in vivo random chemical mutagenesis of the whole organism was the traditional and most often practised approach to induce mutations. The in vitro method of random chemical mutagenesis has been used many times for improving enzyme properties, but it has its own flaws, and hence, it is not gaining popularity as a robust random mutagenesis method for directed evolution studies. In the present work, random mutations have been successfully introduced in the desired gene sequence with a dual approach to random chemical mutagenesis. DuARCheM comprises two different approaches: in vivo chemical mutagenesis and in vitro genetic manipulation (Figure 1). The cells that contained the plasmid construct with the gene of interest were treated with EMS, which was added to the culture medium, and allowed to grow under the mutagenic pressure.

The plasmid construct was isolated from EMS-treated cells and digested with restriction enzymes to excise the gene of interest. The treated gene fragment was then ligated to an untreated expression vector and further transformed into a suitable expression host. Thus, in vivo mutations were induced and genetic manipulations were performed in vitro to ensure that only the defined DNA sequence is affected with EMS-induced mutagenesis.

There was one more finding that indicated that in DuARCheM, transformation efficiencies are not affected unlike the earlier reports that used the in vitro approach. In in vitro EMS-based mutagenesis, the ethylated DNA is not removed. This ethylated DNA is difficult to transform and this is why 60% transformation rates have been reported earlier.[21] Our approach also results in ethylated DNA, but because replication is occurring in the cell, an ethylated base is replaced by a normal base during the next replication cycle. So there is no effect on the transformation rate, and hence it results in a large library of mutants. To the best of our knowledge, there is no such report of chemical mutagenesis of a defined DNA fragment with a chemical mutagen within the cell. The main disadvantage of whole-cell in vivo random chemical mutagenesis is that the mutagenesis is indiscriminate. Thus, the construct that carries the gene of interest as well as the gene itself, and indeed the chromosomal DNA of the host cell, suffer mutation. Mutations can eventually affect essential host genes, which in turn could cause a reduction in genetic stability and cell viability. To address this problem, mutations were carried out in DH-5α cells and the plasmid that was isolated from them was digested to liberate the treated *Pseudomonas aeruginosa* lipase (lip) gene, ligated with untreated pET21b and further transformed into BL-21 competent cells. The indiscriminate nature of random chemical mutagenesis is one of the major reasons why it has not gained popularity among the most frequently used methods for generating random mutations. In the in vitro chemical mutagenesis method, the template is limited and does not increase over time, whereas in an in vivo system, there is no dearth of template because of replication in the cell. This might be another reason why earlier methods could not produce a large library, which in turn affects the quality of the variant molecules. Apart from the above advantages, the high EMS concentration used in the present study was not possible in the earlier method as high

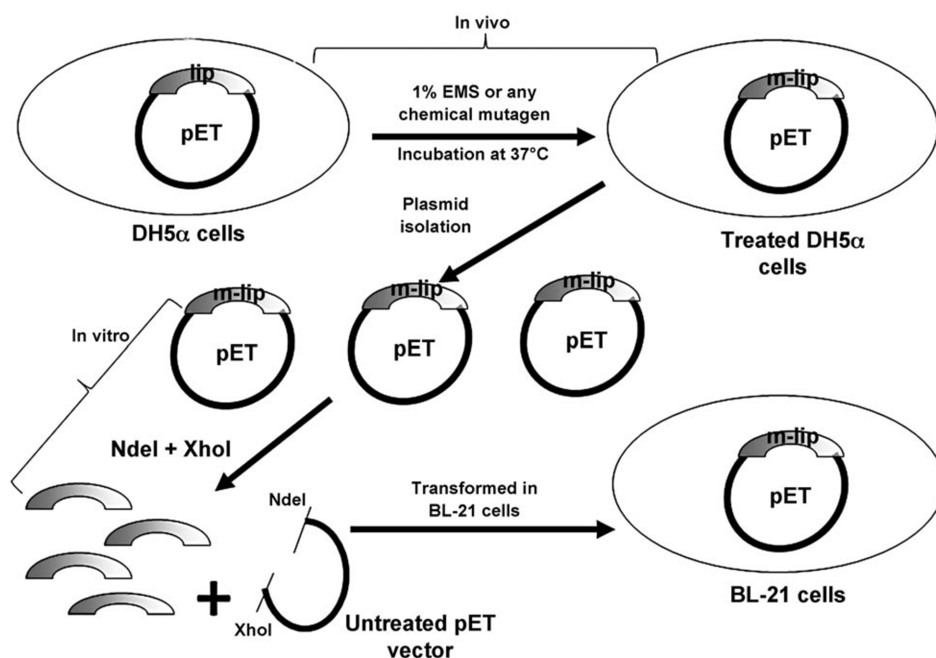

Figure 1. Schematic representation of random chemical mutagenesis of a defined gene sequence by using EMS in the DuARCheM process. Cells that contained the pLip construct were grown in the presence of chemical mutagen (EMS). This comprises the in vivo step of DuARCheM. Treated plasmids were isolated and digested with NdeI and XhoI to remove the treated lipase gene from the treated vector. The treated lipase gene was then ligated with untreated pET21-b vector and transformed into Bl-21(DE-3) cells. This comprises the in vitro step of DuARCheM. As a result, the physicochemical changes that are seen in the enzyme are due to the mutations that were introduced in the gene sequence.

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EMS concentrations in in vitro condition will adversely affect the transformation efficiency.[21]

Optimisation of EMS concentration and incubation time

The reported literature connecting chemical mutagens with DNA fragmentation led us to investigate the effect of EMS on DNA fragmentation. The DuARCheM depends on the concentration and time of growth of cells under EMS pressure. The plasmid that was isolated from the cells was treated with different EMS concentrations, but for the same duration of time, and showed different patterns of DNA fragmentation. It was observed that with the increased concentration of EMS, the DNA fragmentation increased. At 1% EMS concentration, there was no DNA fragmentation. At 2% and 3% EMS concentration, EMS led to DNA fragmentation and the fragmentation was more in the case of 3% EMS concentration (Figure 2A). Next we tried to optimise the time of treatment by keeping the EMS concentration constant (1%). When the cells were incubated with 1% EMS for 1, 2 and 3 h, it was found that DNA fragmentation was visible after 2 h and increased after 3 h (Figure 2B). Therefore, cells that contained the lipase construct (pLip) were grown in the presence of 1% EMS for 1 h for in vivo mutagenesis. The cells were then harvested and the plasmid isolated. Any delay in harvesting the cells and isolation of plasmid led to increased DNA fragmentation.

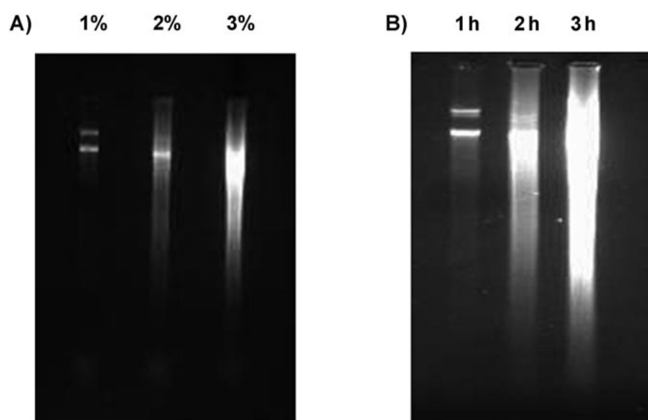

Figure 2. A) Optimisation of EMS concentration for DuARCheM. Cells that contained pLip construct were grown (37 °C, 200 rpm) in the presence of different concentrations of EMS (1, 2 and 3%) for 1 h. The treated plasmid constructs were isolated and run on a 1% gel to check for DNA fragmentation. B) Optimisation of EMS treatment time for DuARCheM. Cells that contained pLip construct were grown (37 °C and 200 rpm) in the presence of 1% EMS for different lengths of time (1, 2 and 3 h). The treated plasmid constructs were isolated and run on 1% gel to check for DNA fragmentation.

Mutation pattern

The mutants were picked from minimal salt media (MSM)/tricaprylin plates based on the colour change that was due to the hydrolytic activity of lipase. In total 26 mutants were sequenced and analysed for any mutation in the defined DNA sequence (Table 1). As expected, we obtained both transition as well as transversion mutations in the variants. We have made an interesting and significant observation that the mutations tend to accumulate when they are forced in vivo (Figure 3A). Some mutants showed accumulation of mutations (Figure 3B). UM03 and UM04 had one mutation at position 760 in the lipase gene. UM06 was found to have mutations at nucleotide positions 526, 610 and 760. UM12 had mutations at nucleotide positions 760, 526, 583 and 610. UM14 was found to have mutations at nucleotide positions 136, 526, 760 and 610. UM01 was found to have mutation at nucleotide positions 526, 610, 631 and 760. UM05 and UM11 were found to have mutations at positions 760 and 748. The mutation at position 760 seems to be the parent mutation. Accumulation of mutations is a general phenomenon that occurs when mutants of one round are further passed through several rounds of mutagenesis. The main significance of this work lies in the fact that DuARCheM results in the accumulation of mutations in a single round of mutagenesis as mutation and cell division take place concomitantly. Accumulation of mutations is not possible in in vitro chemical mutagenesis in a single round of mutagenesis because there is no replication during the mutagenesis procedure. In in vitro chemical mutagenesis, the gene of interest is incubated with a chemical mutagen for a particular time period. Mutations will accumulate in the in vitro random chemical mutagenesis approach only if the mutant of the first generation is passed through several more rounds of mutagenesis; this results in a tedious process of more mutagenesis and screening. DuARCheM leads to the generation of a mutant library in which the mutants share some similar point mutations in addition to other mutations. This is an advantageous situation because it leads to a library in which there are many mutants that differ in one or two mutations, but still carry the parent mutations. So, studying the phenotypic effect of all these mutants might help to ascertain the role of additional mutations. This particular property of dual approach makes it more effective and less tedious because the probability of obtaining the desired mutant increases in a shorter time and with fewer screening rounds. This property is visible because the mutations occur simultaneously with plasmid replication and cell duplication. The fact that each cell carries more than one plasmid copy might also play a role to some extent in such an observation.

Effect on hydrolytic activity of lipase by DuARCheM

We further investigated the effect of the mutations introduced by DuARcheM on the enzymatic activity of lipase. Tricaprylin hydrolysis was used as the primary screening system in which the decrease in pH due to acid formation was monitored by the change of bromothymol dye colour. Colonies were screened by plating the DuARCheM constructs on MSM plates that contained substrate, dye and inducer. Among them, 26 colonies were sequenced. These colonies were further tested for tricaprylin hydrolysis by using a 96-well format (Figure 4). The production of tricaprylic acid due to the enzymatic activity of the lipase results in the green colour of bromothymol to change to yellow. UM01, UM08, UM09 and UM14 showed

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Table 1. Sequence analysis of mutants generated by one round of DuaRCheM.

Mutant	Nucleotide residues		Amino acid residues		Mutant	Nucleotide residues		Amino acid residues	
clone	Position (bp)	Substitution	Position (aa)	Substitution	clone	Position (bp)	Substitution	Position (aa)	Substitution
UM01	526	A→G	176	Thr→Ala	UM15	406	C→A	136	His→Gln
	610	G→A	204	Val→Ile		434	G→C	145	Arg→Pro
	631	G→A	211	Glu→Glu		533	C→G	178	Thr→Arg
	760	C→G	254	Asn→Lys	UM16	237	G→C	79	Ser→Ser
UM02	487	G→A	163	Ser→Asp		259	C→G	87	Leu→Val
UM03	760	C→G	254	Asn→Lys	UM17	406	C→A	136	His→Gln
UM04	760	C→G	254	Asn→Lys		434	G→C	145	Arg→Pro
UM05	748	A→C	250	Asn→Thr	UM18	55	C→A	19	Ala→Asp
	760	C→G	254	Asn→Lys		290	G→C	97	Ser→Thr
UM06	526	A→G	176	Thr→Ala		291	C→G	97	Ser→Thr
	610	G→A	204	Val→Ile	UM19	558	G→C	186	Glu→Asp
	760	C→G	254	Asn→Lys		748	A→C	250	Asn→Thr
UM07	442	A→T	148	Pro→Pro		760	C→G	254	Asn→Lys
UM08	466	A→G	156	Ile→Val	UM20	419	C→A	140	Thr→Asn
UM09	757	G→A	253	Ala→Thr		424	G→A	142	Asp→Asn
UM10	232	C→T	78	Thr→Thr		439	A→G	147	Gln→Arg
UM11	748	A→C	250	Asn→Thr		440	G→A	147	Gln→Arg
	760	C→G	254	Asn→Lys	UM21	929	G→T	310	Ser→Ile
UM12	526	A→G	176	Thr→Ala	UM22	750	C→G	250	Asn→Lys
	283	G→C	195	Ala→Pro		837	C→G	279	Asp→Glu
	610	G→A	204	Val→Ile	UM23	627	C→G	209	Cys→Trp
	760	C→G	254	Asn→Lys	UM24	757	G→A	253	Ala→Thr
UM13	217	G→A	73	Arg→His	UM25	744	C→T	248	Ser→Ile
UM14	136	G→A	46	Asp→Asn	UM26	526	A→G	176	Thr→Ala
	526	A→G	176	Thr→Ala		760	C→G	254	Asn→Lys
	610	G→A	204	Val→Ile					
	760	C→G	254	Asn→Lys					

higher activity than the original lipase gene. UM01 showed maximum hydrolytic activity and was almost yellow in colour. UM07 was the colony that showed the least hydrolytic activity; it did not show any colour change and was greenish. The enzyme activities were quantitated for all the sequenced mutants (Table 2).

Conclusions

We report here a novel approach for random chemical mutagenesis (DuARCheM) that introduces mutations in a defined gene sequence. This approach is advantageous over in vitro random chemical mutagenesis methods,[17–22] and at par with other random point mutagenesis methods in the following ways:

1) *Accumulation of mutations*: the DuARCheM scores better than other known random chemical mutagenesis methods due to the accumulation of point mutations in a single round of mutagenesis.
2) *Large library size*: DuARCheM scores better than other known random chemical mutagenesis methods as transformation efficiency is not affected, unlike the previously reported method.[21]
3) *Mutagenesis is not indiscriminate*: the main disadvantage of in vivo random chemical mutagenesis is that mutagenesis is indiscriminate. The construct that carries the gene of interest as well as the gene itself, and indeed the chromosomal DNA of the host cell, suffer mutation. Mutations might eventually affect essential host genes, which in turn could cause a reduction in genetic stability and cell viability. In our approach, this problem is alleviated.
4) *Availability of template*: there is no dearth of template molecules because replication of the template occurs in the cell concomitantly with the mutation.
5) *Added benefit*: this approach has the added benefit that library size can be increased by increasing culture volume.
6) *At par with PCR-based mutagenesis*: this approach is at par with PCR-based mutagenesis methods as amplification and mutation take place concomitantly.
7) *Transition and transversion mutations*: EMS is capable of introducing transitions and transversions in a given DNA sequence. One single mutagen is enough for generating both types of mutations; this increases the spectrum of variants.

This approach could be further extended to other chemical mutagens to introduce mutations in a defined DNA fragment. Also, we could apply several chemical mutagens in combination with this approach to force a balance between transition and transversion mutations in a defined DNA sequence. The present approach could play a pivotal role in improving existing enzymes, thus driving ahead neo-Darwinism.

Experimental Section

Organism: The *Pseudomonas aeruginosa* strain isolated in our laboratory was deposited at the Microbial Type Culture Collection

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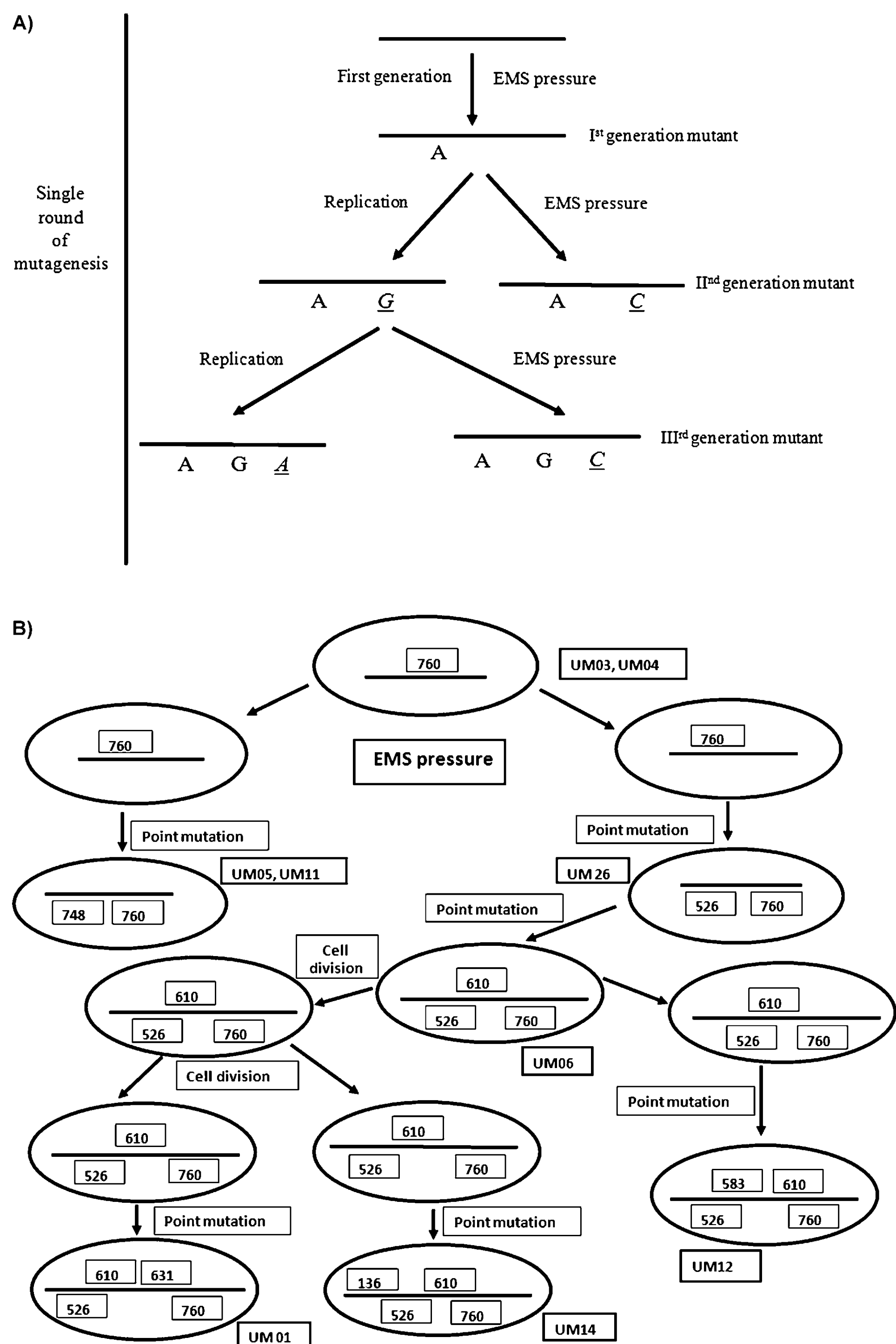

Figure 3. A) Accumulation of mutations in DuARCheM. In the presence of EMS, the wild-type gene undergoes point mutation. These point mutations are passed on to further variants in the same library after cell division and plasmid replication. When the cell with the parent mutation divides, the genetic material is equally divided; this results in two cells that have the same mutation in the gene of interest, but because both cells are grown in the presence of mutagen, separate mutations can occur in the two cells. So, there is an accumulation of point mutations within a library in a single round of mutagenesis. B) Accumulation of mutations by DuARCheM in the mutant library after a single round of mutagenesis; few variants contain one or more common point mutations. The parent mutant has a point mutation at nucleotide position 760 bp; the downstream variants are derived from the parent and mutations are accumulated.

(MTCC, Chandigarh, India; accession number: 5113). *E. coli* DH5α and BL21 (DE-3) cells were used as hosts.

Plasmid constructs and growth conditions: The lipase gene was PCR amplified by using the genomic DNA of *P. aeruginosa* MTCC 5113 as template, and inserted into pDrive vector (Quiagen). The 935 bp lipase gene insert was excised from the pDrive vector by using NdeI and XhoI and subcloned in pET-21b (Novagen). The Lip gene from *P. aeruginosa* that was cloned into the pET-21b plasmid was designated as pLip. *E. coli*, which served as the host organism, were grown, overnight, in Luria–Bertani (LB) medium that contained ampicillin (100 $\mu g\,mL^{-1}$) at 37 °C.

Random chemical mutagenesis and construction of the libraries of variant lipase genes: To generate a library of lipase variants, mutations were introduced by using EMS. The pLip construct was transformed in DH5α cells, and single colonies were grown on LB growth medium for 12 h at 37 °C. Inoculum (1 %) was introduced into a fresh LB vial and allowed to grow until mid-log phase (OD ~1.0). EMS was added to the culture medium (1 %) and cells were incubated at 37 °C. The DH5α cells were then allowed to grow under the EMS pressure for 1 h. Plasmid DNA was isolated from the DH5α cells that contained the pLip construct, digested with NdeI and XhoI, religated into untreated pET21b, which was digested with NdeI and XhoI, and transformed in BL-21 cells. Transformed colonies were treated individually as mutants and were screened for change in activity.

Expression of the pLip construct in BL-21 cells: pET-21b-lip (pLip) construct was expressed in BL-21 cells. The cells were induced with IPTG (1 mM) for 6 h at 37 °C. Each mutant was picked from the LB/ampicillin plates and induced with IPTG (1 mM) for 6 h.

Expression of the mutant pLip construct in BL-21 cells: On transformation of BL-21 cells with EMS-treated pLip construct, the colo-

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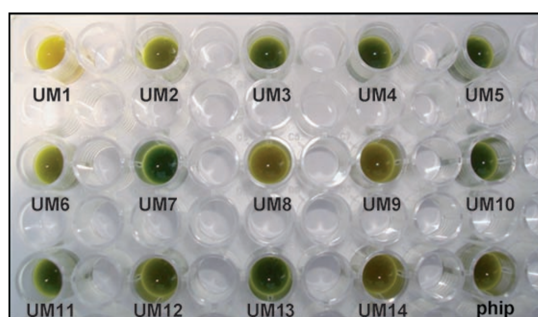

Figure 4. Effect of DuARCheM on the hydrolytic activity of *P. aeruginosa* lipase (lip). The variants were checked for the effect of DuARCheM on the hydrolytic activity of the lipase. A cell suspension (100 μL; 200 mg mL^{-1}) was added to a buffer solution (150 μL; 2.5% tricaprylin, 2.0% Triton X-100 and bromothymol) and the reaction was allowed to continue.

Table 2. Enzyme activities of all sequenced mutants.

Mutant	Enzyme activity [U mL^{-1}]	Mutant	Enzyme activity [U mL^{-1}]
wild-type lipase	55.2	UM14	64.6
UM01	76.4	UM15	57.9
UM02	53.2	UM16	36.3
UM03	26.8	UM17	58.1
UM04	28.0	UM18	54.3
UM05	22.3	UM19	40.1
UM06	49.8	UM20	45.7
UM07	5.70	UM21	55.3
UM08	62.1	UM22	59.4
UM09	60.1	UM23	54.9
UM10	22.7	UM24	60.3
UM11	25.4	UM25	53.4
UM12	54.0	UM26	56.3
UM13	39.2		

nies that appeared on LB/ampicillin plates were treated as the mutant colonies. These colonies were picked and allowed to grow in LB medium in the presence of ampicillin (100 μg mL^{-1}) for 12 h at 37 °C. Vials with LB medium (10 mL) were freshly inoculated with culture (1%). Each vial contained a separate colony, and was incubated to OD 0.6. Cells were induced with IPTG (1 mм) when the OD reached 0.6. The cells in each of the vials were allowed to grow in the presence of IPTG for 6 h.

High-throughput screening of mutants for increased enzyme activity (specific activity): High-throughput screening was done by using a pH-based method. Tricaprylin hydrolysis was monitored by the change in the colour of the pH-based dye bromothymol. The gene constructs from the DuARCheM experiments were transformed into BL-21 cells and plated on MSM plates that contained tricaprylin (2%) and IPTG (1 mм). The 96-well plate screening was also based on the same principle. Cell suspension (100 μL; 200 mg mL^{-1}) was added to a buffer solution (150 μL; 2.5% tricaprylin, 2.0% Triton X-100 and bromothymol) and the reaction was allowed to continue.

Sequence analysis of lip variants: The sequencing of the mutant DNA was performed at Bangalore Genie, India, by using an ABI PRISM® 3100 Genetic Analyzer sequencer. DNA sequencing data were processed by using the DNASTAR software package to find the point mutations in the variants. The DNA sequences were then translated to the corresponding proteins and compared to the wild-type protein.

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Keywords: directed evolution · DNA · DuARCHeM · ethyl methane sulfonate · mutagenesis

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