"Cell engineering and antibiotic-free selection for Vaccinal antigens production in *E. coli*: the ultimate sophistication to combine safety and productivity"

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What makes vaccine production so specific?

- Vaccine candidates are always very different
  - Origin
  - Nature
  - Complexity
- Vaccine manufacturing needs are
  - Versatile expression platforms
  - Low Cost Of GoodS
  - Safety

Why *E. coli* ?

- A large number of vaccine candidates are bacterial antigens
- Some viral antigens, VLP or VLP-like structures can be produced in *E. coli*
- *E. coli* engineering will give access to increasing possibilities in a short and mid-term future
  - **Knock-in or Knock-out strategies**
  - **Genomic reconstruction including metabolic pathways from other microorganisms**
- *E. coli* is well known and accepted from health authorities and adaptable to antibiotic-free selection

Why do we have to consider alternatives to antibiotic-based selection?

- The Increasing regulatory requirements to which biotherapeutics are subjected will have a great impact on industrial protein production.
  - **There may be "zero tolerance" towards antibiotic-based selection in production systems.**
- Besides the antibiotic itself, the antibiotic resistance gene is an important consideration.
  - **The complete absence of antibiotic-resistance gene being the only way to ensure that there is no propagation in the environment or transfer of resistance to pathogenic strains.**
  - **Complete absence is required for DNA immunisation or Gene therapy vectors**

The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 24 April 2001
CPMP/BWP/3088/99

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

Plasmid DNA products (eg. Plasmid DNA)
"The use of all specific elements or region of DNA should be justified. Special attention should be given to the nature of the selection marker. The use of certain selection markers, such as resistance to antibiotics, which may adversely impact on other clinical therapies in the target population, should be avoided."

Guidance for Industry

Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Vaccine or Related Product

be toxins, carcinogens, teratogens, or allergens. Antibiotics and other components (e.g., growth factors, antibodies) used in the culture but neither required nor specifically intended to be in the final vaccine product should be removed before use. Procedures to

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research (CBER)
January 1999

Guidance for Industry

Guidance for Human Somatic Cell Therapy and Gene Therapy

"Concerning environmental impact and the use of drug resistance traits, consult the NIH Guidelines for Research Involving Recombinant DNA Molecules, Section III-A-1-a (59 FR 34496, amended 61 FR 59732). Non-antibiotic selection systems can also be used."

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
March 1998

Characterization of the Metabolic Burden on *Escherichia coli* DH1 Cells Imposed by the Presence of a Plasmid Containing a Gene Therapy Sequence

A. Rozkov,[1]* C.A. Avignone-Rossa,[1] P.F. Ertl,[4] P. Jones,[2] R.D. O'Kennedy,[3] J.J. Smith,[2] J.W. Dale,[1] M.E. Bushell[1]

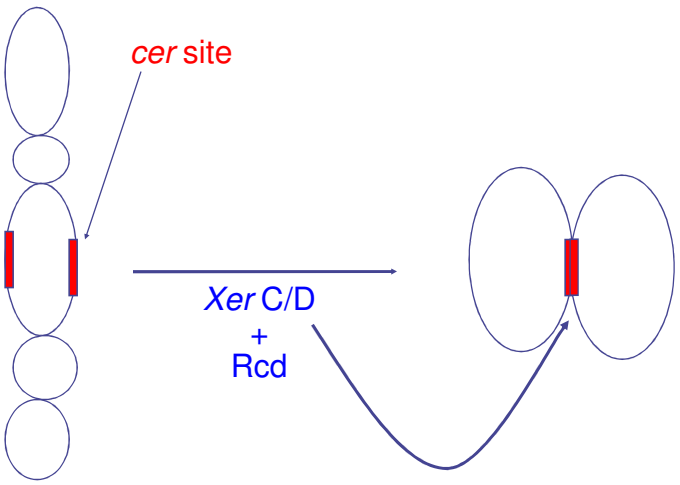
How?
A stepwise strategy was applied

- Vector stabilsation
  - Reduce plasmid loss during fermentation
- Antibio-free selection
  - Several alternatives
  - Poison-Antidote system ccdA / ccdB as a model
- Complete elimination of antibio-resistance gene after cloning steps
  - Homologous recombination
  - Can impact protein production
  - Mandatory for DNA immunisation

Vector stabilisation

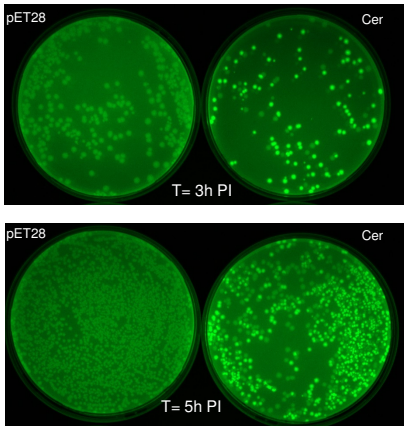
- Avoid or reduce plasmid loss during cell division
  - Plasmid are randomly distributed to daugther cells during bacterial division
  - Plasmid are multimeric, often dimeric
  - Making them monomeric will reduce the probability of obtaining empty cells
  - Multimer "resolution" can be obtained using the *Cer* fragment

The *cer* locus
Multimer resolution



Cer and plasmid stability

	pET28	Cer
T=0	100	100
T=induction	100	100
Ti+1h	100	100
Ti+3h	16	72
Ti+5h	3	62



Presence and orientation of the *Cer* locus: influence on plasmid stability?

- The case of Urease (*H. pylori*) produced in Erlen flasks in absence of Kanamycin

CULTURE TIME	PLASMID WITHOUT CER	WITH CER ORIENTATION 1	WITH CER ORIENTATION 2
1h	**87 %**	**100 %**	**100 %**
2h	**IPTG addition**		
3h	**67 %**	**100 %**	**100 %**
5h	**1 %**	**50 %**	**34 %**
25h	**0 %**	**9 %**	**8 %**

Antibiotic-free selection
Different possibilities

- Complementation of an essential gene
  - **Several examples: Dap D, infA**
  - **Need specific medium**
- Auxotrophy (ex: Glycine auxotrophy)
  - **Disruption of the *glyA* gene in *E. coli* M15**
  - **Plasmid contains *glyA* under control of a weak promote**
    - Use of a specific medium (not always optimal for over-expression)
    - Active antibiotic-free selection / decrease in expression level (lab scale)
- Post-Segregational Killing
  - **Cell Death is induced upon plasmid loss**
  - **Poison / Antidote (ccdA / ccdB as a model)**



Poison-antidote
Genes ccdA/ccdB

Separate-component-stabilization system for protein and DNA production without the use of antibiotics
(Szpirer/Milinkovitch)
BioTechniques® May 2005
Volume 38, Number 5: pp *775-781*

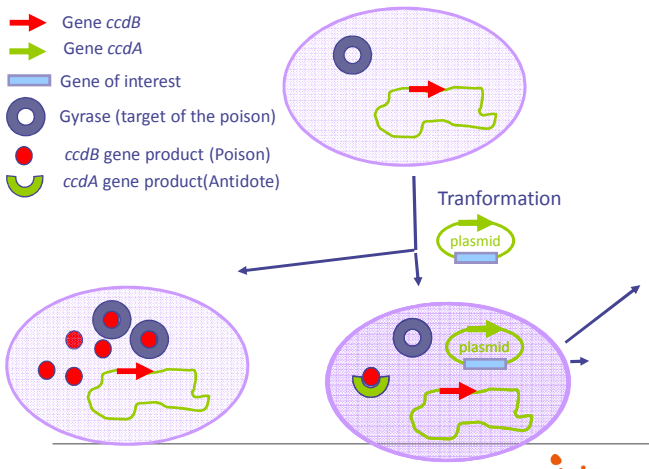
- Commercial system from Delphigenetics (Belgium)
- System already used in Gateway cloning system (Invitrogen)

Mode of action

- Gene *ccdB* (the poison) ,
  - **Insertion into the bacterial genome.**
  - **Encodes a stable protein (100aa), binding gyrase (essential for cell division) inducing cell death**
- Gene *ccdA* (the antidote)
  - **Plasmid-borne**
  - **Under control of a weak promoter, encodes an instable protein (90aa)**

How does it works?

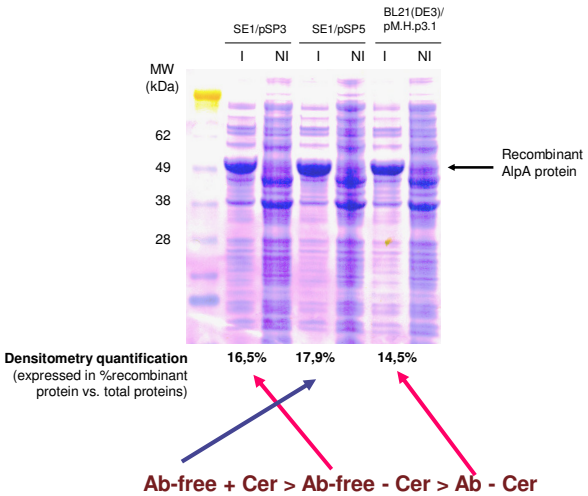
- Commercially available strain (Delphigenetics)



Evaluation in Shake-Flasks

- Transformation of *E. coli* SE1 by pSP1
  - The Km resistance gene is eliminated by digestion and self-ligation
- Evaluation of protein expression in Erlen flask cultures:
  - Test of Km / Cer system as a control
  - Evaluate the ccd system
  - Combine ccd with or without Cer
- Results:
  - No difference between the two systems regarding growth profile and some difference in expression level.
  - No plasmid loss (96 clones analysed – pDNA extracted by an automate)

Production of recombinant protein in flasks, in absence of kanamycine: *Cer* effect (effet de *Cer*)

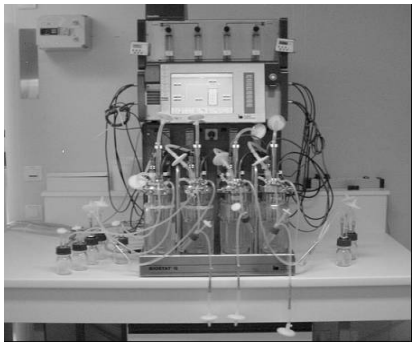


A Synergy effect is observed

and measured by scanning

Evaluation in fermenter at 1L scale

- Mimic the actual pilot-scale process: same values of the culture parameters (pO_2, T°, pH)
- Test two induction times (standard – OD=25 and earlier - OD=1)
- In parallel, cultivate the original strain (antibiotic-based system) as an internal control



Culture parameters can be adjusted separetly

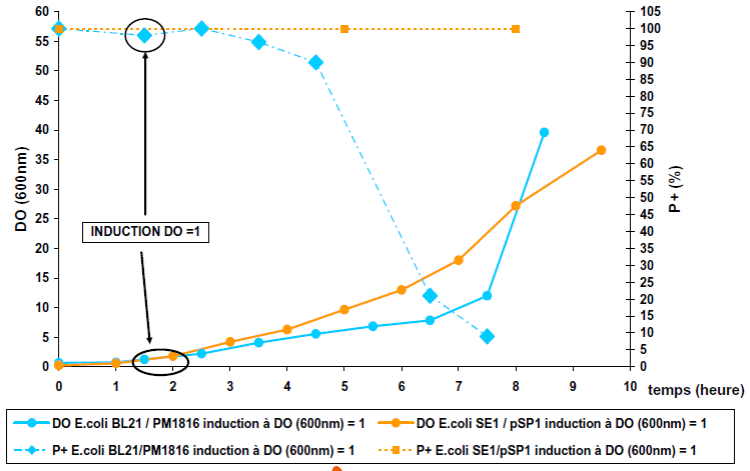
Evaluation in fermenter at 1L scale

OD=1
To mimick "High Stress" Conditions

OD=25
Standard Fermentation Conditions

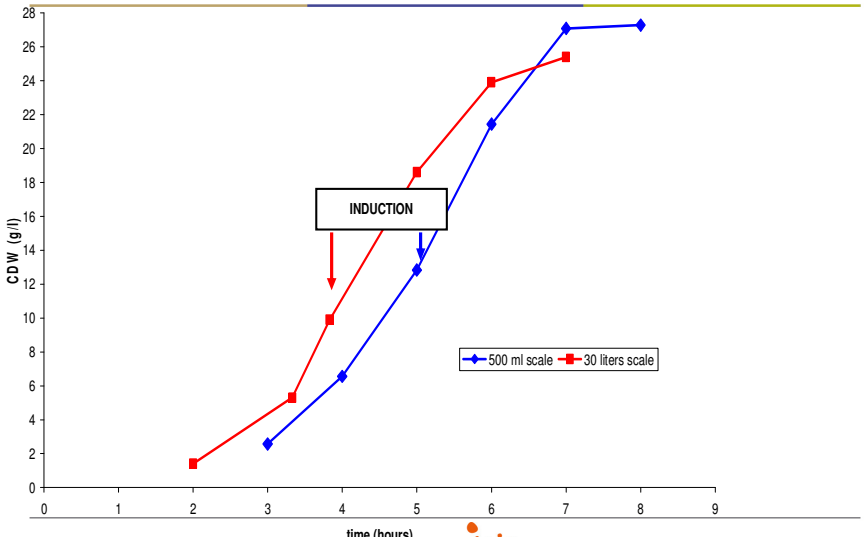
	Induction at early stage of growth		Induction at advanced stage of growth	
	System based on kana resistance	Antibiotic free system	System based on kana resistance	Antibiotic free system
Cell Dry Weight (g/L)	28	23	22	24
Plasmid retention (%)	5	100	90	98
Product yield (mg/L)	36	350	280	603
Specific productivity (mg product/ g CDW)	1	15	13	25

Plasmid maintenance / bacterial growth according to “high stress” conditions (early induction)



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kinetics of cell growth between 500ml scale and 30 liter scale with antibiotic-free system and a late induction



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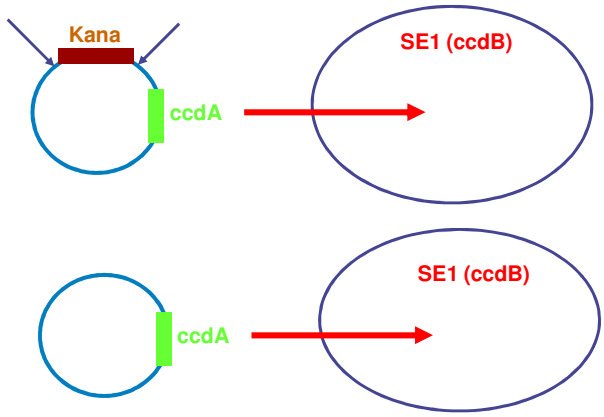
The ccd System - Conclusion

- Functional at 1L and 30L scale
  - No plasmid loss whatever the time of induction
  - protein yield at least similar or often better especially in case of late induction time or difficult to express protein.

Further improvement

- Antibiotic-based selection is required for the construction steps of the expression plasmid
  - **Prevent from external contaminations**
  - **In an ideal situation, the antibiotic-resistance gene must be eliminated just before fermentation**
- Elimination of the Kanamycin resistance gene
  - **Through restriction digestion and re-ligation**
    - No positive selection afterwards (next slide)
    - Tedious (plating, restriction map, PCR)
  - **Simply by homologous recombination**
    - Positive selection

Digestion and religation is not an optimal process



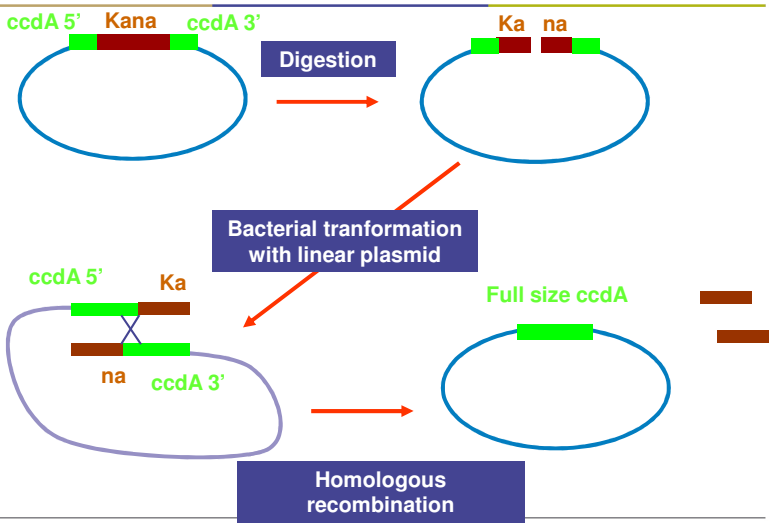
No direct selection of KanR – plasmids upon tranfection of the SE1 strain

No difference between Kana+ and Kana- plasmids

Need isolation of clones and further analysis (restriction map, PCR, plating…)

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Homologous recombination process



ccdA is functional only if the KanaR gene is eliminated

Conclusion & Perspectives:

- A combination of plasmid stabilisation and antibiotic-free selection can improve recombinant protein production.
- Complete elimination of antibiotic resistance gene with a positive selection can be obtained through homologous recombination
  - Positive selection
  - Reduce the size of the vector
  - Can improve production of protein / plasmid
- Regulatory status for antibiotic-free selection
  - Preferred
  - Highly recommended
  - Mandatory
- Ongoing Improvement
  - Combine anti-bio free selection with new engineered strains
    - Improved solubility / secretion capacity

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References

SELF-REPLICATING VECTOR LACKING AN ANTIBIOTIC-RESISTANCE GENE

BREVETS (PCT)

(19) Organisation Mondiale de la Propriété Intellectuelle
Bureau international

(43) Date de la publication internationale
21 janvier 2010 (21.01.2010)

PCT

(10) Numéro de publication internationale
WO 2010/007246 A1

Peubez et al. Microbial Cell Factories 2010, 9:65
http://www.microbialcellfactories.com/content/9/1/65

MICROBIAL CELL FACTORIES

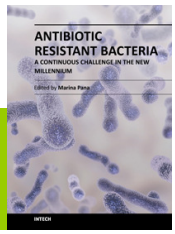
RESEARCH Open Access

Antibiotic-free selection in E. coli: new considerations for optimal design and improved production

Highly accessed

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Isabelle Peubez[1], Nicolas Chaudet[2], Charlotte Mignon[1], Géraldine Hild[1], Stéphanie Husson[1], Virginie Courtois[1], Karelle De Luca[1], Denis Speck[2], Régis Sodoyer[1*]



Antibiotic Resistance (ISBN 979-953-307-855-6)
Antibiotic-Free Selection for Bio-Production: Moving Towards a New Gold Standard
Regis Sodoyer, Virginie Courtois, Isabelle Peubez and Charlotte Mignon

pathogens

ANTIBIOTIC-FREE SELECTION IN BIOTHERAPEUTICS: « NOW AND FOREVER »
Charlotte Mignon, Régis Sodoyer & Bettina Werle
Special Issue "Alternatives to Antibiotics: Current Strategies and Future Prospects" In preparation

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What Else?

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"Simplicity is the ultimate sophistication"

Leonardo Da Vinci

Thank you for your attention

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