

EMGEN* Newsletter

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Eastern Mediterranean Health Genomics and Biotechnology Network (EMHGBN) was created in 2004 with collaboration of representatives of selected centre of excellence in (health related) molecular biology, biotechnology & genomics in the Eastern Mediterranean region by recommendations and efforts of WHO/EMRO.

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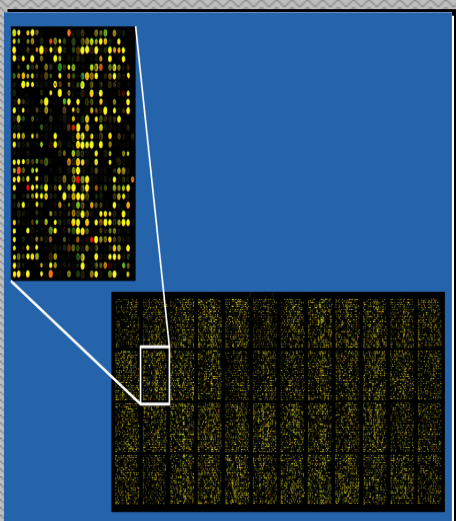
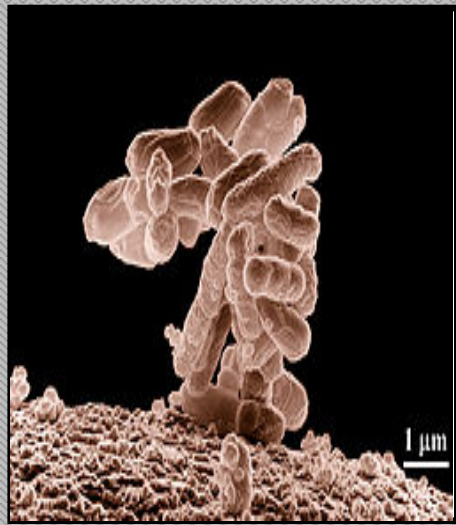
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Article

Identification and over-expression of a thermostable lipase from *Geobacillus thermoleovorans* Toshki in *Escherichia coli*



Prof. Yasser R. Abdel-Fattah



Ass. Prof. Ahmed A. Gaballa

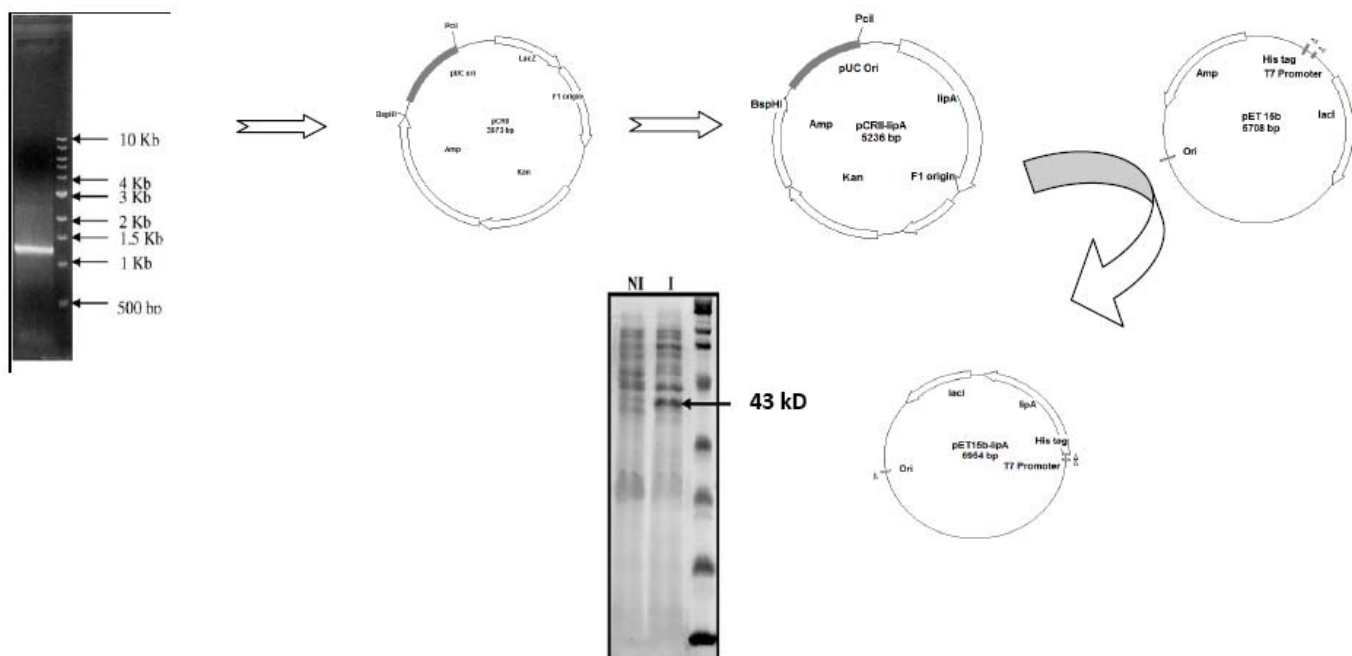
The paper entitled: Identification and over-expression of a thermostable lipase from Geobacillus thermoleovorans Toshki in Escherichia coli which is published in Microbiological Research, Vol. 163 (2008) 13-20, described the isolation of thermophilic bacteria identified as Geobacillus thermoleovorans according to 16S rDNA sequence analysis that was able to produce thermostable lipase with outstanding characteristics. The enzyme thermal stability at 60°C and retaining 30% of activity for 1 h at 100°C was an indication of industrial applicability in the field of detergents. The study was carried out by Yasser R. Abdel-Fattah and Ahmed A. Gaballa. The two authors were equally contributed to this work. Prof. Yasser R. Abdel-Fattah is now the Dean of the Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and technology Applications; and Dr. Ahmed A. Gaballa, Associate professor in the same institute and presently in a sabbatical leave at Microbiology Dept., Cornell University, USA.

Enzymes produced by extremophiles (Extremozymes) represent a significant milestone for their various applications in food, industry as well as energy production. Egypt is famous of great microbial biodiversity and especially of extremophiles microflora due to the presence of many hot springs, deserts, salterns and soda lakes. Such environment is rich in bacteria producing hydrolytic enzymes that could be a good start for re-exploring the microbial resources in Egypt and to build up capabilities in enzyme bioprocessing. Thermostable enzymes isolated mainly from thermophilic microorganisms have received attention due to their potential commercial applications because of their overall inherent stability and high reaction rates at high temperatures (Demirijan et al., 2001). A number of thermophilic microorganisms have been isolated from different exotic ecological zones of the planet earth in order to be used for such applications (Kohilu et al., 2001; Abdel-Fattah, 2002). Thermostable lipases play an important role in industrial processes employed at temperatures exceeding 50 °C (Sharma et al., 2002). Many thermostable lipases have been purified, characterized and cloned (Lee et al., 1999; Cho et al., 2000; Sinchaikul et al., 2001; Ewis et al., 2004) for industrial uses in detergents and treatment of domestic sewage.



To clone the target gene by PCR, different bacilli thermostable lipases were aligned using ClustalX and degenerative primers were designed in the 5' and the 3' conserved ends of the genes. The open reading frame (ORF) of the thermostable lipase gene was amplified by PCR from the genomic DNA of *G. thermoleovorans* Toshki with Taq DNA polymerase. The amplified product was purified from gel and ligated into T/A cloning vector pCR II TOPO (Invitrogen). The lipase gene was further sub-cloned to over expression vector pET 15b (contains the strong T7 promoter (IPTG induced) and His-tagged to facilitate the purification steps afterwards). After transformation and selection of the positive clone, some experiments on factors affecting gene expression were carried out on a bench top fermentor scale. Fermentation results illustrated that the best induction medium for the construct was 2xYT, and the maximum induction rate was obtained on adding IPTG after 70 minutes of growth start.

Overall, the paper represents a fast and efficient method for cloning genes using PCR, and illustrates the advantages of thermozymes due to their high degree of tolerance to harsh environmental conditions, which is exactly needed in industrial applications.



Schematic drawing shows the cloning and over-expression of lipase gene from *G. thermoleovorans* Toshki, with the subsequent SDS-PAGE in the presence of IPTG as inducer (I) and in its absence (NI).





Article

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Note to the Readers

Dear Readers, Researchers, Biotech Industries,

We are interested in reflecting on your research papers (from 2007 until present) or any reports about your work/research in laboratories, institutes or companies. We appreciate if you send us a brief description of your recent work in maximum 500 words and pictures. In addition, we require two pictures related to you and/or one or all of the contributors. Please send them in “doc” or “docx” format to one of the following email addresses. In addition, we may put your advertisement in our far reaching newsletter, for which the same below emails can receive them.

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Training

Current techniques in studying of protein-protein and protein-DNA interactions

Two-hybrid screening is a molecular biology technique to investigate protein-protein interactions and protein-DNA interactions on the bases of physical interactions between two proteins or a single protein and a DNA molecule.

First step in this discovering technique is the activation of downstream reporter gene by the binding of a transcription factor onto an upstream activating sequence (UAS).

For the purposes of two-hybrid screening, the transcription factor is split into two separate fragments, called the binding domain (BD) and activating domain (AD). The BD is the domain responsible for binding to the UAS and the AD is the domain responsible for activation of transcription. Theoretically, any living cell might be used as the background to a hybrid analysis, but yeast and *E. coli* are the only two common host organism used in this discovery technique. Drug and poison discovery, determination of protein function and Zinc finger protein selection are most desirable applications of this technique.

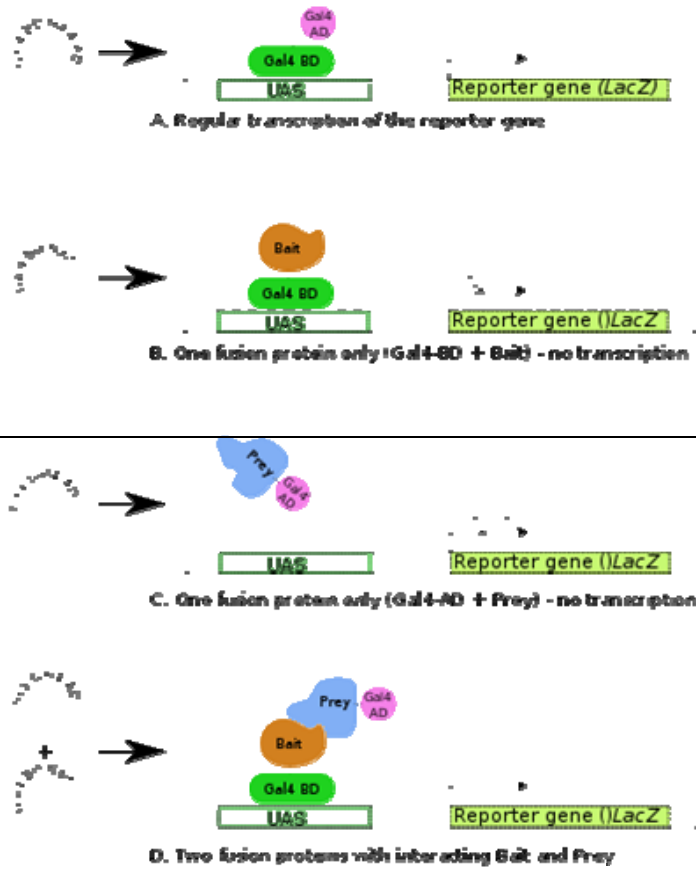
On the other hand, Phage display is another applicable method for the study of protein, protein peptide, and protein DNA interactions that utilizes bacteriophage to connect proteins with the genetic information that encodes them. This connection between genotype and phenotype enables large libraries of proteins to be screened and amplified in a process called *in vitro* selection, which is analogous to natural selection. The most common bacteriophages used in phage display are M13 and fd filamentous phage, though T4, T7, and λ phage have also been used.

Like the two-hybrid system, phage display is used for the high-throughput screening of protein interactions.

By immobilizing a relevant DNA or protein target to the surface of a well, a phage that displays a protein that binds to one of those targets on its surface will remain while others are removed by washing. Those that remain can be eluted, used to produce more phages (by bacterial infection with helper phage) and so produce a phage mixture that is enriched with relevant (i.e. binding) phage. The repeated cycling of these steps is referred to as "panning", in reference to the enrichment of a sample of gold by removing undesirable materials.

Phage eluted in the final step can be used to infect a suitable bacterial host, from which the phagemids can be collected and the relevant DNA sequence excised and sequenced to identify the relevant, interacting proteins or protein fragments.





Schema of two-hybrid assay, checking for interactions between two proteins

One-hybrid

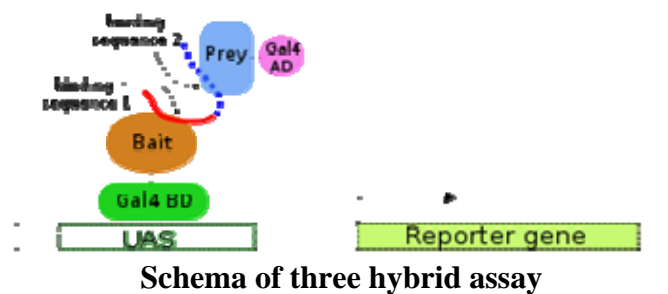
The one-hybrid variation of this technique is designed to investigate protein-DNA interactions and uses a single fusion protein in which the AD is linked directly to the binding domain. The binding domain in this case, however is not necessarily of fixed sequence as in two-hybrid protein-protein analysis but may be constituted by a library. This library can be selected against the desired target sequence which is inserted in the promoter region of the reporter gene construct. In a positive-selection system, a binding domain which successfully binds the UAS and allows transcription will thus be selected.

Note that selection of DNA-binding domains is not necessarily performed using a one-hybrid system, but also can be performed using a two-hybrid system in which the binding domain is varied and the bait and prey proteins are kept constant.



Three-hybrid

RNA-protein interactions have been investigated through a three-hybrid variation of the two-hybrid technique. In this case, a hybrid RNA molecule serves to join together the two protein fusion domains which are not intended to interact with each other but rather the intermediary RNA molecule (through their RNA-binding domains). Techniques involving non-fusion proteins that perform a similar function, may also be referred to as three-hybrid methods.



One-two-hybrid

Simultaneous use of the one- and two-hybrid methods (that is, simultaneous protein-protein and protein-DNA interaction) is known as a one-two-hybrid approach and is expected to increase the stringency of the screen.

Bacterial Display

Bacteria display or bacterial surface display is a protein engineering technique used for *in vitro* protein evolution. Libraries of polypeptides displayed on the surface of bacteria can be screened using flowcytometer or iterative selection procedures (biopanning).

Yeast display

Yeast display or yeast surface display is a technique used in the field of protein engineering. The yeast display technique was first published by the laboratory of Professor K. Dane Wittrup. The technology was sold to Abbott Laboratories in 2001.

A protein of interest is displayed as a fusion to the Aga2p protein on the surface of yeast. The Aga2p protein is naturally used by yeast to mediate cell-cell contacts during yeast cell mating. As such, display of a protein via Aga2p projects the protein away from the cell surface, minimizing potential interactions with other molecules on the yeast cell wall. The use of magnetic separation



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and flowcytometer in conjunction with a yeast display library is a highly effective method to isolate high affinity protein ligands against nearly any receptor through directed evolution.

Advantages of yeast display over other *in vitro* evolution methods include eukaryotic expression and processing, quality control mechanisms of the eukaryotic secretory pathway, minimal avidity effects, and quantitative library screening through fluorescent-activated cell sorting (FACS).

Disadvantages include smaller mutagenic library sizes compared to alternative methods and differential glycosylation in yeast compared to mammalian cells. It should be noted that these disadvantages have not limited the success of yeast display for a number of applications, including engineering the highest monovalent ligand-binding affinity reported to date for an engineered protein (Boder, E.T. 2000).

Ribosome Display

Ribosome Display is a technique used to perform *in vitro* protein evolution to create proteins that can bind to a desired ligand. The process results in translated proteins that are associated with their mRNA progenitor which is used, as a complex, to bind to an immobilized ligand in a selection step. The mRNA-protein hybrids that bind well are then reverse transcribed to cDNA and their sequence amplified via PCR. The end result is a nucleotide sequence that can be used to create tightly binding proteins.

Ribosome display either begins with a DNA sequence or naive library of sequences coding for a specific protein. The sequence is transcribed, and then translated *in vitro* into protein. However, the DNA library coding for a particular library of binding proteins is genetically fused to a spacer sequence lacking a stop codon. This spacer sequence, when translated, is still attached to the peptidyl tRNA and occupies the ribosomal tunnel, and thus allows the protein of interest to protrude out of the ribosome and fold. What results is a complex of mRNA, ribosome, and protein which can bind to surface-bound ligand. This complex is stabilized with the lowering of temperature and the addition of cations such as Mg^{2+} . During the subsequent binding, or panning stages, the complex is introduced to surface-bound ligand. This can be accomplished in several ways, for example using an affinity chromatography column with a resin bed containing ligand, a 96-well plate with immobilized surface-bound ligand, or magnetic beads that have been coated with ligand. The complexes that bind well are immobilized. Subsequent elution of the binders via high salt concentrations, chelating agents, or mobile ligands which complex with the binding motif of the protein allow dissociation of the mRNA. The mRNA can then be reverse transcribed back into cDNA, undergo mutagenesis, and iteratively fed into the process with greater selective pressure to isolate even better binders.

mRNA display

mRNA display is a display technique used for *in vitro* protein, or peptide evolution to create molecules that can bind to a desired target. The process results in translated peptides or proteins that are associated with their mRNA progenitor via a puromycin linkage. The complex then binds to an





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immobilized target in a selection step (affinity chromatography). The mRNA-protein fusions that bind well are then reverse transcribed to cDNA and their sequence amplified via a polymerase chain reaction. The end result is a nucleotide sequence that encodes a peptide with high affinity for the molecule of interest. Puromycin is an analogue of the 3' end of a tyrosyl-tRNA with a part of its structure mimics a molecule of adenosine, and the other part mimics a molecule of tyrosine. Compared to the cleavable ester bond in a tyrosyl-tRNA, puromycin has a non-hydrolysable amide bond. As a result, puromycin interferes with translation, and causes premature release of translation products.

To synthesize an mRNA-polypeptide fusion, the fused puromycin is not the only modification to the mRNA template. Oligonucleotides and other spacers need to be recruited along with the puromycin to provide flexibility and proper length for the puromycin to enter the A site. Ideally, the linker between the 3' end of an mRNA and the puromycin has to be flexible and long enough to allow the puromycin to enter the A site upon translation of the last codon. This enables the efficient production of high-quality, full-length mRNA-polypeptide fusion. Linkers longer than 40 nucleotides and shorter than 16 nucleotides showed greatly reduced efficiency of fusion formation. Also, when the sequence rUrUP presented adjacent to the puromycin, fusion did not form efficiently.

In addition to providing flexibility and length, the poly dA portion of the linker also allows further purification of the mRNA-polypeptide fusion due to its high affinity for dT cellulose resin. The mRNA-polypeptide fusions can be selected over immobilized selection targets for several rounds with increasing stringency. After each round of selection, those library members that stay bound to the immobilized target are PCR amplified, and non-binders are washed off.

Comparing of techniques

Two-hybrid can provide an important first hint for the identification of interaction partners. Moreover, the assay is scalable, which makes it possible to screen for interactions among many proteins.

The main criticism applied to the yeast two-hybrid screen of protein-protein interactions is the possibility of a high number of false positive and negative identifications. The exact rate of false positive results is not known, but estimates are as high as 50%. The reason for this high error rate lies in the principle of the screen: The assay investigates the interaction between (1) over expressed (2) fusion proteins in the (3) yeast (4) nucleus. Each of these points (1-4) alone can give rise to false results. For example, over expression can result in non-specific interactions. Moreover, a mammalian protein is sometimes not correctly modified in yeast, which can also lead to false results. Finally, some proteins might specifically interact when they are co-expressed in the yeast, although in reality they are never present in the same cell at the same time. Due to the combined effects of all error sources the overall confidence of the yeast two-hybrid assay is rather low. However, yeast two-hybrid data is shown to be of similar quality to data generated by the alternative approach of coaffinity purification followed by mass spectrometry (AP/MS). The probability of generating false positives means that all interactions should be confirmed by a high





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confidence assay, for example co-immunoprecipitation of the endogenous proteins, which is difficult for large scale protein-protein interaction data.

As mentioned, there are many other molecular display technologies to study protein-protein and protein-DNA interactions, such as phage display, bacterial display, yeast display, and ribosome display and mRNA display. From these current techniques, mRNA display technology has many advantages over the others. The first three biological display libraries listed have polypeptides or proteins expressed on the respective microorganism's cell surface and the accompanying coding information for each polypeptide or protein is retrievable from the microorganism's genome. However, the library size for these three *in vivo* display systems is limited by the transformation efficiency of each organism. For example, the library size for phage and bacterial display is limited to $1-10 \times 10^9$ different members. The library size for yeast display is even smaller. Moreover, these cell-based display systems only allow the screening and enrichment of peptides/proteins containing natural amino acids. In contrast, mRNA display and ribosome display are *in vitro* selection methods. They allow a library size as large as 10^{15} different members. The large library size increases the probability to select very rare sequences, and also improves the diversity of the selected sequences. In addition, *in vitro* selection methods remove unwanted selection pressure, such as poor protein expression, and rapid protein degradation, which may reduce the diversity of the selected sequences. Finally, *in vitro* selection methods allow the application of *in vitro* mutagenesis and recombination techniques throughout the selection process.

Although both ribosome display and mRNA display are *in vitro* selection methods, mRNA display has some advantage over the ribosome display technology. mRNA display utilizes covalent mRNA-polypeptide complexes linked through puromycin; whereas, ribosome display utilizes stalled, noncovalent ribosome-mRNA-polypeptide complexes. For ribosome display, selection stringency is limited to keep ribosome-mRNA-polypeptide in a complex because of the noncovalent ribosome-mRNA-polypeptide complexes. This may cause difficulties in reducing background binding during the selection cycle. Also, the polypeptides under selection in a ribosome display system are attached to an enormous rRNA-protein complex, a ribosome, which has a molecular weight of more than 2,000,000 Da. There might be some unpredictable interaction between the selection target and the ribosome, and this may lead to a loss of potential binders during the selection cycle. In contrast, the puromycin DNA spacer linker used in mRNA display technology is much smaller compared to a ribosome. This linker may have less chance to interact with an immobilized selection target. Thus, mRNA display technology is more likely to give less biased results.

Source: various sections of the article were collected and picked selectively from different Wikipedia pages





Announcement

The 2nd Iranian Proteomics Conference

IPS
Iranian Proteomics Society

ROYAN INSTITUTE
پژوهشگاه رویان

انجمن پروتئومیکس ایران و پژوهشگاه رویان برگزار می‌کند:
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پژوهشگاه رویان، تهران
۳ و ۴ اردیبهشت ۱۳۸۸

The Second Iranian
Proteomics Congress

Royan Institute,
Tehran, Iran
April 23-24 2009

Congress Secretariat:
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Biotechnology Research Institute of Iran,
Post Office Box (31535-1897), Karaj, Iran

Telefax: +98261 2702893
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www.proteomics.ir/congress

Invited Speakers:

- Professor Young-Ki Paik
President of Human Proteome Organisation
- Professor Max C.M. Chung
Secretary General of Asia Oceania Human
Proteome Organisation
- Professor Pier Giorgio Righetti
Department of Chemistry, Materials and
Chemical Engineering "Giulio Natta"
- Dr. Hans-Peter Mock
Institute for Plant Genetics and
Crop Plant Research

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Cover Picture

Cover Pictures Description (From top to bottom)

Title: *E. coli* bacteria

Description: *E. coli* is Gram-negative, facultative anaerobic and non-sporulating. The cells are about 2 micrometres (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 μm . It can live on a wide variety of substrates. *E. coli* uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon dioxide.

Source: en.wikipedia.org/wiki/E._coli

Title: *E. coli* bacteria with flagella

Description: *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding shiga toxin from *Shigella* to *E. coli* O157:H7, carried by a bacteriophage.

Source: en.wikipedia.org/wiki/E._coli

Title: A DNA microarray

Description: A DNA microarray (also commonly known as *gene or genome chip*, *DNA chip*, or *gene array*) is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon chip forming an array for the purpose of gene expression profiling, genotyping, copy number analysis, loss of heterozygosity (LOH) analysis, or DNA-protein interaction (ChIP). The principal advantage of DNA microarrays over previous technologies is that DNA microarrays allow the biological status of thousands of genes or genomic loci to be assayed simultaneously.

Source: en.wikipedia.org/wiki/User:Paphrag/sandbox

