# Engenharia de enzimas e células. Engenharia de reguladores de transcrição.

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MICROBIOLOGIA
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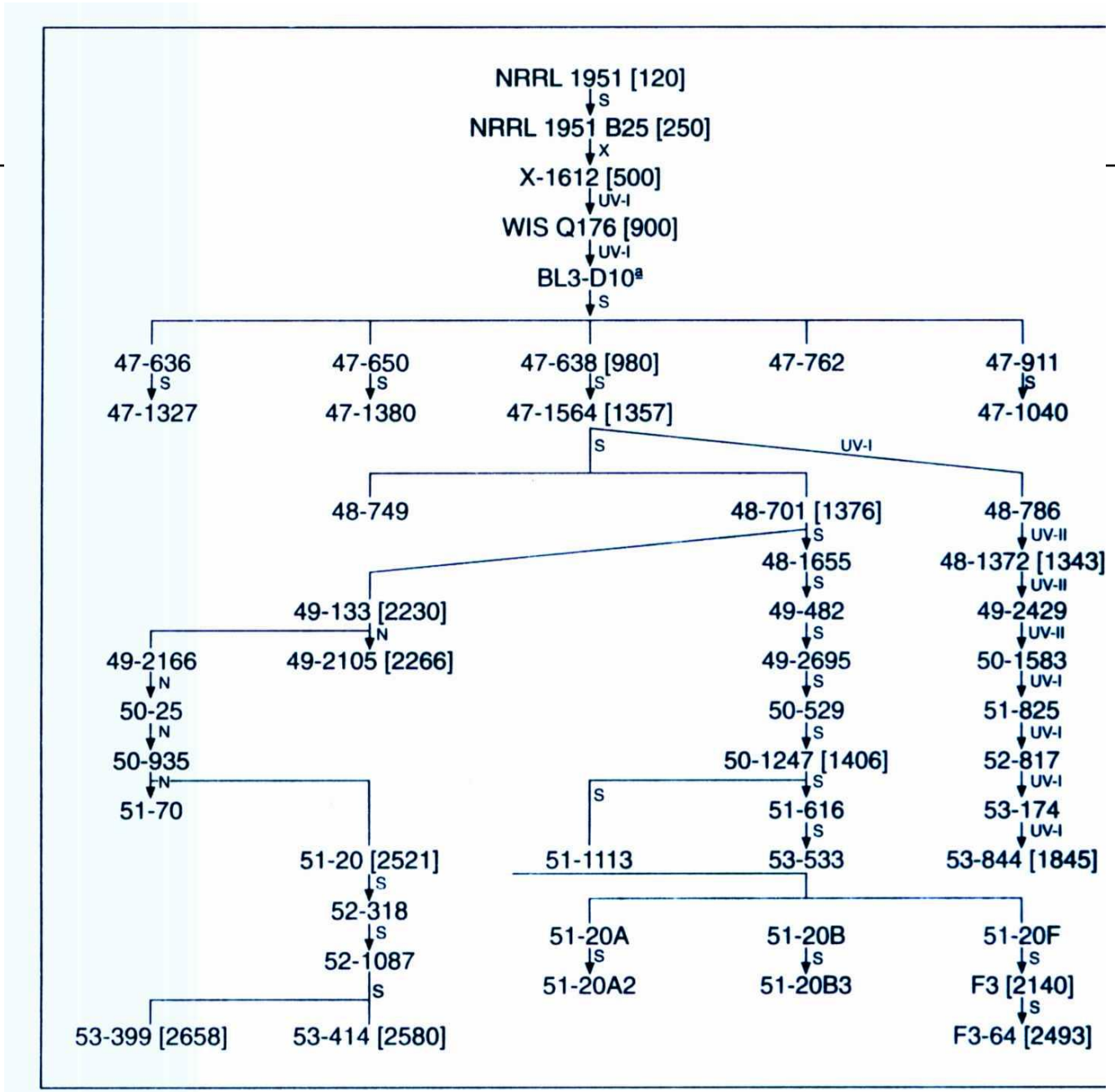
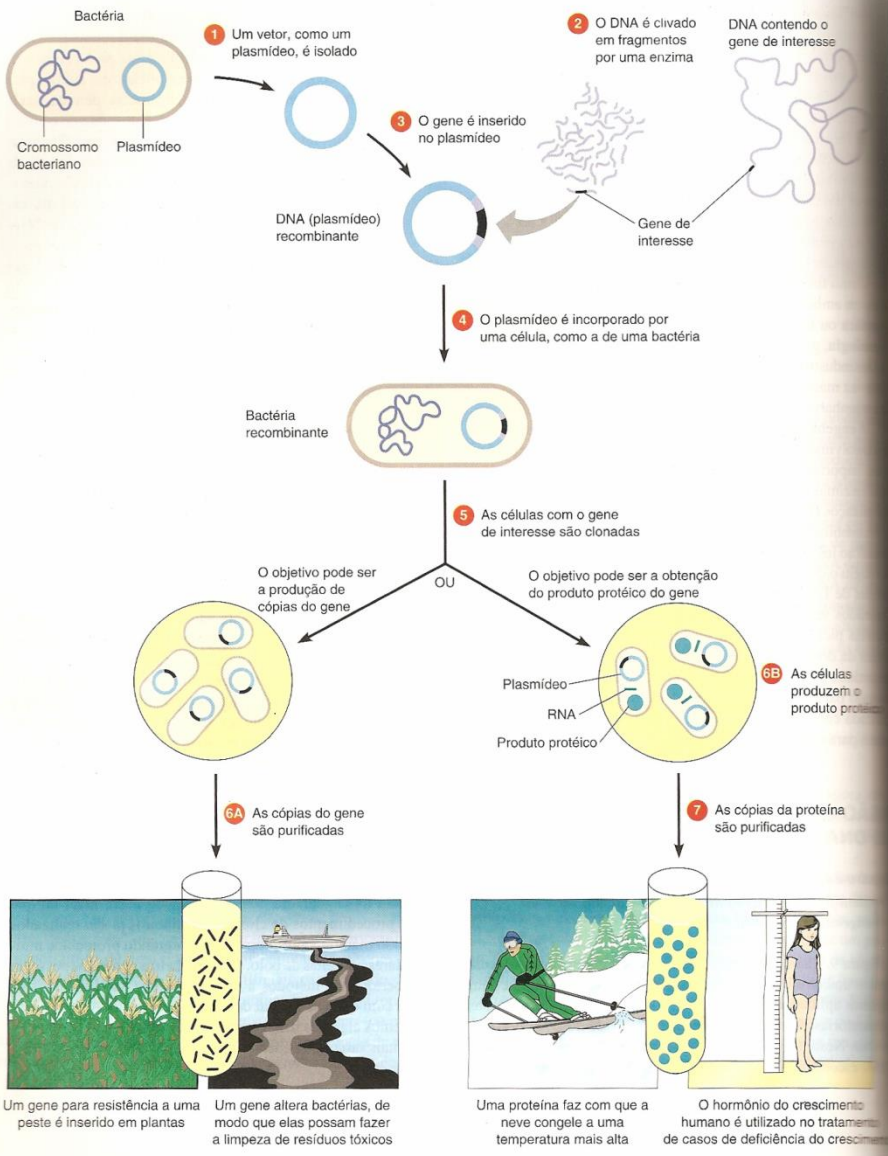
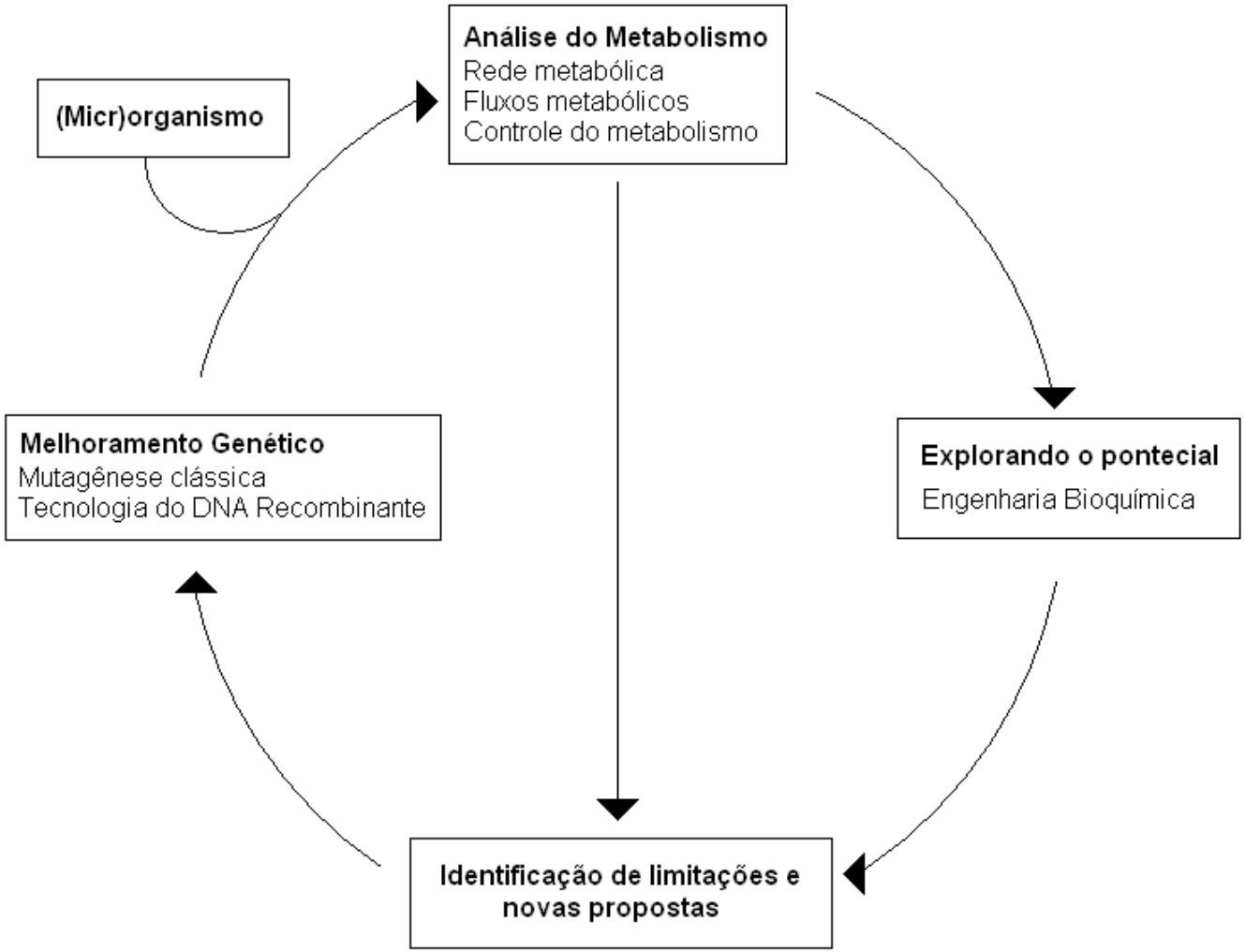
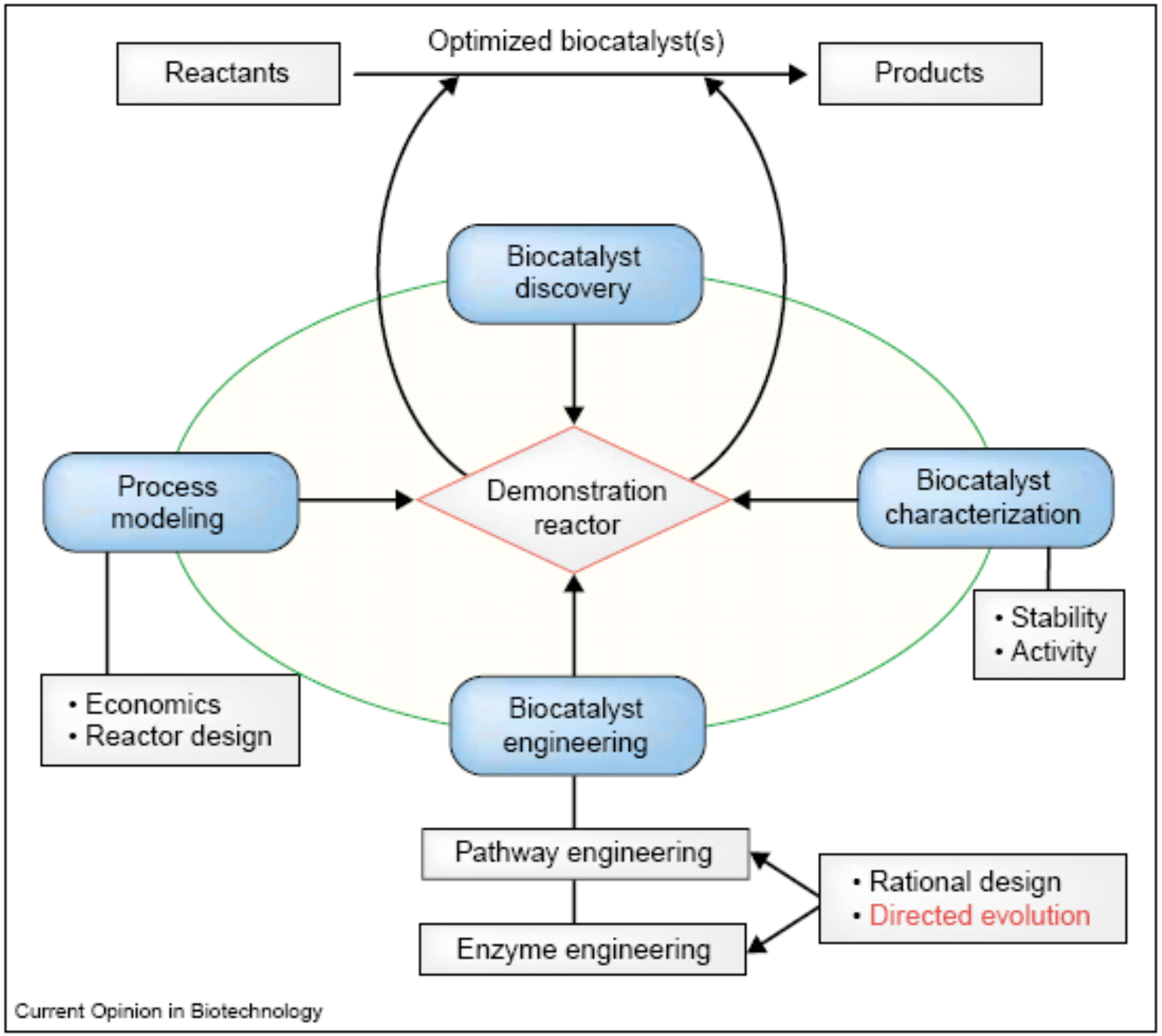
# Mutagênese clássica




Bactéria
1 Um vetor, como um plasmídeo, é isolado
2 O DNA é clivado em fragmentos por uma enzima
DNA contendo o gene de interesse
3 O gene é inserido no plasmídeo
Cromossomo bacteriano
Plasmídeo
DNA (plasmídeo) recombinante
Gene de interesse
4 O plasmídeo é incorporado por uma célula, como a de uma bactéria
Bactéria recombinante
5 As células com o gene de interesse são clonadas
O objetivo pode ser a produção de cópias do gene
OU
O objetivo pode ser a obtenção do produto protéico do gene
Plasmídeo
RNA
Produto protéico
6B As células produzem o produto proteico
6A As cópias do gene são purificadas
7 As cópias da proteína são purificadas
Um gene para resistência a uma peste é inserido em plantas
Um gene altera bactérias, de modo que elas possam fazer a limpeza de resíduos tóxicos
Uma proteína faz com que a neve congele a uma temperatura mais alta
O hormônio do crescimento humano é utilizado no tratamento de casos de deficiência do crescimento

# Engenharia metabólica



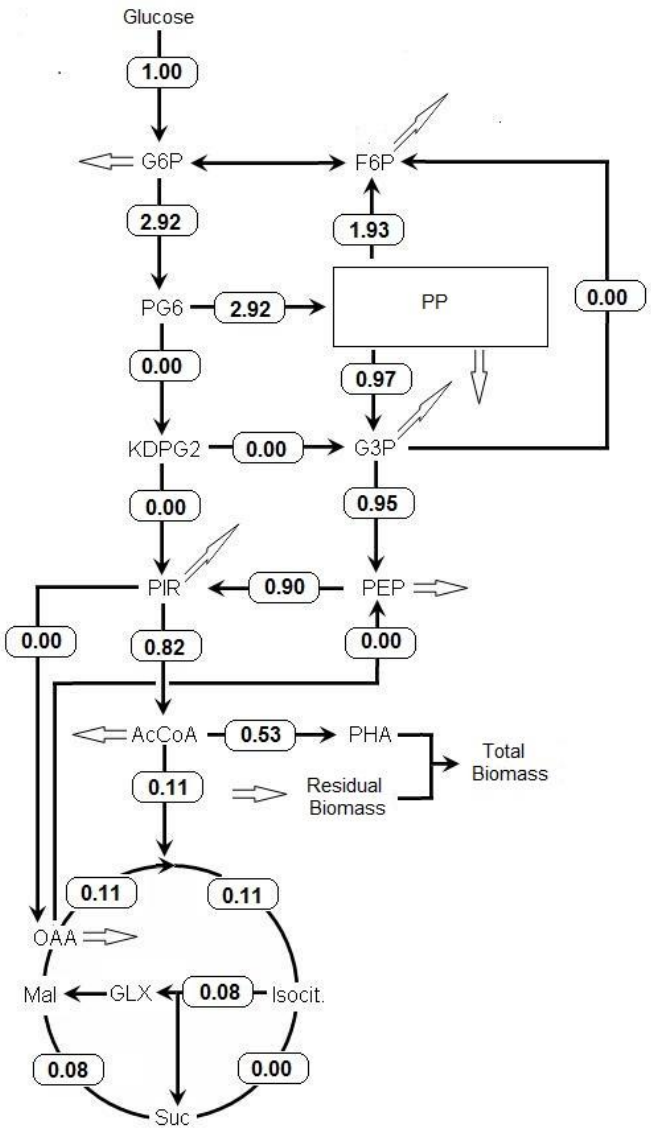

Reactants
Optimized biocatalyst(s)
Products
Biocatalyst discovery
Process modeling
Demonstration reactor
Biocatalyst characterization
• Stability
• Activity
• Economics
• Reactor design
Biocatalyst engineering
Pathway engineering
Enzyme engineering
• Rational design
• Directed evolution
Current Opinion in Biotechnology

# Evolução dirigida

**Porque?** Modificar as propriedades da enzima para atender melhor aos propósitos de um bioprocesso (mudar a especificidade pelo substrato, velocidade de processamento, etc).

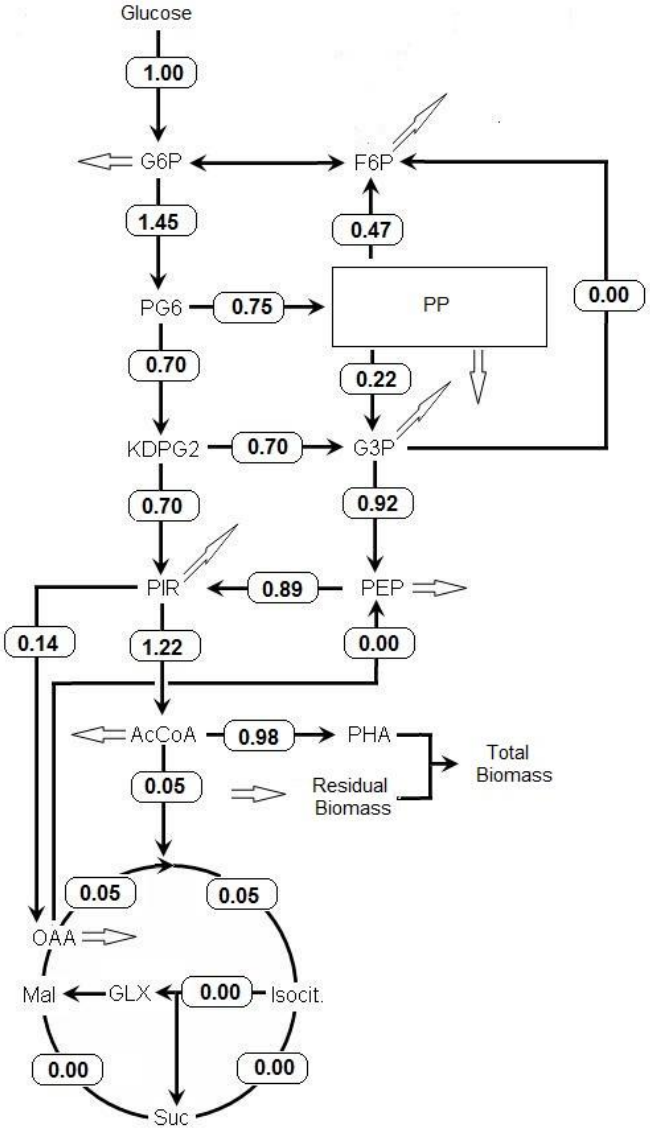
**Como?** Uso de linhagens mutadoras ou técnicas de PCR.

## Metabolic flux analysis




Fluxes distribution for PHA production by *Pseudomonas* sp. from glucose.

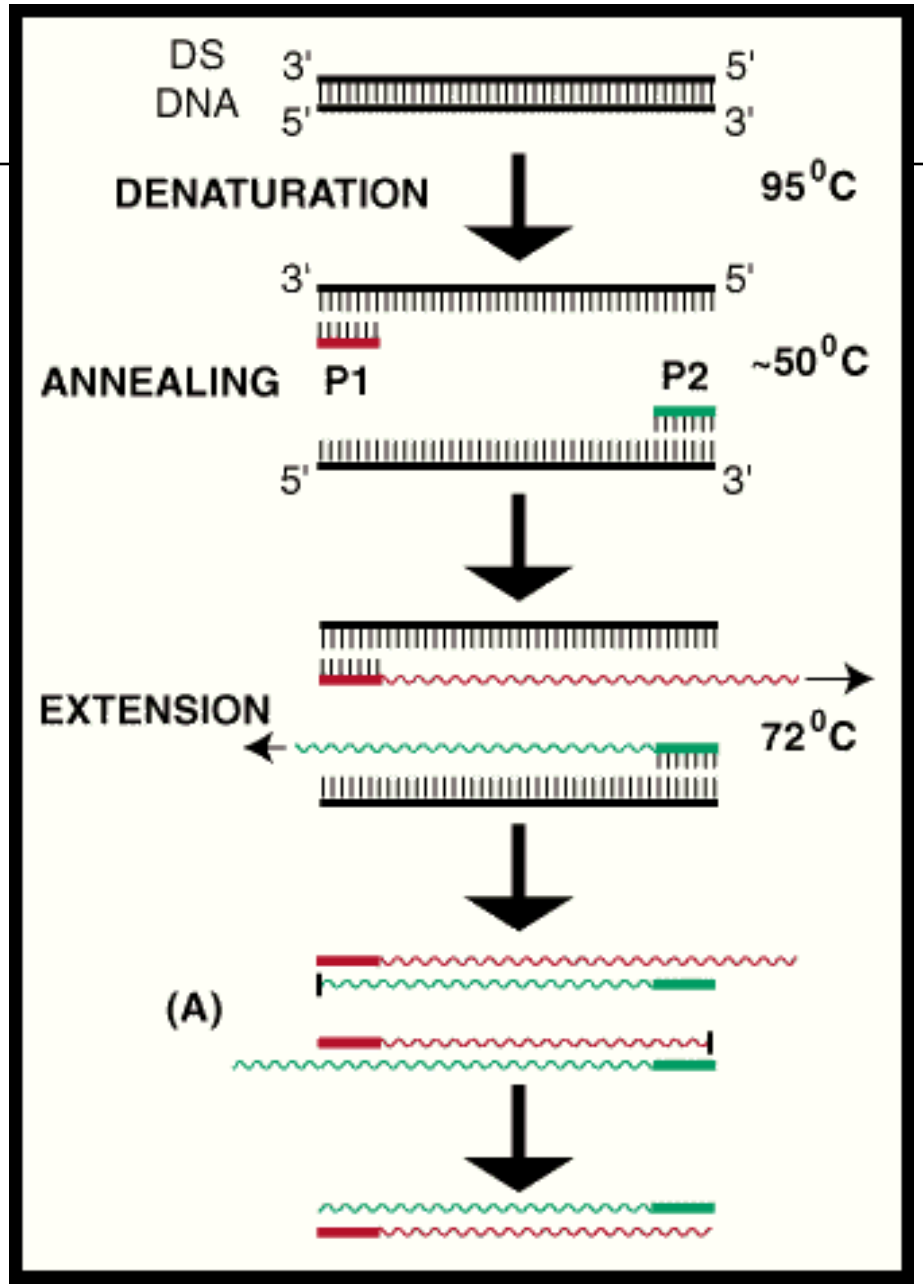
## Elementary (flux) analysis



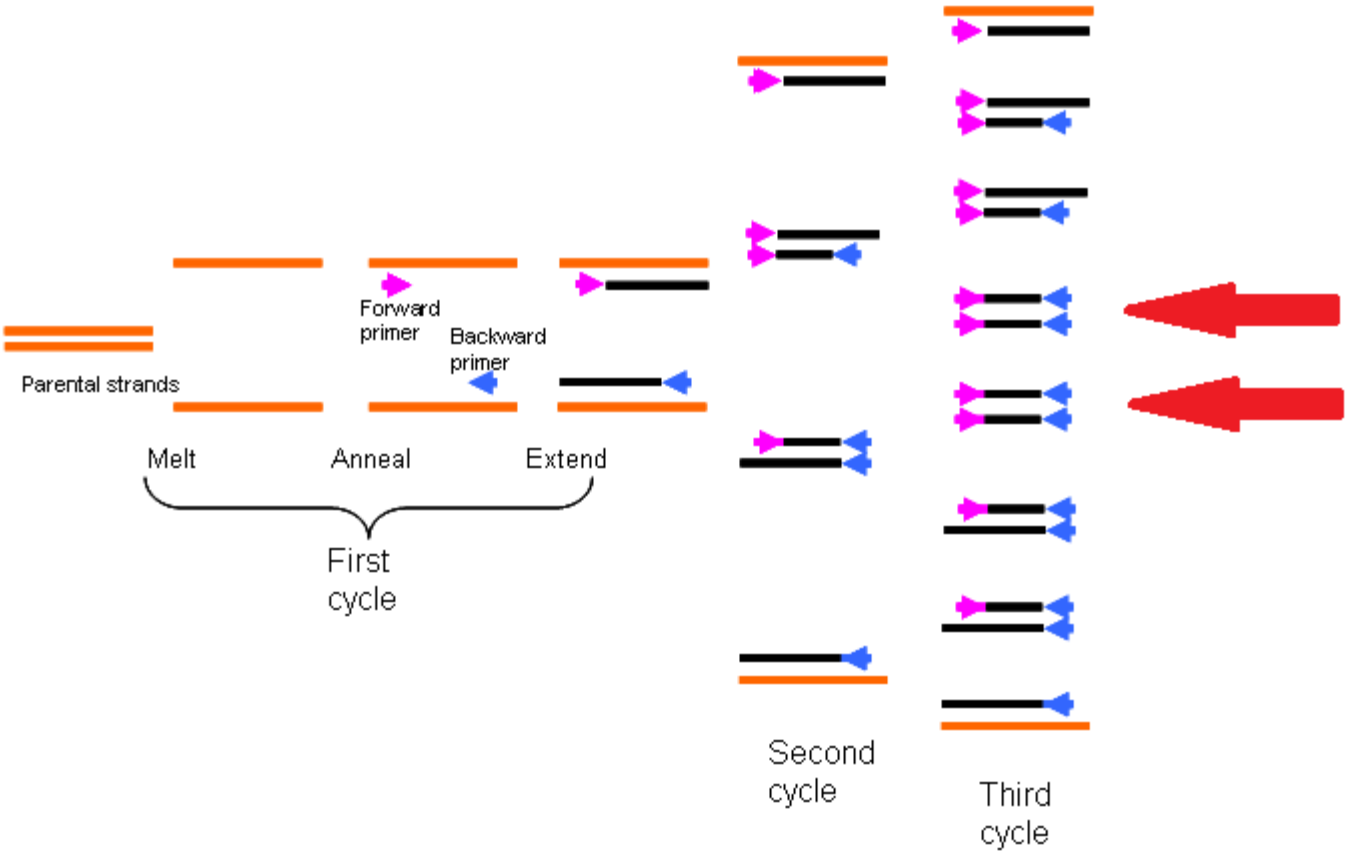

Optimal fluxes distribution for PHA production by *Pseudomonas* sp. from glucose.

# PCR

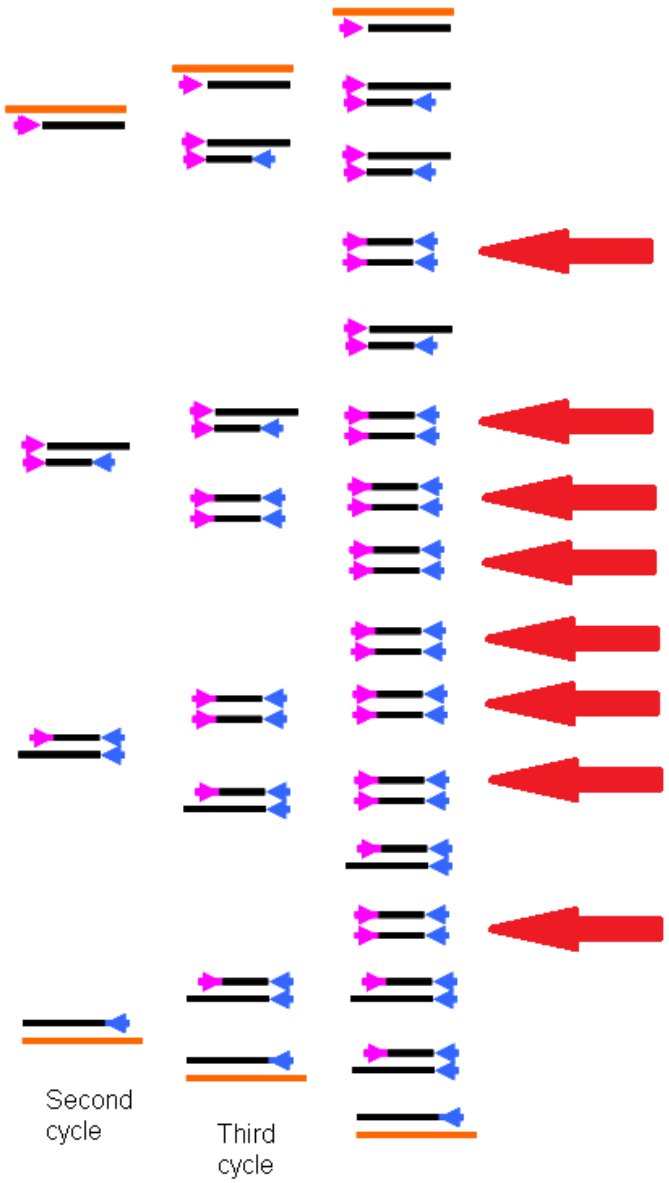
# PCR


DS
DNA
3'
5'
5'
3'
DENATURATION
95⁰C
3'
5'
ANNEALING
P1
P2
~50⁰C
5'
3'
EXTENSION
72⁰C
(A)

# PCR


Forward primer
Backward primer
Parental strands
Melt
Anneal
Extend
First cycle
Second cycle
Third cycle

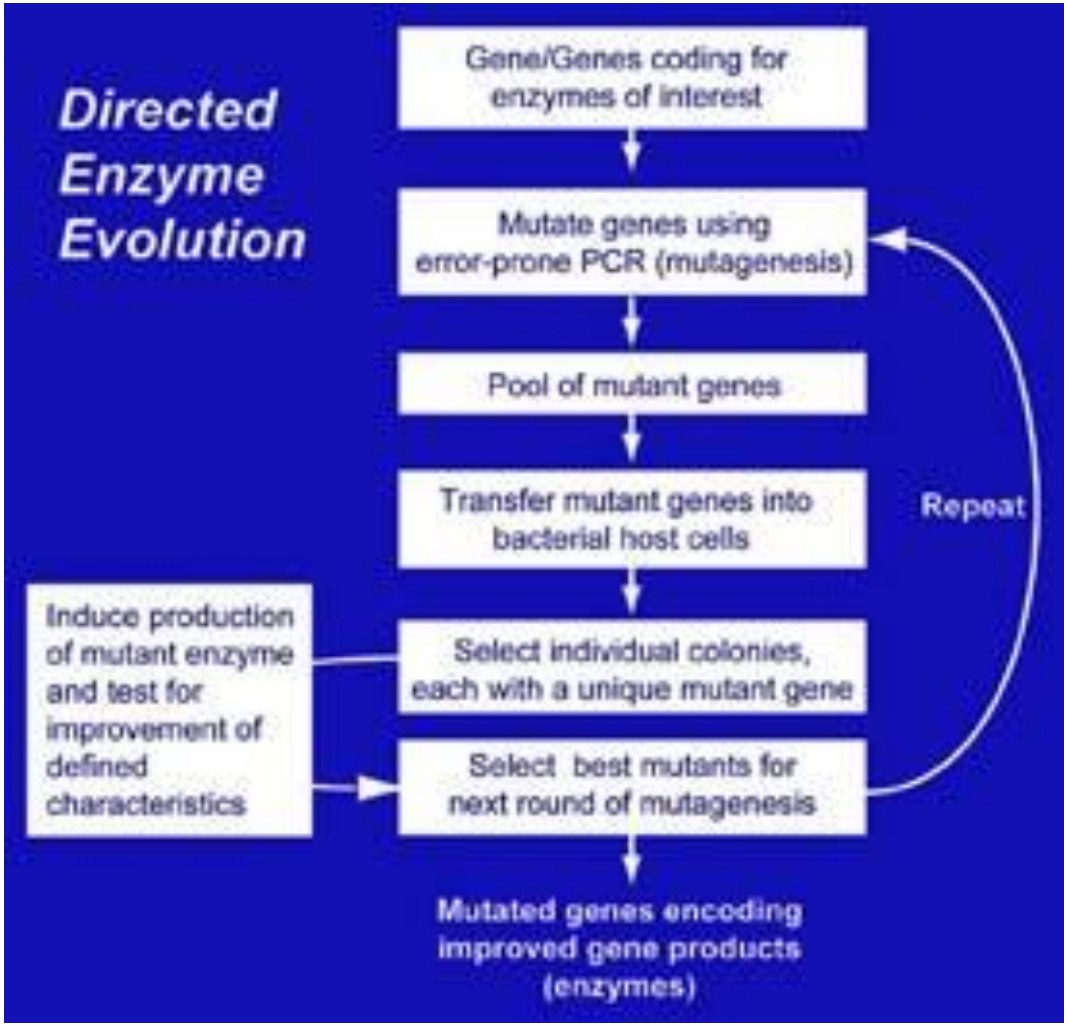
# PCR



# PCR

| Ciclos | DNA | Alvo | não alvo |
|---|---|---|---|
| 0 | 1 | 0 | 1 |
| 1 | 2 | 0 | 2 |
| 2 | 4 | 0 | 4 |
| 3 | 8 | 2 | 6 |
| 4 | 16 | 8 | 8 |
| 5 | 32 | 22 | 10 |
| 6 | 64 | 52 | 12 |
| 7 | 128 | 114 | 14 |
| 8 | 256 | 240 | 16 |
| 9 | 512 | 494 | 18 |
| 10 | 1024 | 1004 | 20 |
| 11 | 2048 | 2026 | 22 |
| 12 | 4096 | 4072 | 24 |
| 13 | 8192 | 8166 | 26 |
| 14 | 16384 | 16356 | 28 |
| 15 | 32768 | 32738 | 30 |
| 16 | 65536 | 65504 | 32 |
| 17 | 131072 | 131038 | 34 |
| 18 | 262144 | 262108 | 36 |
| 19 | 524288 | 524250 | 38 |
| 20 | 1048576 | 1048536 | 40 |
| 21 | 2097152 | 2097110 | 42 |
| 22 | 4194304 | 4194260 | 44 |
| 23 | 8388608 | 8388562 | 46 |
| 24 | 16777216 | 16777168 | 48 |
| 25 | 33554432 | 33554382 | 50 |
| 26 | 67108864 | 67108812 | 52 |
| 27 | 134217728 | 134217674 | 54 |
| 28 | 268435456 | 268435400 | 56 |
| 29 | 536870912 | 536870854 | 58 |
| 30 | 1073741824 | 1073741764 | 60 |

# Evolução dirigida



# Linhagem bacteriana mutadora

*Escherichia coli* XL1 RED

Deficiente em três mecanismos de reparo primários;

*mutS* – reparo de erros de pareamento.

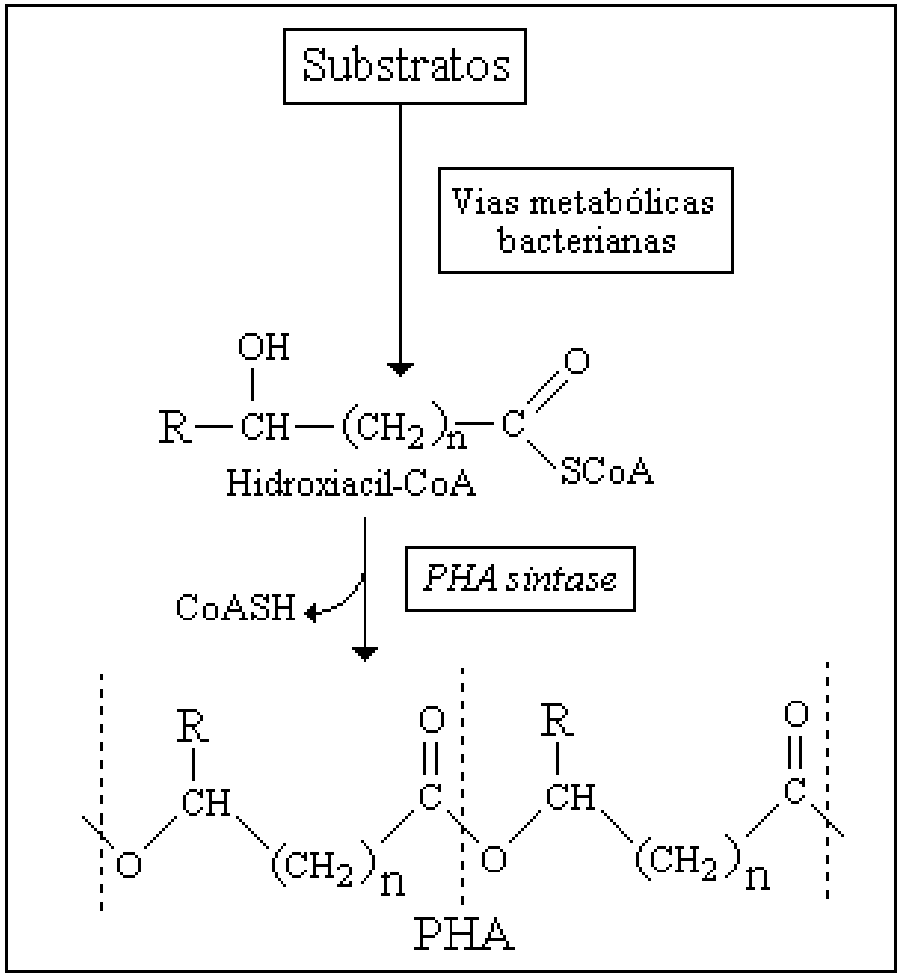
*mutD* – deficiente na atividade exonuclease 3´ - 5´ da DNA polimerase III.

*mutT* – incapaz de hidrolisar 8-oxodGTP.

A taxa de mutação ao acaso é ~5000 vezes maior que na linhagem selvagem.

# Embaralhamento de DNA

# (DNA shuffling)


Substratos
Vias metabólicas bacterianas
OH
R — CH — (CH2)n — C
O
SCoA
Hidroxiacil-CoA
CoASH
PHA sintase
R
CH
O
C
O
(CH2)n
PHA

# Evolução dirigida

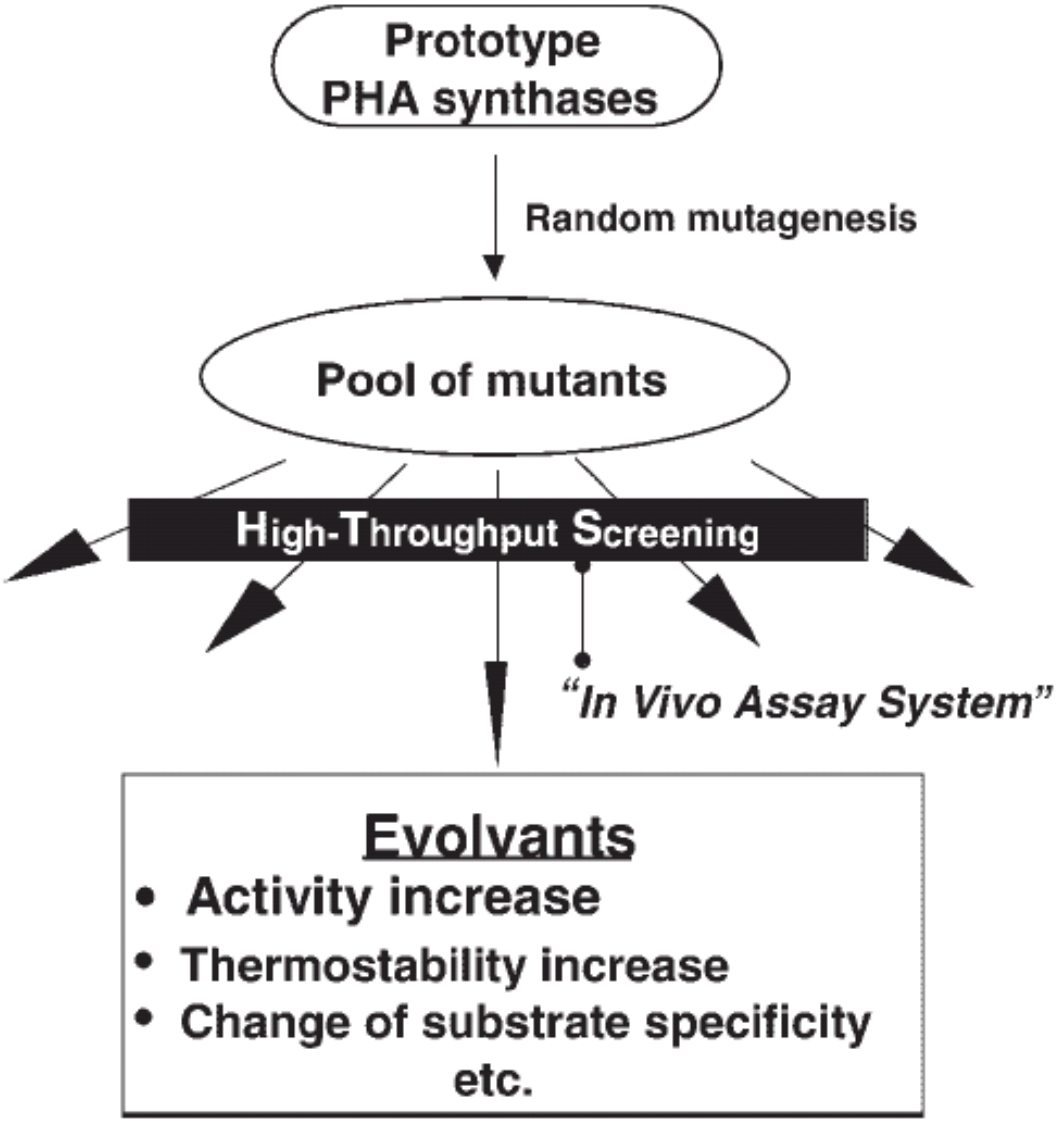



Figure 3. Outline of the in vitro evolution of PHA synthase.

# Evolução dirigida

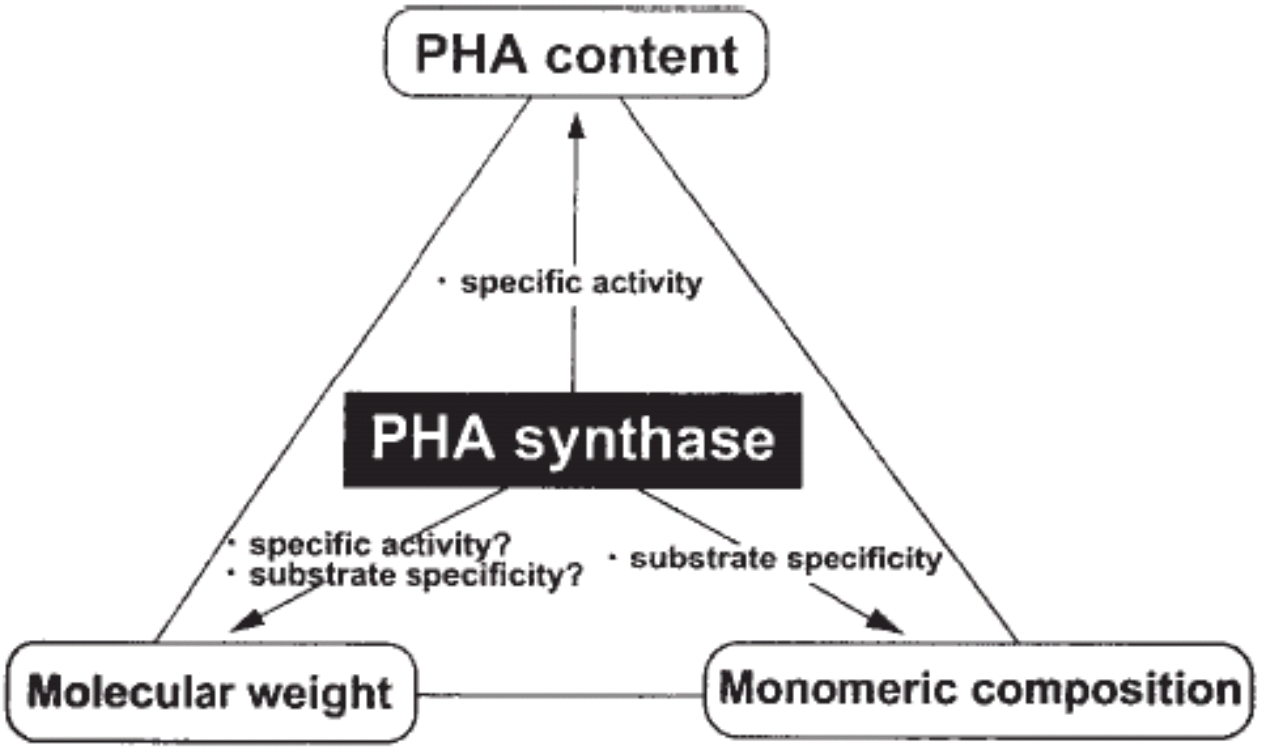



Figure 2. Properties of PHA related to the PHA synthase function.

# Evolução dirigida

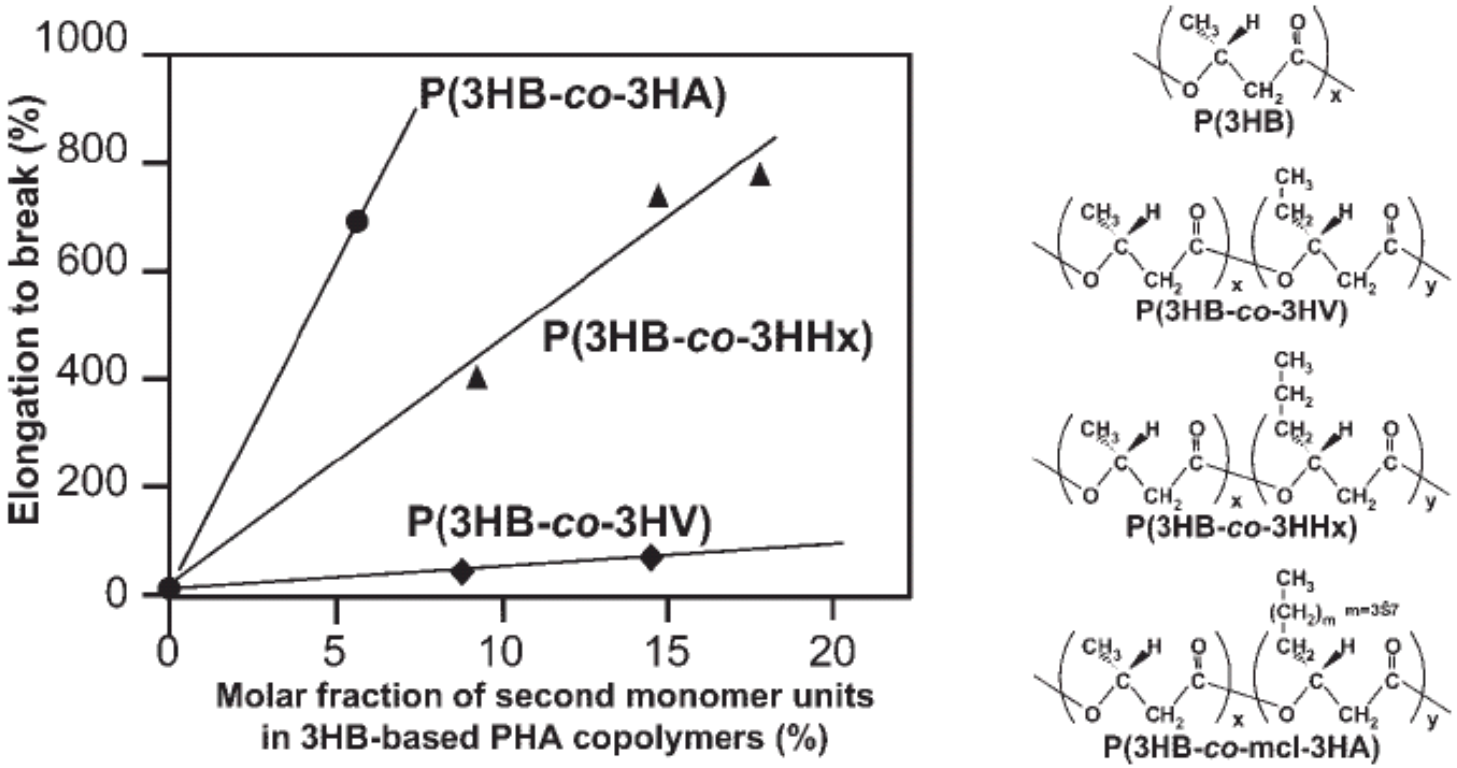



Figure 5. Effects of second monomer units on the elongation to break of 3HB-based PHA copolymers (left part) and the chemical structures of PHA copolymers (right part).

# Evolução dirigida

ELSEVIER

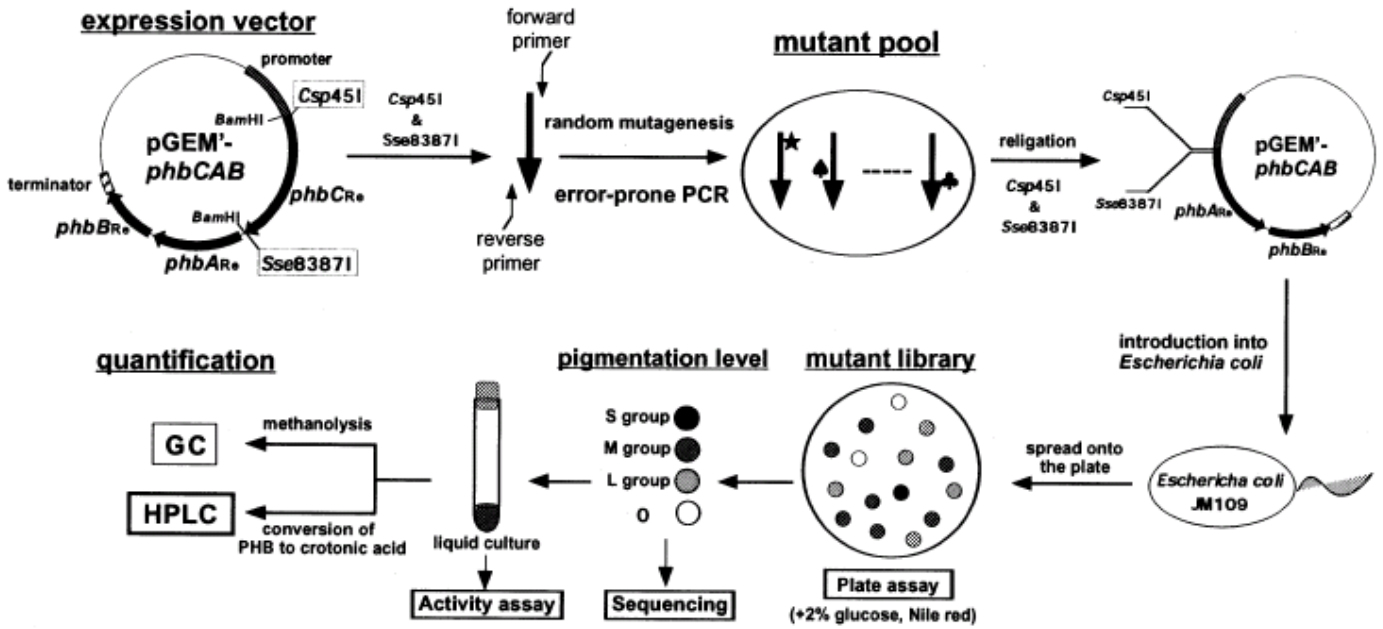
FEMS Microbiology Letters 198 (2001) 65–71

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

## Analysis of mutational effects of a polyhydroxybutyrate (PHB) polymerase on bacterial PHB accumulation using an in vivo assay system

Seiichi Taguchi *, Akira Maehara, Kazuma Takase, Maki Nakahara,



# Evolução dirigida

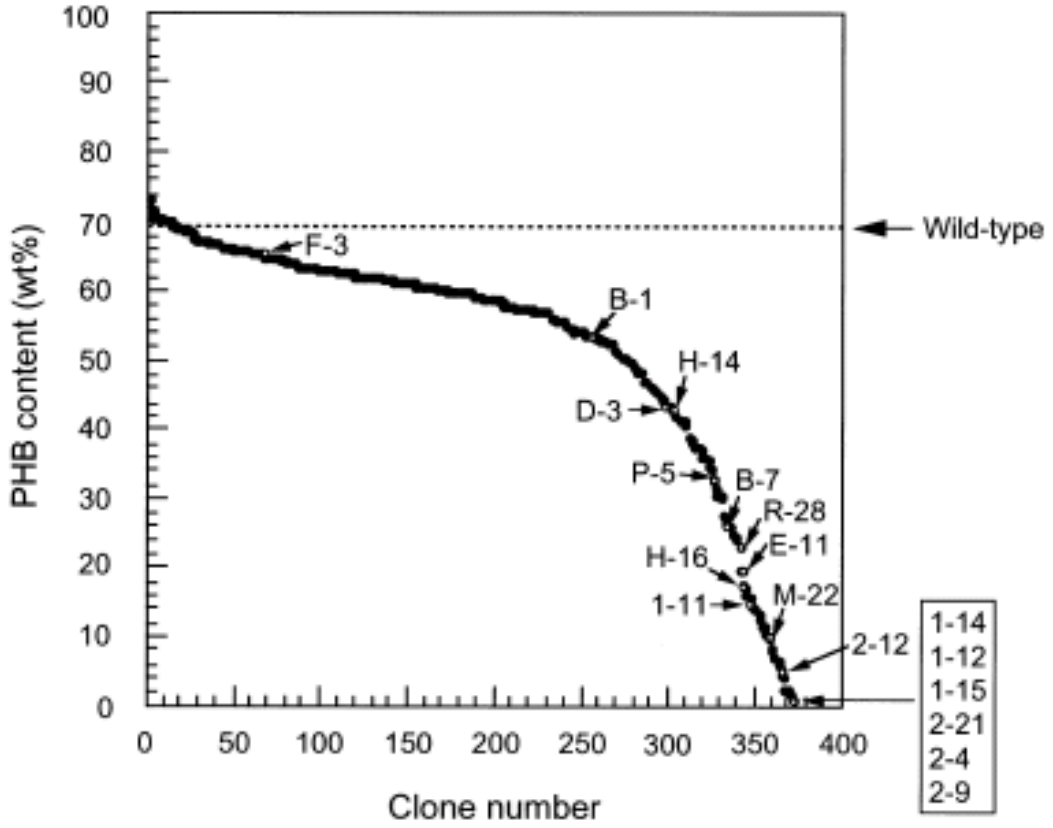



Fig. 2. Distribution pattern of the level of PHB accumulation of the random mutant population. A total of 378 clones containing all the 48 S-group clones and arbitrarily selected clones, 310 clones from M-group and 20 clones from L-group, were used to make the distribution curve (generally called 'fitness landscape' [24]), plotted in descending order. The broken line denotes the averaged level of PHB accumulation by the wild-type clone. Eighteen mutants which were subjected to nucleotide sequence analysis (see Fig. 3) are marked by arrows.

# Evolução dirigida

A.A. Amara · A. Steinbüchel · B.H.A. Rehm

## In vivo evolution of the *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolation and characterization of modified PHA synthases with enhanced activity

Received: 14 January 2002 / Revised: 18 April 2002 / Accepted: 26 April 2002 / Published online: 22 June 2002

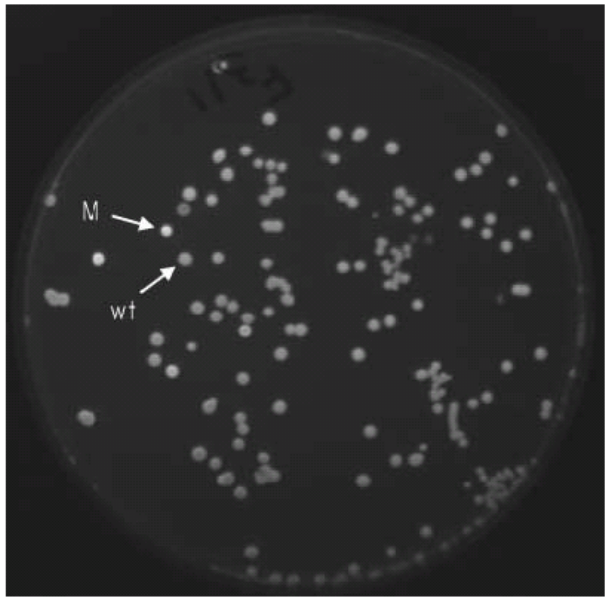




**Fig. 1** Polyhydroxyalkanoate (PHA)-overproducing mutant isolated as colony from Nile red-containing agar plates. Nile red screening was performed as described by Spiekermann et al. (1999). This agar plate shows an *Escherichia coli* JM109 colony (*white arrow*) harboring plasmid pPS2-M1 (*M*, mediating overproduction of PHA) and another colony harboring the wild-type PHA synthase-encoding plasmid pPS2 (*wt*)

**Table 2** In vitro and in vivo activity of *A. punctata* polyhydroxyalkanoate (PHA) synthase mutants. Recombinant *E. coli* harboring plasmid pBHR69 and the mutated pPS2 was cultivated for 24 h in LB medium containing 2% (w/w) glucose, 75 μg ampicillin $ml^{-1}$ and 1 mM isopropyl-β-D-thiogalactopyranoside. The relative specific PHA synthase activity of crude extracts was provided as the percentage of wild-type activity corresponding to 0.16 units $mg^{-1}$ protein. The relative PHA content was provided as the percentage of wild-type PHA accumulation corresponding to about 20% (w/w) of CDW. Only the homopolyester polyhydroxybutyrate was accumulated under these conditions (Antonio et al. 2000)

| Mutation | Relative specific PHA synthase activity (%) | Relative PHA content (%) |
|---|---|---|
| M1 | 480 | 120 |
| M2 | 216 | 107 |
| M3 | 74 | 45 |
| M4 | 106 | 111 |
| M5 | 110 | 126 |

# Evolução dirigida

**Table 3** Analysis of the in vivo substrate specificity of the various *A. punctata* PHA synthase mutants and the weight average molar masses of the respective PHA. *E. coli* JM109 harboring the various plasmids was grown for 48 h at 37 °C in LB medium containing 0.4% (w/v) dodecanoate and 0.2 mg acrylic acid $ml^{-1}$ (Qi et al. 1998; Antonio et al. 2000). The PHAs were isolated and their molecular weights determined as described in Materials and methods. Means and standard deviations from three independent measurements are presented. *3HB* 3-hydroxybutyrate, *3HHx* 3-hydroxyhexanoate, $M_W$ weight average molar mass

| Plasmid | PHA composition (mol%) | | $M_W$ (g $mol^{-1}$) |
|---|---|---|---|
| | 3HB | 3HHx | |
| pPS2 (wild type) | 76 | 24 | 205,000±5,000 |
| pPS2-M1 | 81 | 19 | 264,000±10,000 |
| pPS2-M2 | 82 | 18 | 218,000±4,000 |
| pPS2-M3 | 88 | 12 | 339,000±10,000 |
| pPS2-M4 | 80 | 20 | 358,000±7,000 |
| pPS2-M5 | 80 | 20 | 277,000±5,000 |

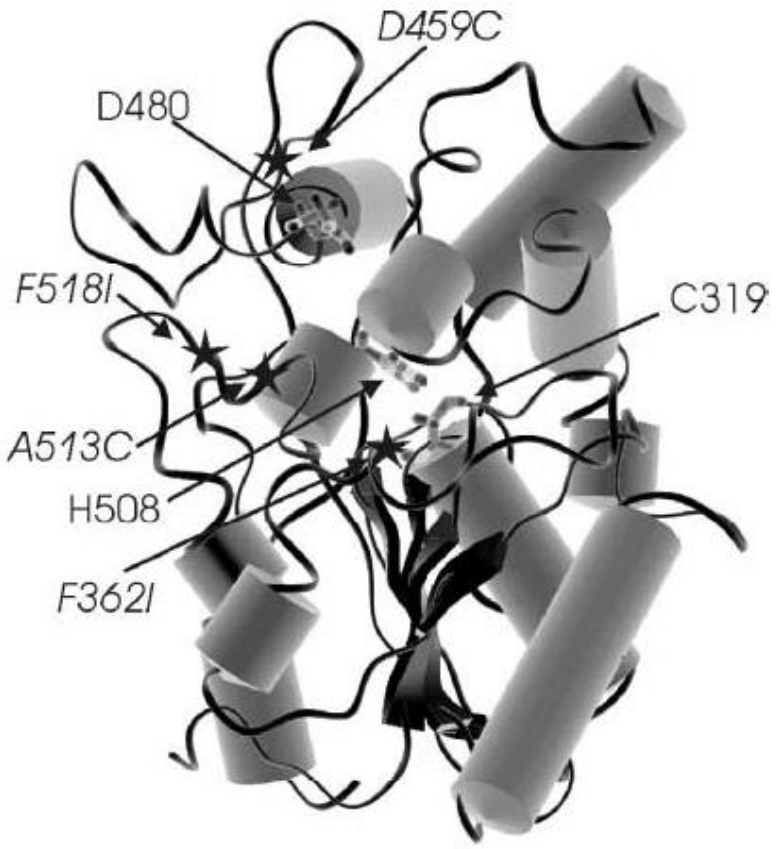



**Fig. 2** Localization of mutations sites of the *Aeromonas punctata* PHA synthase gene mutants M1 (F518I), M4 (F362I, F518I) and M5 (D459V, A513C) in the threading model of the *Ralstonia eutropha* PHA synthase (Rehm et al. 2002b)

# Evolução dirigida

Production of polyhydroxyalkanoate (PHA) from renewable carbon sources in recombinant *Ralstonia eutropha* using mutants of original PHA synthase

Seiichi Taguchi [a,b,*], Hirofumi Nakamura [c], Tomoyasu Kichise [a], Takeharu Tsuge [d], Ichiro Yamato [c], Yoshiharu Doi [a,d]

**Enhanced Synthesis of Poly(3-hydroxybutyrate) in Recombinant *Escherichia coli* by Means of Error-Prone PCR Mutagenesis, Saturation Mutagenesis, and *In Vitro* Recombination of the Type II Polyhydroxyalkanoate Synthase Gene**

**Kazuma Takase[1], Seiichi Taguchi[*,1,2] and Yoshiharu Doi[1,3]**

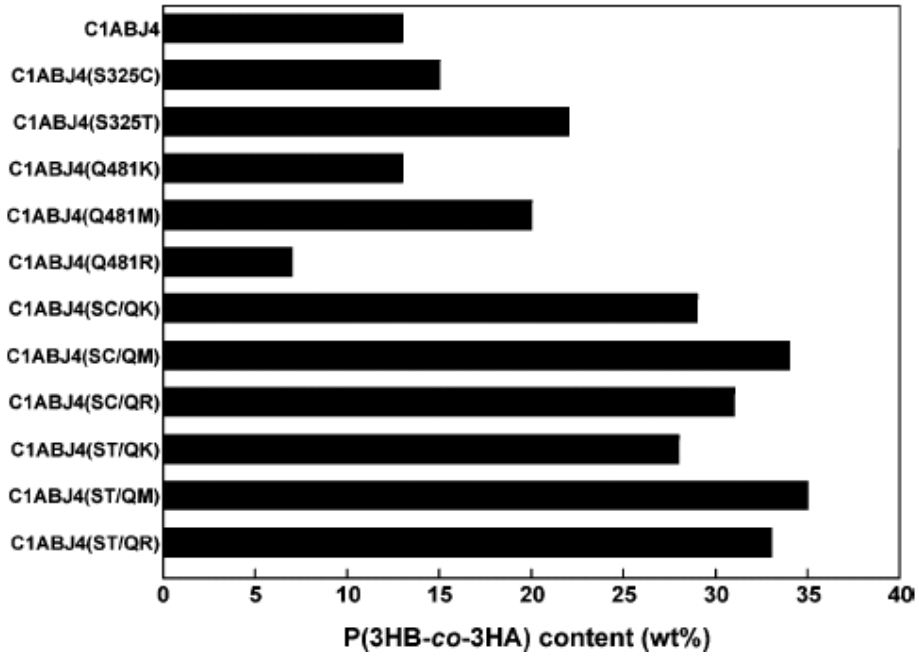
Alteration of Chain Length Substrate Specificity of *Aeromonas caviae* *R*-Enantiomer-Specific Enoyl-Coenzyme A Hydratase through Site-Directed Mutagenesis

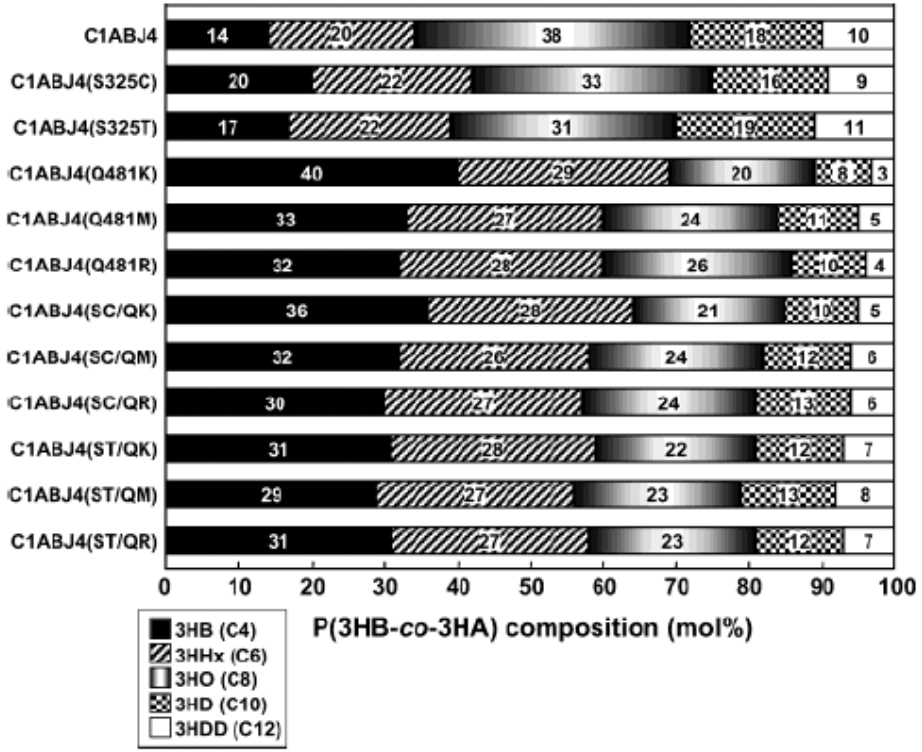
Takeharu Tsuge,[1,2]* Tamao Hisano,[3] Seiichi Taguchi,[2]† and Yoshiharu Doi[1,2]

# Evolução dirigida

## Alteration of Substrate Chain-Length Specificity of Type II Synthase for Polyhydroxyalkanoate Biosynthesis by in Vitro Evolution: in Vivo and in Vitro Enzyme Assays

Kazuma Takase,† Ken'ichiro Matsumoto,† Seiichi Taguchi,*,†,‡ and Yoshiharu Doi†,§



# Evolução dirigida

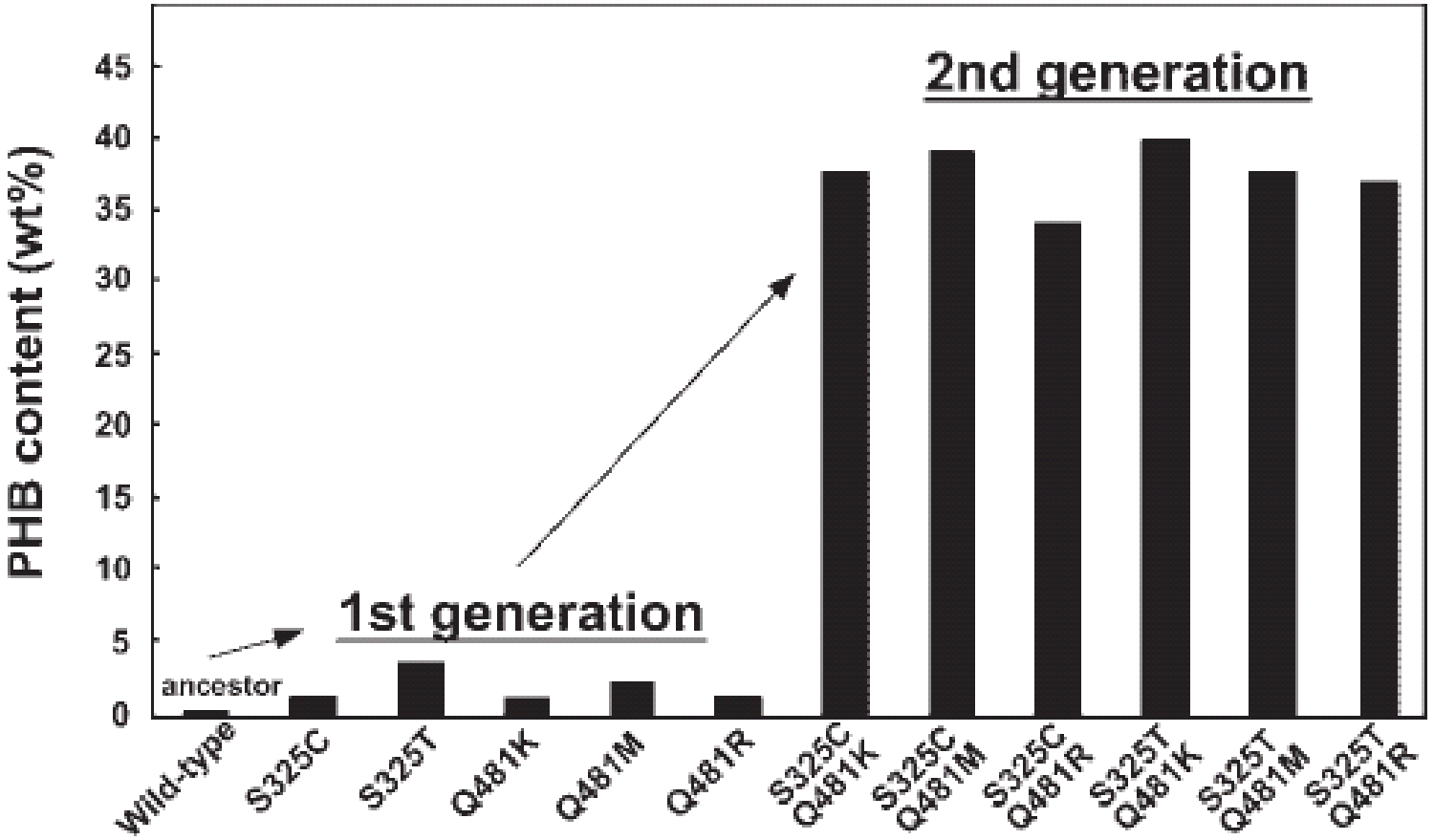



Figure 7. Evolutionary lineage throughout systematic molecular engineering of the PHA synthase from *Pseudomonas* sp. 61-3.

# Evolução dirigida

## In Vivo and in Vitro Characterization of Ser477X Mutations in Polyhydroxyalkanoate (PHA) Synthase 1 from *Pseudomonas* sp. 61−3: Effects of Beneficial Mutations on Enzymatic Activity, Substrate Specificity, and Molecular Weight of PHA

Ken'ichiro Matsumoto,[†,‡] Emi Aoki,[§] Kazuma Takase,[‡] Yoshiharu Doi,[‡] and Seiichi Taguchi*[,‡,§]

**Table 2.** PHA Contents and Monomer Compositions of P(3HB-*co*-3HA) Copolymers Produced by the Recombinant *E. coli* LS5218 Expressing the S477X Mutant Genes

| | | monomer composition (mol %) | | | | |
|---|---|---|---|---|---|---|
| PHA synthase | PHA content[a] (wt %) | 3HB ($C_4$) | 3HHx ($C_6$) | 3HO ($C_8$) | 3HD ($C_{10}$) | 3HDD ($C_{12}$) |
| wild-type | 11 | 13 | 18 | 39 | 19 | 11 |
| S477A mutant | 11 | 16 | 23 | 35 | 17 | 9 |
| S477C mutant | 1 | 15 | 23 | 39 | 16 | 7 |
| S477F mutant | 19 | 27 | 30 | 26 | 12 | 5 |
| S477G mutant | 15 | 18 | 32 | 29 | 15 | 6 |
| S477H mutant | 11 | 23 | 24 | 32 | 14 | 7 |
| S477K mutant | 1 | 36 | 33 | 19 | 8 | 4 |
| S477M mutant | 1 | 15 | 27 | 36 | 15 | 7 |
| S477N mutant | 6 | 13 | 20 | 40 | 18 | 9 |
| S477Q mutant | 1 | 17 | 30 | 34 | 14 | 5 |
| S477R mutant | 9 | 30 | 35 | 24 | 8 | 3 |
| S477Y mutant | 6 | 28 | 29 | 26 | 12 | 5 |

[a] Cells were grown on M9 medium containing 0.3% sodium dodecanoate at 37 °C for 72 h.

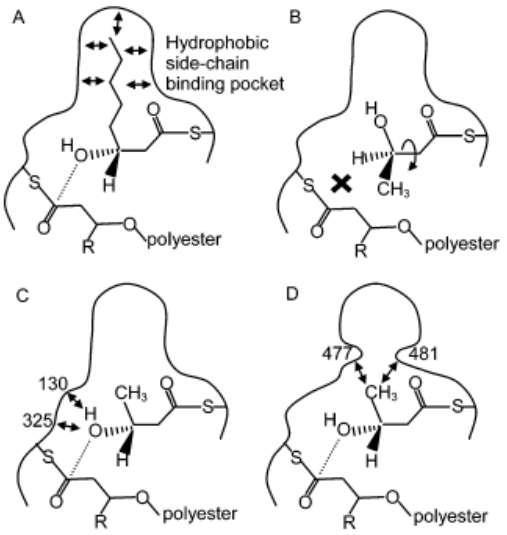



**Figure 3.** Proposed mechanism accounting for the effect of amino acid substitutions in the PHA synthase on activity and substrate specificity. (A) Binding of MCL side chain may hold the 3-hydroxyl group of the incoming substrate in the proper orientation. (B) Class II PHA synthase has little activity toward SCL substrates, since mobility of the enzyme-bound substrate causes a low rate of elongation. (C) Interaction with the 3-hydroxyl group, which holds the 3-hydroxyl group to the proper orientation, leads to an enhancement in total enzymatic activity. (D) Blockage of the MCL substrate and/or stabilization of SCL side chain leads to a change in substrate specificity toward smaller substrates.

# Evolução dirigida

RESEARCH LETTER

## Combination of N149S and D171G mutations in *Aeromonas caviae* polyhydroxyalkanoate synthase and impact on polyhydroxyalkanoate biosynthesis

Takeharu Tsuge[1], Shinko Watanabe[1], Daisuke Shimada[1], Hideki Abe[2], Yoshiharu Doi[2] & Seiichi Taguchi[3]

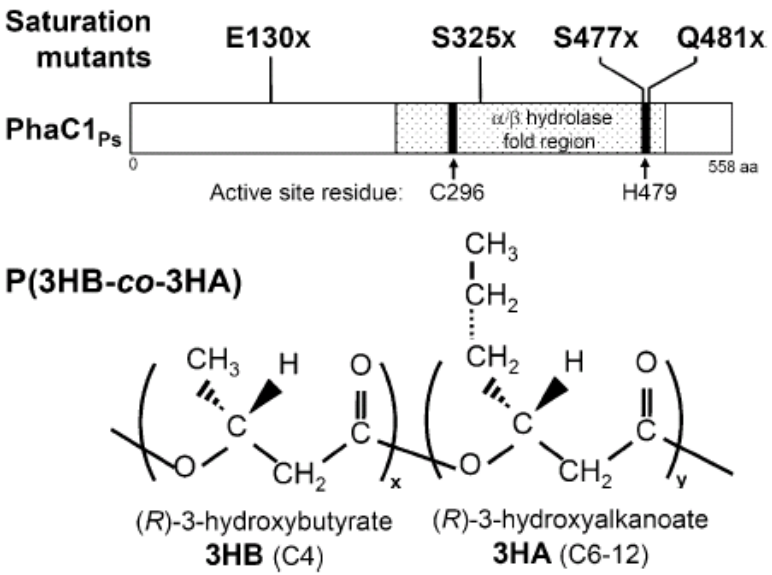
[1]Department of Innovative and Engineered Materials, Tokyo Institute of Technology, Yokohama, Japan; [2]RIKEN Institute, Wako-shi, Saitama, Japan; and [3]Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo, Japan

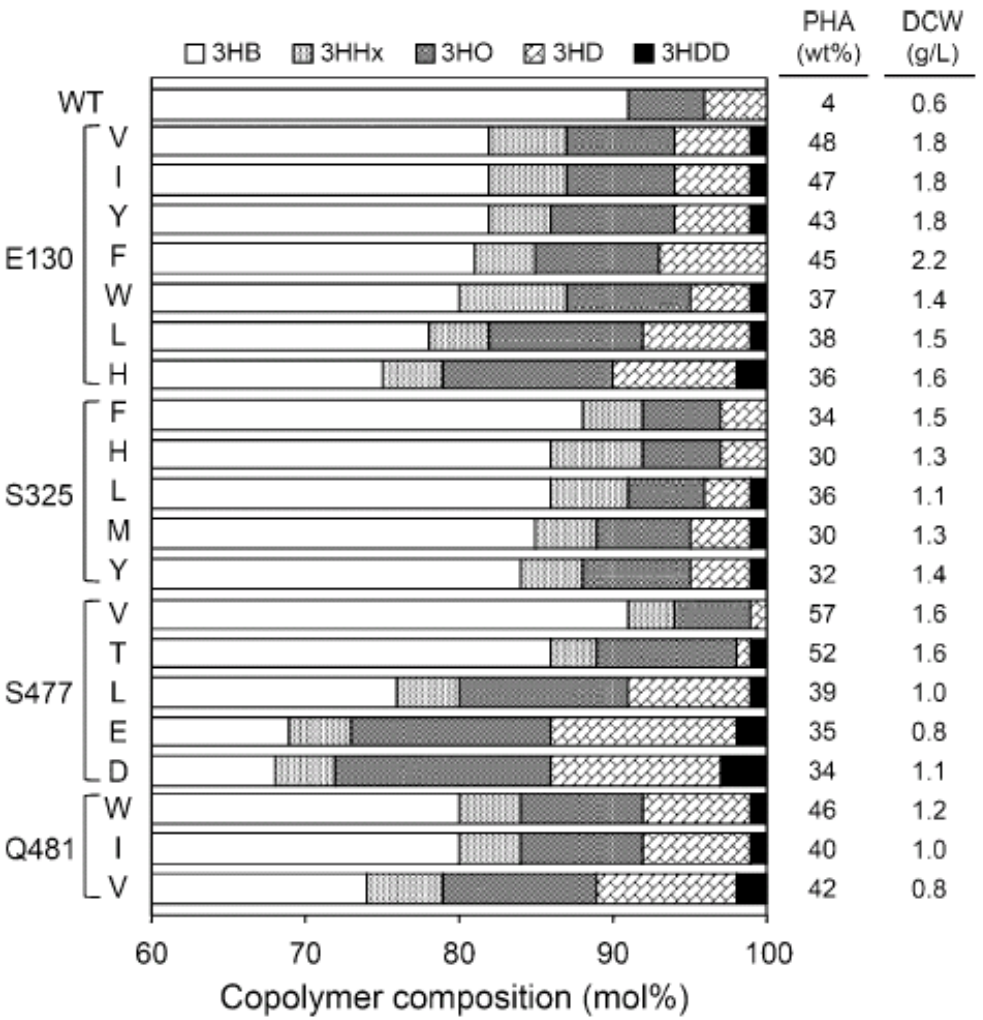
**Table 1.** Polyhydroxyalkanoate production by recombinant *Ralstonia eutropha* PHB$^{-}$4 harboring mutated *phaC*$_{Ac}$ genes from octanoate, their molecular weight and thermal properties

| | | | Polyhydroxyalkanoate composition | | | Molecular weight | | | Thermal properties | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PhaC$_{Ac}$ | Dry cell weight (g L$^{-1}$) | PHA content (wt%) | 3HB (mol%) | 3HHx (mol%) | 3HO (mol%) | $M_n$ ($\times 10^4$) | $M_w$ ($\times 10^4$) | $M_w/M_n$ | $T_g$ (°C) | $T_m$ (°C) | $\Delta H_m$ (J g$^{-1}$) |
| Wild-type | 3.1 ± 0.1 | 82 ± 2 | 87.8 ± 0.2 | 12.2 ± 0.2 | 0 | 44 | 255 | 5.8 | −7 | 128 131 | 18 |
| N149S | 2.8 ± 0 | 82 ± 3 | 83.0 ± 0.1 | 16.8 ± 0.1 | 0.2 ± 0 | 69 | 359 | 5.2 | −7 | 124 142 | 5 |
| D171G | 2.4 ± 0.1 | 86 ± 1 | 86.2 ± 0.4 | 13.8 ± 0.4 | 0 | 21 | 141 | 6.7 | −7 | 132 147 | 4 |
| NSDG* | 3.4 ± 0 | 87 ± 2 | 81.5 ± 0.2 | 18.1 ± 0.2 | 0.4 ± 0 | 44 | 290 | 6.6 | −8 | 111 137 | 7 |

Cells were cultivated in nitrogen-limited mineral salt medium containing sodium octanoate (0.1% w/v × 5, every 12 h) as a sole carbon source.
The values of dry cell weight, polyhydroxyalkanoate content and polyhydroxyalkanoate composition were average of three independent experiments.
*Double mutation of N149S and D171G.

# Evolução dirigida

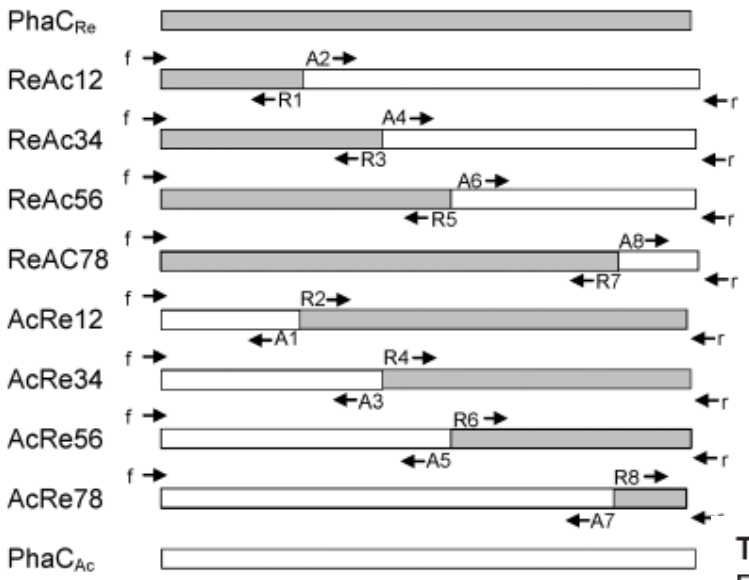



Tsuge et al., 2008

*R. eutropha* recombinante

# Evolução dirigida



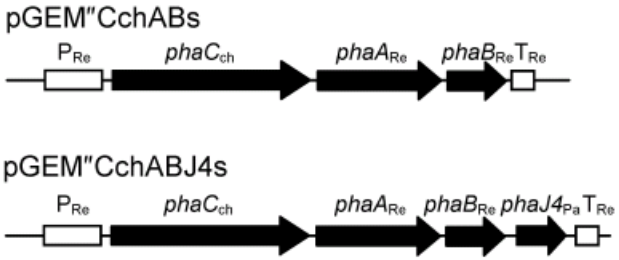




**Figure 2.** Construction of chimeric genes composed of PHA synthase genes from *R. eutropha* and *A. caviae*. R1−R8, A1−A8, f and r, indicate the primers used for amplification of the truncated fragments of the PHA synthase genes; f and r, pETMCS-f and pETMCS-r primers, respectively. $P_{Re}$ and $T_{Re}$, the promoter and terminator regions from *R. eutropha phb* operon, respectively; *phaC*$_{ch}$, chimeric PHA synthase gene; *phaA*, β-ketothiolase gene; *phaB*, acetoacetyl-CoA reductase gene; *phaJ4*, enoyl-CoA hydratase gene.

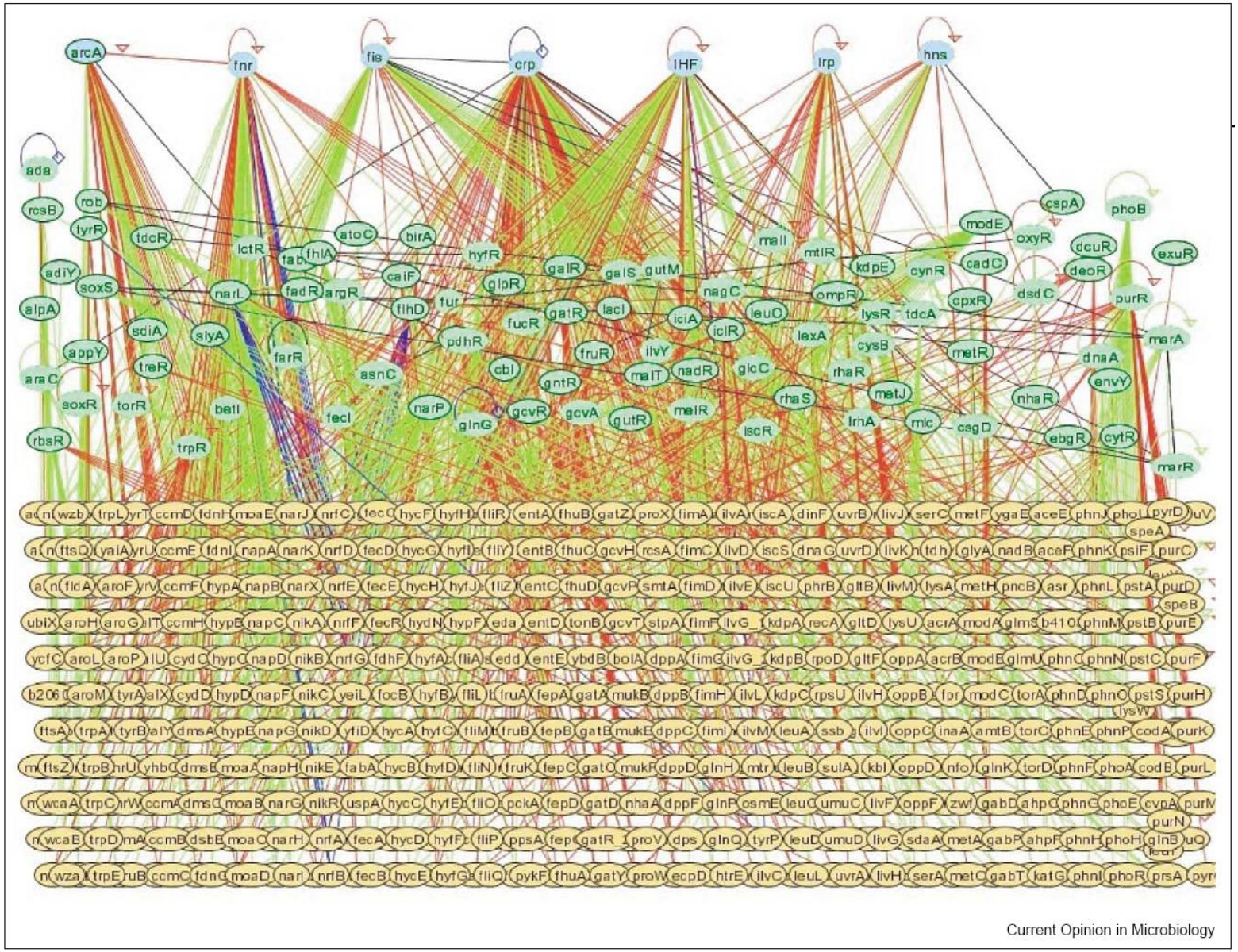
**Table 2.** Content and Composition of the PHAs Produced in Recombinant *E. coli* Harboring Parental and Chimeric PHA Synthase Genes[a]

| | | monomer composition (mol %)[b] | | | molar mass | | |
|---|---|---|---|---|---|---|---|
| PHA synthase | SCL/MCL PHA content (wt %) | 3HB ($C_4$) | 3HHx ($C_6$) | 3HO ($C_8$) | $M_n$ ($10^5$) | $M_w$ ($10^5$) | $M_w/M_n$ |
| PhaC$_{Re}$ | 13 | 100 | 0 | 0 | 2.9 | 8.3 | 3.0 |
| AcRe12 | 50 | 98 | 2 | trace | 1.1 | 2.3 | 2.1 |
| PhaC$_{Ac}$ | 40 | 92 | 8 | 0 | 2.0 | 3.8 | 1.9 |

[a] *E. coli* LS5218 harboring pGEM″CchABJ4s were grown on dodecanoate for SCL/MCL production. [b] 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate.

Matsumoto et al., 2009

*E. coli* recombinante




**Agustino Martínez-Antonio and Julio Collado-Vives** (2003) *Curr. Opin. Microbiol.* **6**: 482

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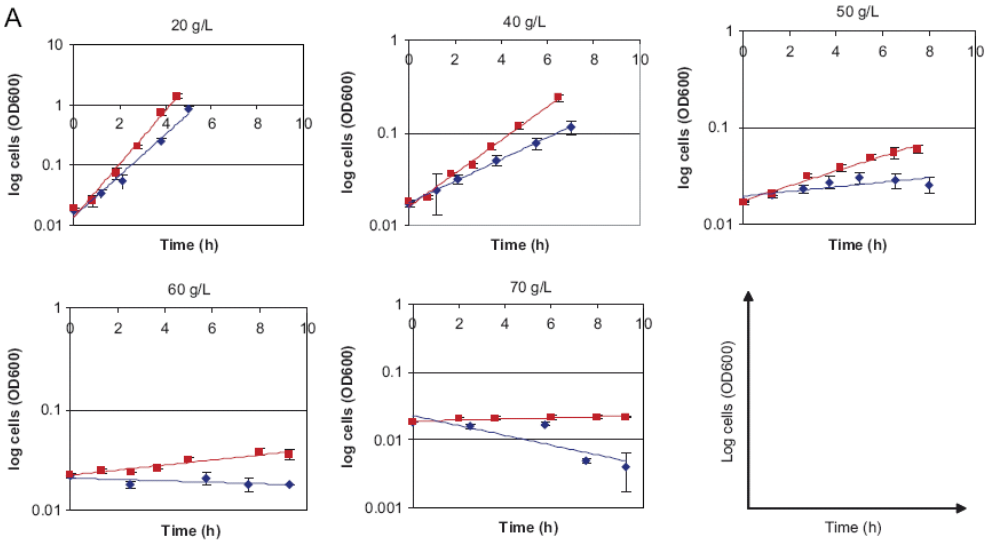
# Global transcription machinery engineering: A new approach for improving cellular phenotype

Hal Alper, Gregory Stephanopoulos*



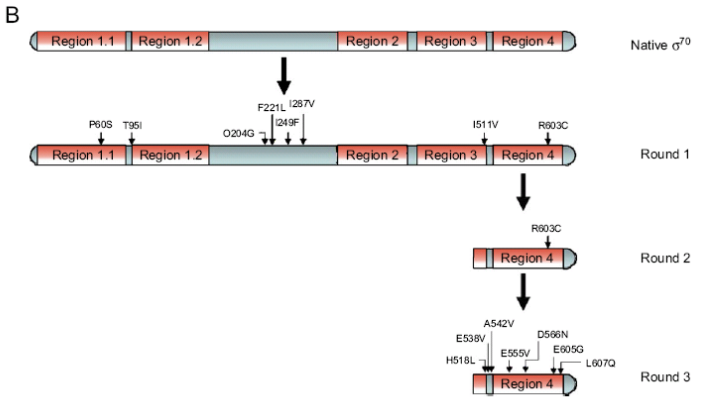




Fig. 1. Isolation of ethanol-tolerant sigma factor mutants. Strains were isolated containing mutant sigma factors, which increased the tolerance to ethanol. (A) Growth curves are presented for the strain harboring the best $\sigma^{70}$ mutant isolated from the third round of mutagenesis and selection (Red) and control strains harboring the wild-type $\sigma^{70}$ (Blue). This round 3 mutant has significantly improved growth rates at all tested ethanol concentrations. Error bars present the standard deviation of independent growth analysis conducted using biological replicates of independent *rpoD* mutant transformants. (B) The location of mutations in the best mutant from each round of mutagenesis is shown on the $\sigma^{70}$ protein in relation to previously identified critical functional regions (Gruber and Gross, 2003). The second round mutagenesis resulted in the identification of a truncated factor containing only one of the two prior mutations in that region.

# Global Transcription Machinery Engineering – Gtm Engineering

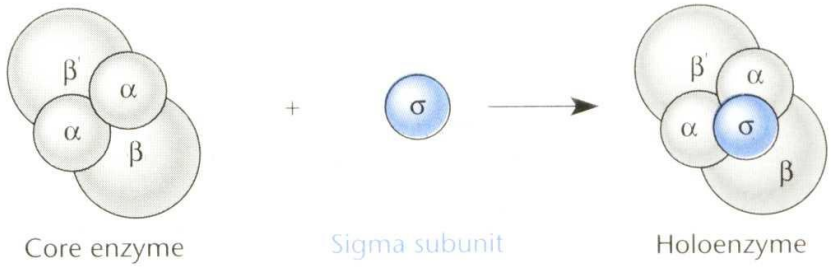
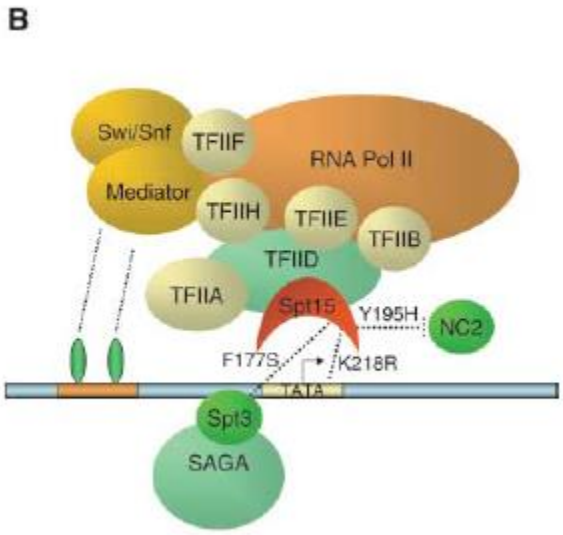



**Figure 2.4** The composition of a typical bacterial RNA polymerase. The core enzyme contains two α subunits, a β subunit, and a β' subunit. The fifth subunit, the σ factor, cycles off after initiation of RNA synthesis.

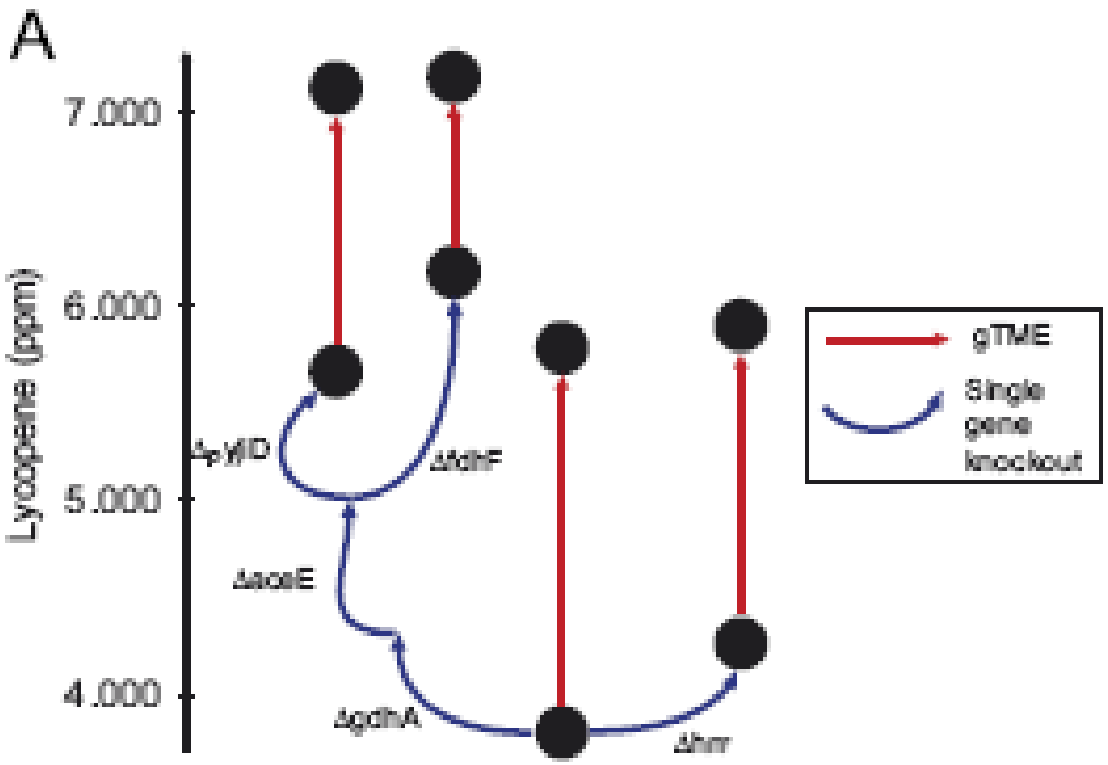
*Table* 13.2 **Some of the Alternative Sigma ($\sigma$) Factors in Bacteria and Their Cognate Promoter Sequences**

| $\sigma$ Factor | Promoter Recognized | Genes Transcribed |
|---|---|---|
| $\sigma^{70}$ | TTGACA-17bp-TATAAT | Many and diverse |
| $\sigma^{32}$ | CNCTTGAA-14bp-CCCCATNT | Heat shock response |
| $\sigma^{54}$ | CTGGNA-7bp-TTGCA | Many and diverse |
| $\sigma^{28}$ | TAA-15bp-GCCGATAA | Chemotaxis, motility, flagellar components |
| $\sigma^{29}$ | TTNAA-17bp-CATATT | Sporulation in *B. subtilis*[1] |

[1]Several different sigma factors control different genes involved in sporulation in *B. subtilis*.



# Global Transcription Machinery Engineering – gTME



# ômicas

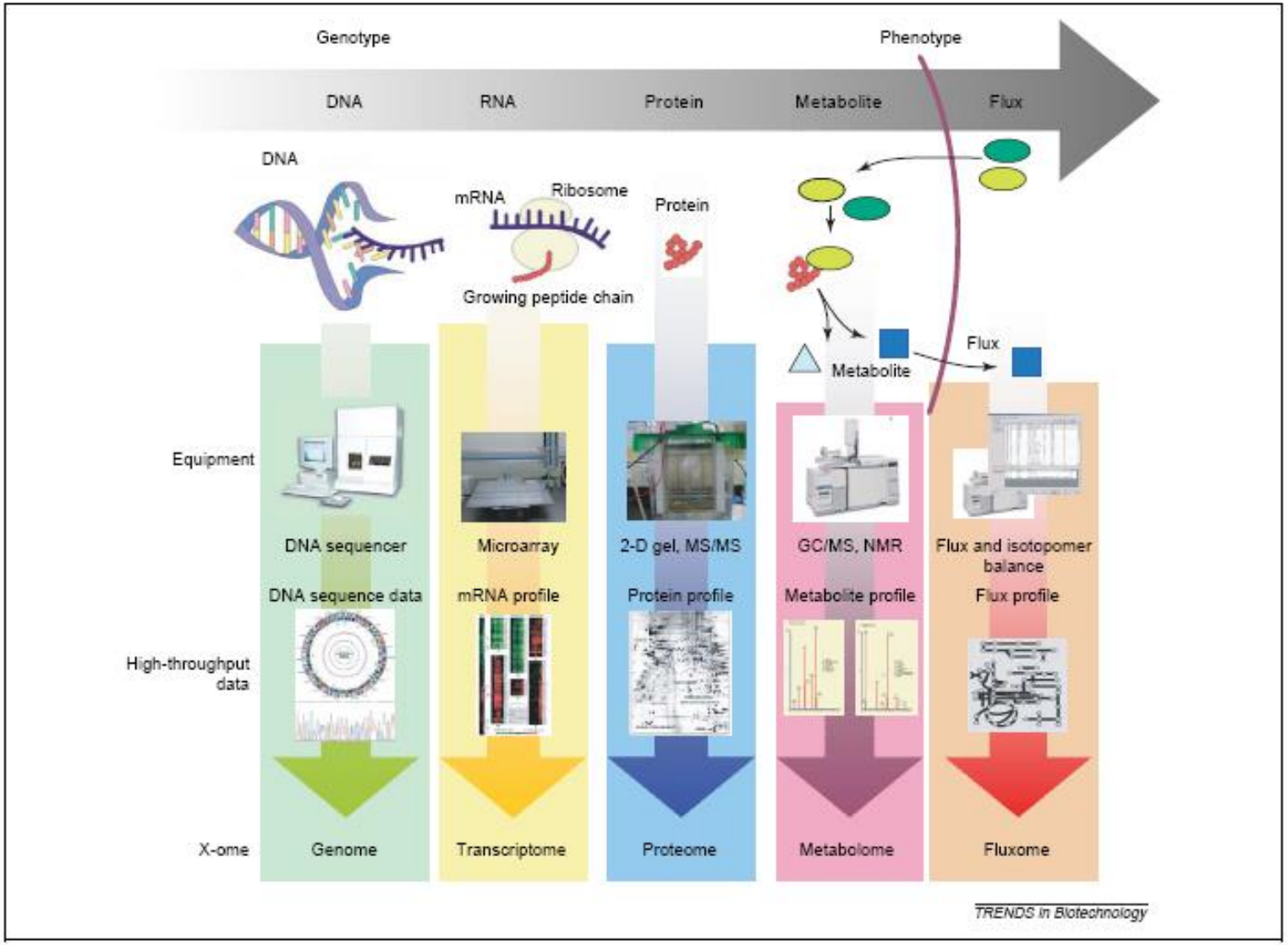
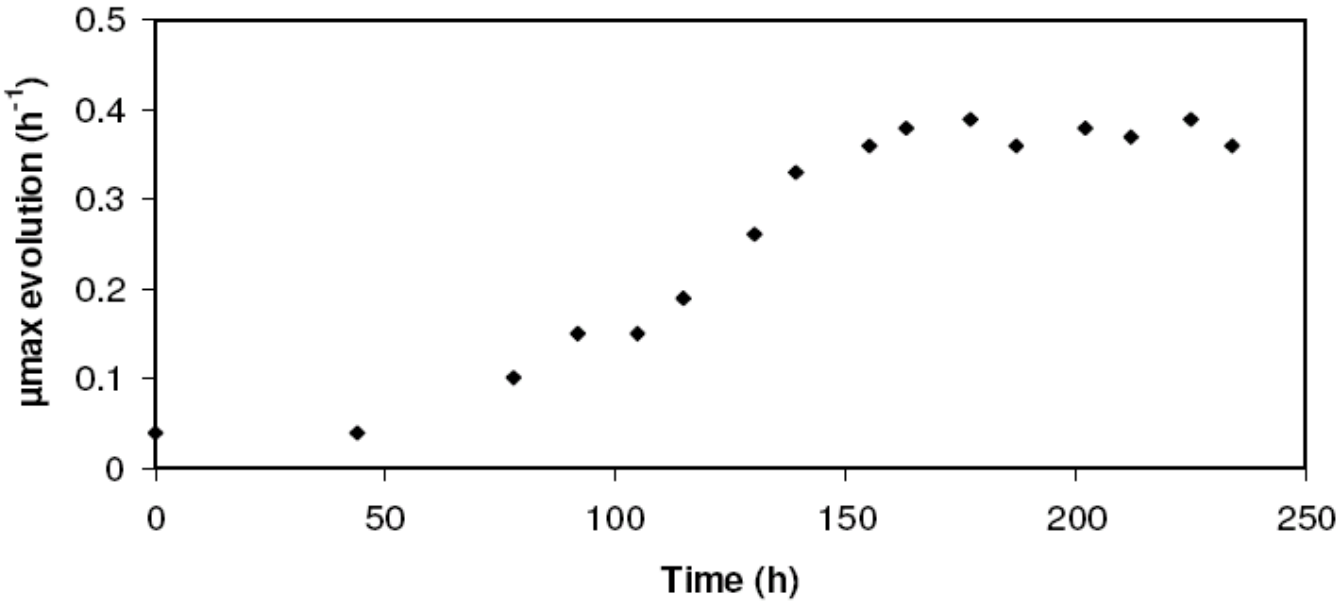



**Figure 1.** High-throughput omics research. Genomics advanced by the development of high-speed DNA sequencing is now accompanied by transcriptome profiling using DNA microarrays. Proteome profiling is joining the high-throughput race as 2D-gel electrophoresis combined with mass spectrography is advancing. Metabolome profiling is also rapidly advancing with the development of better GC/MS, LC/MS and NMR technologies. Isotopomer profiling followed by challenging with isotopically labeled substrate allows determination of flux profiles in the cell (fluxome).

# Engenharia evolutiva

1 Figure 1.

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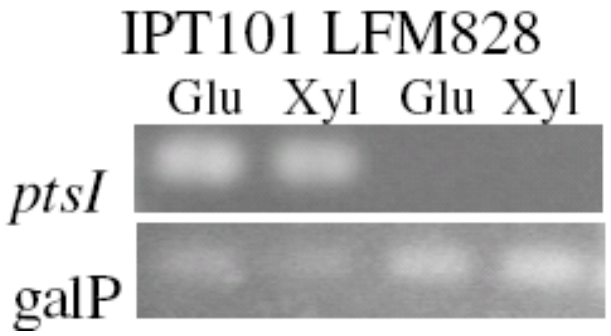
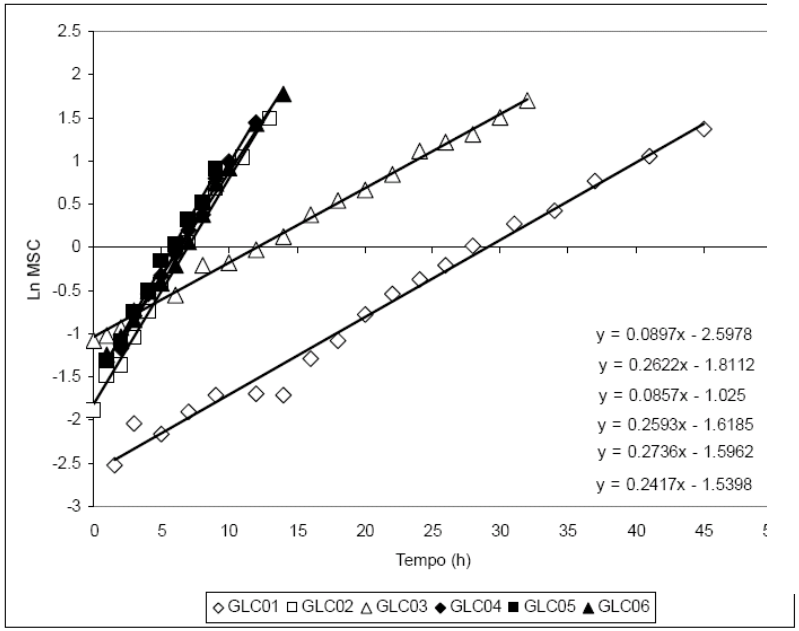


# Engenharia evolutiva



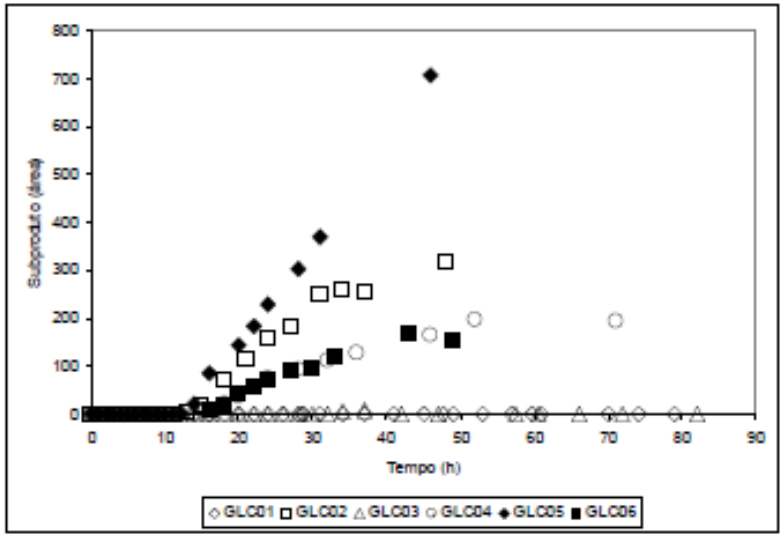




**Figura 33.** Perfil cinético de área de pico observado por HPLC.

With the advent of next-generation fluorescent DNA sequencing[10], our ability to sequence genomes has greatly outpaced our ability to modify genomes. Existing cloning-based technologies are confined to serial and inefficient introduction of single DNA constructs into cells, requiring laborious and outdated genetic engineering techniques.

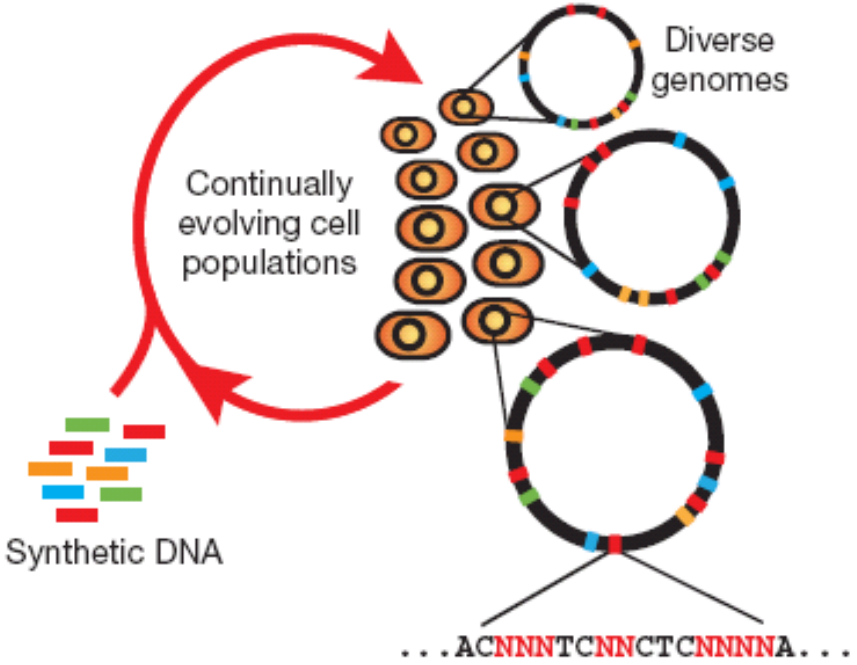



**Figure 1 | Multiplex automated genome engineering enables the rapid and continuous generation of sequence diversity at many targeted chromosomal locations across a large population of cells through the repeated introduction of synthetic DNA.** Each cell contains a different set of mutations, producing a heterogeneous population of rich diversity (denoted by distinct chromosomes in different cells). Degenerate oligo pools that target specific genomic positions enable the generation of a diverse set of sequences at each chromosomal location.