

Antibiotic-Free Selection for Bio-Production: Moving Towards a New „Gold Standard“

Régis Sodoyer, Virginie Courtois, Isabelle Peubez and Charlotte Mignon
*Sanofi Pasteur,
France*

1. Introduction

Antibiotics have shown a proven efficiency profile in therapy against some infectious agents for several decades. Nevertheless, a large-scale spreading of antibiotics in the environment, and emergence of resistant or even multi-resistant pathogenic bacterial strains has become a general concern promise to even further increase. Besides therapeutic applications, antibiotics are often used as a selection pressure to avoid bio-contamination in production processes such as fermentation. In this particular context the problem can show two distinct facets: the antibiotic molecule itself, seen as a contaminant product in a given biological and the antibiotic resistance gene used as a selection marker.

The increasing regulatory requirements to which biological agents are subjected will hopefully have a great impact in the field of industrial protein expression and production. There is an expectation that in a near future, there may be “zero tolerance” towards antibiotic-based selection and production systems. Besides the antibiotic itself, the antibiotic resistance gene is a major subject of consideration. The complete absence of antibiotic-resistance gene being the only way to ensure that propagation in the environment or transfer of resistance to pathogenic strains will not happen.

In order to address these issues, different and complementary approaches can be applied. The first would be to design more stable host/vector couples allowing to set-up and conduct fermentation processes in complete absence of antibiotics. A more achieved strategy would be to substitute the antibiotic-based selection by an alternative mean such as the complementation of an essential gene product, not expressed by the host or sophisticated post-segregational killing mechanism.

For specific therapeutic agents or fields of application such as DNA vaccination or gene therapy the presence of an antibiotic resistance gene in the vector backbone is seen as undesirable to health authorities. In that case the problem is the possibility of horizontal transfer of antibiotic resistance to circulating microbial population.

2. Current selection methods

2.1 Different antibiotic-based systems

Most commercialized vectors use antibiotic-based selection markers. Ampicillin and kanamycin resistance genes are two widely used selection markers.

Ampicillin resistance gene, Amp^R also known as bla_{TEM1}, is derived from *Salmonella paratyphi*. It allows the synthesis of the beta-lactamase enzyme, which neutralizes antibiotics of the penicillin group, such as ampicillin. Surprisingly, ampicillin that is the most popular selection marker used in research laboratories is in fact a very inefficient selection mean in liquid cultures. The antibiotic-resistance gene product, β -lactamase, efficiently secreted into the culture supernatant rapidly eliminates the antibiotic, even if used at high concentration.

Chloramphenicol is more rarely used, or for some specific applications, for instance, large plasmid or cosmid DNA amplification. The spectra of activity of this antibiotic being variable between true bactericidal and bacteriostatic effect according to the gram character or nature of the bacterial strain considered (Rahal and Simberkoff 1979).

Kanamycin resistance gene, Kan^R also known as *ntpII* (neomycin phosphotransferase II) was initially isolated from the transposon Tn5 that was present in the bacterium strain *Escherichia coli* K12. The gene encodes the aminoglycoside 3'-phosphotransferase enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as neomycin and kanamycin.

2.2 Advantages and drawbacks

Even if antibiotic resistance gene are widely use for DNA production and recombinant protein expression in bacteria, regulatory agencies tend to restrict their use because of potential horizontal transfer to environmental bacteria. Indeed, due to several mechanisms of gene transfer between bacteria, a potential risk of antibiotic resistance spread exists.

3. Potential concern

3.1 Safety issue

Antibiotic resistance genes are the most commonly used selectable markers for plasmid production (i.e. for vaccine or therapeutic DNA or for production of recombinant proteins as biotherapeutics). To date, kanamycin resistance gene is the most commonly used as selectable antibiotic marker. Ampicillin resistance gene is not acceptable due to concerns for patients which have reactivity to β -lactam antibiotics. Tetracyclin resistance gene is toxic for *E.coli* (Williams et al., 2009)

The major issue is the horizontal genetic transfer of antibiotic resistance gene to prokaryotic organisms present in the environment for biotherapeutic production or in commensal flora.

3.2 Horizontal transfer

Horizontal genetic transfer (HGT) is the passage of genetic elements between organisms (Tuller et al., 2011). This HGT is a major driving force in bacterial evolution by facilitating the diversity of bacteria. An essential element in HGT is to determine which factors influence the fixation of transferred genes. Some of these factors have already been identified (Tuller et al., 2011) and correspond to the advantage conferred by the transferred gene, the toxicity of its product, the ability of the transferred gene to be integrated into the host genome and to be stabilized, the number of interactions of the transferred gene product and the compatibility of codon usage between the transferred gene and the host. Tuller et

al., 2011, have shown a correlation between the number of horizontally transferred gene with different organisms and the similarity between their tRNA pools. Moreover, organisms present in a same ecological environment have similar tRNA pools. These two points increase the probability of integration and fixation of a HTG into a new host genome.

Acquisition of antibiotic resistance is one element of this evolution by HTG. For example, Datta and Kontomichalou in 1965 have shown the importance of the penicillin resistance transfer across the *Enterobacteriaceae*. More recently, acquisition of the virulence factors that distinguish *Salmonella* from *Escherichia coli* has been shown as the result of HTG (Wiedenbeck & Cohan, 2011).

Moreover, one element favoring HTG is the length of DNA and this is the case with plasmid harboring antibiotic resistance gene used in recombinant protein (biotherapeutic) expression.

These elements are potential concerns that have to be taken into account in production of therapeutics or vaccine plasmid products and of biotherapeutics to restrict safety issues.

4. Regulatory point of view

A large number of guidance for industry, have been released by the FDA. Among these some are directly applied to the use of antibiotic resistance marker genes in different contexts such as transgenic plants at large or crops for animal feed. Here we have deliberately decided to restrict our focus on vaccines and biological therapeutics.

The market of “biotherapeutics”, derived from recombinant DNA technologies, is entering an exponential growing phase. As much as 34 monoclonal antibodies have been, to date, approved by the FDA for various therapeutic applications. Besides antibodies, other products such as next generation recombinant vaccines and gene therapy constructs are progressively invading new therapeutic areas. As a result of growth in existing markets and the opening of new opportunities, the global demand is largely projected to further increase. A direct consequence is a progressive adaptation and strengthening of the existing regulation. A reasonable expectation is a move towards a “zero tolerance” for antibiotic based selection in production systems.

4.1 North American & European regulation

As soon as in July 1993, the FDA drafted some points to consider in the characterization of cell lines used to produce biological products.

‘Penicillin or other beta lactam antibiotics should not be present in production cell cultures. Minimal concentrations of other antibiotics or inducing agents may be acceptable [21 CFR 610.15(c)]. However, the presence of any antibiotic or inducing agent in the product is discouraged.’

The WHO technical report series N° 878: “Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals”, published in 1998 goes exactly in the same direction.

‘Penicillin or other β -lactam antibiotics shall not be present in production cell cultures.’

Minimal concentration of other antibiotics may be acceptable. However the presence of any antibiotic in a biological process or product is discouraged.'

Over the years the recommendation became more precise or specific to some categories of biological products such as DNA vaccines. The potential issue of allergic responses to some classes of antibiotics is evoked, the necessity to document the trace amount of antibiotics in the final product clearly seen as mandatory. And finally appears an interesting allusion to novel strategies to replace antibiotic-based selection.

In December 1996 FDA issued a draft guidance entitled - Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications

'Antibiotic resistance is commonly employed as a selection marker. In considering the use of an antibiotic resistance marker, CBER is advising manufacturers against the use of penicillin or other beta-lactam antibiotics as these antibiotics can, in certain individuals, result in allergic reactions ranging in severity from skin rashes to immediate anaphylaxis. When an antibiotic resistance marker is required in a plasmid DNA vaccine construct, CBER advises the use of an antibiotic such as kanamycin or neomycin. These aminoglycoside antibiotics are not extensively used in the treatment of clinical infections due to their low activity spectrum, prevalence of kanamycin-resistant bacteria, and their problematic therapeutic index with toxicities including irreversible ototoxicity and nephrotoxicity. Specifications for the level of antibiotic present in the final container should be established and should consider the minimum level of antibiotic that will give an unintentional clinical effect. The use of alternative antibiotic resistance markers or the use of suppressor tRNA genes in a plasmid construct intended as plasmid DNA vaccine should be discussed with CBER prior to full scale development of a new vaccine product.'

Several updates (2006 and 2009) of this guidance are accessible on the web.

The EMEA is perfectly in line with its North American counterpart, as a matter of example, a 2001 guidance indicates:

'lack of expression in mammalian cells should be verified due to regulatory concerns'

This comment is an illustration of the concern applied to the antibiotic-resistance gene itself.

In a draft review, released from the FDA in November 2004, it is clearly specified that the use of beta-lactams should be avoided or at least very clearly documented in terms of safety for the patient. This recommendation, even if not prohibitive, appears as extremely dissuasive.

Content and Review of Chemistry, Manufacturing and Control (CMC) Information for Human Gene Therapy Investigational New Drug Application (INDs)

'Because some patients may be sensitive to penicillin, we recommend that you, a sponsor, do not use beta-lactam antibiotics during the manufacturing of a therapeutic product for humans. If beta-lactam antibiotics are used, we recommend that you take and describe precautions to prevent hypersensitivity reactions.'

In a recent release from the FDA (October 23, 2009) dedicated to Vaccines, Blood & Biologics, entitled: Common Ingredients in U.S. Licensed Vaccines: Why are antibiotics in some vaccines?

'Certain antibiotics may be used in some vaccine production to help prevent bacterial contamination during manufacturing. As a result, small amounts of antibiotics may be present in some vaccines. Because some antibiotics can cause severe allergic reactions in those children

allergic to them (like hives, swelling at the back of the throat, and low blood pressure), some parents are concerned that antibiotics contained in vaccines might be harmful. However, antibiotics most likely to cause severe allergic reactions (e.g., penicillins, cephalosporins and sulfa drugs) are not used in vaccine production, and therefore are not contained in vaccines.'

In the issue of July 7th 2011 of: Common Ingredients in U.S. Licensed Vaccines, some comments on antibiotics used in vaccine manufacturing processes are somewhat moderated.

'Examples of antibiotics used during vaccine manufacture include neomycin, polymyxin B, streptomycin and gentamicin. Some antibiotics used in vaccine production are present in the vaccine, either in very small amounts or they are undetectable. For example, antibiotics are used in some production methods for making inactivated influenza virus vaccines. They are used to reduce bacterial growth in eggs during processing steps, because eggs are not sterile products. The antibiotics that are used are reduced to very small or undetectable amounts during subsequent purification steps. The very small amounts of antibiotics contained in vaccines have not been clearly associated with severe allergic reactions.'

4.2 Conclusion and future rules

According to the above mentioned information:

- It is not yet prohibited but strongly advised to avoid or minimize the use of any kind of antibiotics in cell or bacterial culture,
- If antibiotics are used, it is mandatory to minimize their amount and to control for the presence of traces in the final product,
- The rationale for the use of antibiotics must be clearly documented in the CTD
- Penicillin, more generally β -lactams and streptomycin must not be used in reason of potential concerns with hyper reactivity of some patients to antibiotics of the β -lactam family
- Kanamycin and neomycin are the preferred choice and still tolerated.

The use of antibiotic resistance markers is generally discouraged, and if used the *in vivo* effect needs to be evaluated.

There are specific mentions for the nature of the gene encoding resistance to kanamycin, as reviewed by Williams et al., (2009). The gene neomycin phosphotransferase III [npt-III, *aph* (3')-III] should be avoided, since it also confers resistance to amikacin, a reserve antibiotic (EMA, 2008).

As a final comment it is easy to anticipate, what might be the future requirements from health authorities: constructs have to be completely devoid of antibiotic resistance genes in their final structure, even if in use at early stages of construction. Alternative solutions would be available and validated soon.

5. Alternatives to antibiotics in bio-production

5.1 Vector stabilization

One aspect to be considered during recombinant biopharmaceutical expression is the stability of the plasmid used. More than 20 years ago, several studies on natural plasmids have highlighted that some plasmids naturally display regions necessary for their stability.

Ogura & Higara, 1983a, have shown that plasmid F, that exists only as one to two copies per chromosome and is stably inherited to daughter cells during cell growth, contains stabilization sequences. They did show that these sequences were independent from plasmid replication function. They first identify 3 regions essential for plasmid maintenance: *SopA* and *SopB* that acts in trans and *SopC* that acts in cis to stabilize the plasmid by probably interacting with cellular components. These authors also put in evidence that *SopA*, *SopB* and *SopC* were not sufficient for full stability of mini-F plasmid, and identified the *ccd* (control of cell death) region that seemed to control cell division when copy number carrying *ccd* segment decreases (Ogura & Higara, 1983b). The so-called *ccd* region is divided into two functional regions: *ccdB*, which product inhibits the host cell division and *ccdA*, which product is able to inhibit the *ccdB* function. Two years after, Jaffé et al., 1985, demonstrated that cell division is not immediately inhibited and that residual division could take place in the plasmid free-cells before finally being inhibited. Authors concluded that *ccd* region guarantees that plasmid carrying cells could grow preferentially in a population by killing plasmid free daughter cells, introducing the concept of post-segregational killing.

Plasmid R1 has also been shown to contain a stabilization system (Gerdes et al., 1985). As for plasmid F, the stabilization system is based on post segregational killing due to the *parAB*+ locus. This locus is composed of two genes *Hok* (Host killing) and *Sok* (suppression of killing). The translation of the *Hok* messenger, encoding a toxin lethal to the bacteria, is completely blocked by the anti-messenger *Sok*. In the absence of plasmid, *Sok*, which is less stable than *Hok*, is lost first, allowing the translation of the *Hok* mRNA and expression of the toxin lethal to the cell.

Concerning plasmid maintenance, it has been shown that factors reducing multimerization of plasmid could increase plasmid stability (Summers & Sherratt, 1984). ColE1 plasmid contains a region, *cer* that seems to be necessary for a recombination event converting multimers to monomers, allowing the plasmid to be more stable. Multimer resolution is achieved through action of the XerCD site-specific recombinase at the *cer* site (see Figure 1). Cloning of the *cer* locus into various expression vectors has been extensively documented and the proof of principle largely established in high-cell density cultures.

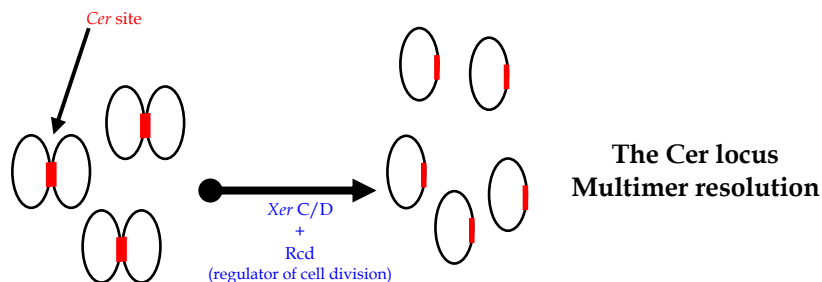


Fig. 1. Multimers resolution of high copy plasmids by XerC/D recombinase at *cer* locus.

5.2 Genomic integration

If mutation and deletion into *E.coli* genome are now widely used, it seems that genomic integration is not the preferred way to express a recombinant protein without antibiotic selection. However, some plasmid-free system have been described (see Figure 2).

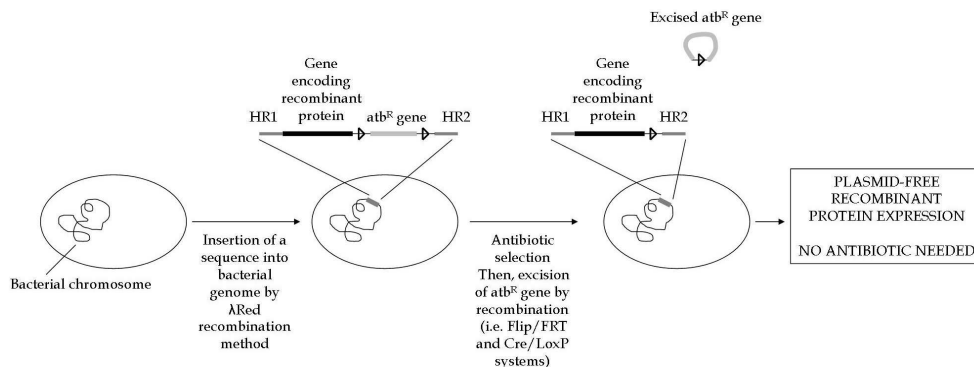


Fig. 2. Establishment of a plasmid-free expression system, illustration adapted from Striedner et al., 2010.

In 2009, a “plasmid-free T7-based *E. coli* expression system” has been developed by inserting a region of a pET plasmid into bacterial genome by λ Red recombination and P1 transduction (Datsenko & Wanner, 2000, as cited in Striedner et al., 2010). The study compared protein expression between plasmid-based and plasmid-free expression system showing an improved protein production with chromosome-based system. This system also conferred a high stability and simple upstream processing as well as high flexibility in process design. (Striedner et al., 2010).

More recently, Lemuth et al., 2011, reported the construction of the first plasmid-free *E. coli* strain that produces astaxanthin. This engineered *E. coli* strain harbors 5 heterologous biosynthetic genes from *P. ananatis* and one from *N. punctiforme* that are required for the formation of astaxanthin. Furthermore, a plasmid-free *E. coli* strain that accumulated astaxanthin as the exclusive carotenoid was engineered. This system presents many advantages compared to a plasmid-based strategy: a reduced metabolic burden, a better stability and obviously the absence of selection markers such as antibiotic resistance genes.

5.3 Complementation of essential gene product and auxotrophy markers

Essential gene complementation requires the engineering of a bacterial strain lacking an essential gene. The activity of the lacking gene product can be complemented by the culture medium or by transforming the bacteria with a plasmid having this gene as selection marker (see Figure 3).

Several antibiotic-free selection systems are based on gene complementation. One of the first systems was based on *dapD* gene (Degryse, 1991). The *dapD* gene, which has a role in the lysine biosynthetic pathway as well as cell wall assembly, has been selected as a preferred candidate by several authors, knowing that mutations in the DAP pathway are lethal. The limitations, in that case, are the intrinsic difficulty in construction of a *dapD* mutant strain and the dependence towards defined culture media composition.

Based on the same gene, a more elaborated strategy called “operator repressor titration” emerged in 2001. In this system, *dapD* gene is engineered in order to be under the control of lac Operon. When not supplemented with IPTG or DAP, *dapD* gene is not expressed

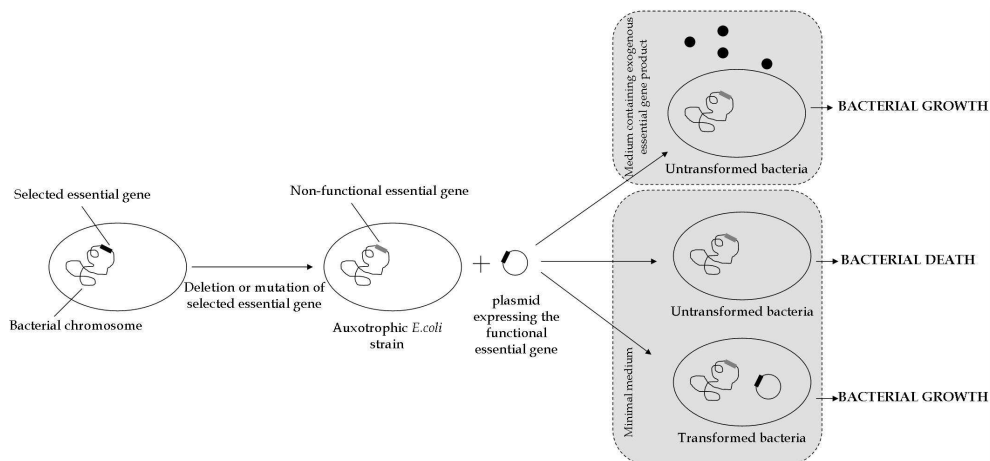


Fig. 3. Antibiotic-free selection system based on essential gene complementation.

inducing bacterial death. When the strain is transformed by a lac operator containing multi copy plasmid, the operator competitively titrates the Lac I repressor and allows the expression of *dapD* from the lac promoter allowing bacterial growth. (Cranenburgh et al., 2001 & 2004)

Other essential genes have been selected for the same purpose, such as *infA*, coding for translation initiation factor 1 (IF1), an essential protein for cell viability. In 2004, Hägg et al. generated a strain in which the *infA* gene has been deleted by a plasmid-based gene replacement method. They used a plasmid encoding a functional IF1 as selection marker and show that the system is tightly regulated and that no cross feeding is observed since initiation factors released into the media from lysed cells are not absorbed by plasmid-free cells.

The *fabI* essential gene has been used in an original way because of its property to reduce the *E. coli* susceptibility to triclosan when overexpressed. In this system, only plasmid containing cells overexpress *fabI* gene and can grow in presence of triclosan. Even if triclosan is a biocide, it is considered as non-antibiotic and regulatory agencies approve the use of triclosan for clinical use (Goh & Good, 2008).

More recently, Dong et al., 2010, developed a novel antibiotic-free selection system based on complementation of host auxotrophy in the NAD synthesis pathway. NAD can be *de novo* synthesized from tryptophan and aspartate with the quinolinic acid phosphoribosyltransferase (QAPRTase) or synthesized using the salvage pathway according to different substrates such as nicotinamide. Authors constructed a bacterial strain depleted for QAPRTase gene that can only grow if the NAD synthesis pathway is complemented by addition of salvage pathway substrate or QAPRTase gene present on a plasmid. The results obtained with this novel selection system show that the QAPRTase selection marker does not represent a metabolic burden for bacterial growth and the stability of all plasmid harboring this system were 100% in the Δ QAPRTase strain even after 6 days of continuous growth. In this study, researchers went further by complementing for the first time in an antibiotic-free selection system a bacterial strain by a mammalian QAPRTase gene with success.

A strain is auxotrophic for one amino-acid if it carries a genetic mutation that renders it unable to synthesize the amino-acid. Such a strain will be able to grow only if the amino-acid is present in the environment or if the functional gene product is expressed from a plasmid. Amino-acid auxotrophy markers had been investigated as novel antibiotic-free selection markers.

In 2001, Fiedler & Skerra developed expression vectors containing the *proAB* gene, in order to complement their proline-auxotrophic K12 strain. Their aim was not to develop an antibiotic-free selection system but to use their strain to obtain a better expression of recombinant antibody Fab fragment. For this reason, the plasmid-mediated complementation is used simultaneously with beta-lactamase selection to completely abolish plasmid loss during high scale fermentation.

In 2008, Vidal et al. described a plasmid selection system, devoid of antibiotic resistance gene and based on glycine auxotrophy. Researchers generated an *E.coli* strain that contains a deletion in the *glyA* gene, which encode for serine hydroxymethyl transferase, an enzyme involved in glycine biosynthesis pathway in *E.coli*. This strain can grow fast on a defined media only if glycine is added to the culture medium or if the bacteria harbor a plasmid expressing a functional *glyA* gene. They show comparable amount of recombinant protein with their system compared to a classical beta-lactamase selection system.

5.4 RNA-based antibiotic-free selection systems

Several antibiotic-free selection strategies are based on RNA, using antisense or anti-messenger properties or using suppressor tRNA.

The principle of down regulating an essential gene upon plasmid loss has been exploited in a very original way for the design of new vectors for gene therapy (Mairhofer et al., 2008). The expression of the essential gene *murA* encoding an enzyme essential for the biosynthesis of cell wall is under control of the Tet repressor, TetR expression is inhibited by an RNA-RNA antisense interaction with RNAI derived from plasmid origin of replication ColE1 (see Figure 4A). The major advantage of this system is that no additional sequence is required on the plasmid. (Pfaffenzeller et al., 2006; see figure 4B)

In a recent paper, RNA based selectable marker, not restricted to ColE1 containing vectors is described (Luke et al., 2009). Briefly, a counter-selectable marker (*sacB*) levansucrase from *Bacillus subtilis*, under control of the RNA-IN promoter is integrated into the bacterial chromosome induces cell death in presence of sucrose. Plasmid maintenance is ensured by the presence of the plasmid-borne regulator RNA-OUT anti-messenger acting as a down regulator of the expression of levansucrase (see Figure 4C).

Another vector system so-called pCOR, based on the complementation of an amber mutation using a suppressor tRNA, and conditional origin of replication has also been established (Soubrier et al., 1999). The original feature of the model is that an additional degree of refinement was introduced, since the dependence created between the host and the vector has become bilateral (see Figure 4D).

Nevertheless, the requirement for a minimal medium for culture means these systems are more likely to be used for DNA production rather than recombinant protein over expression.

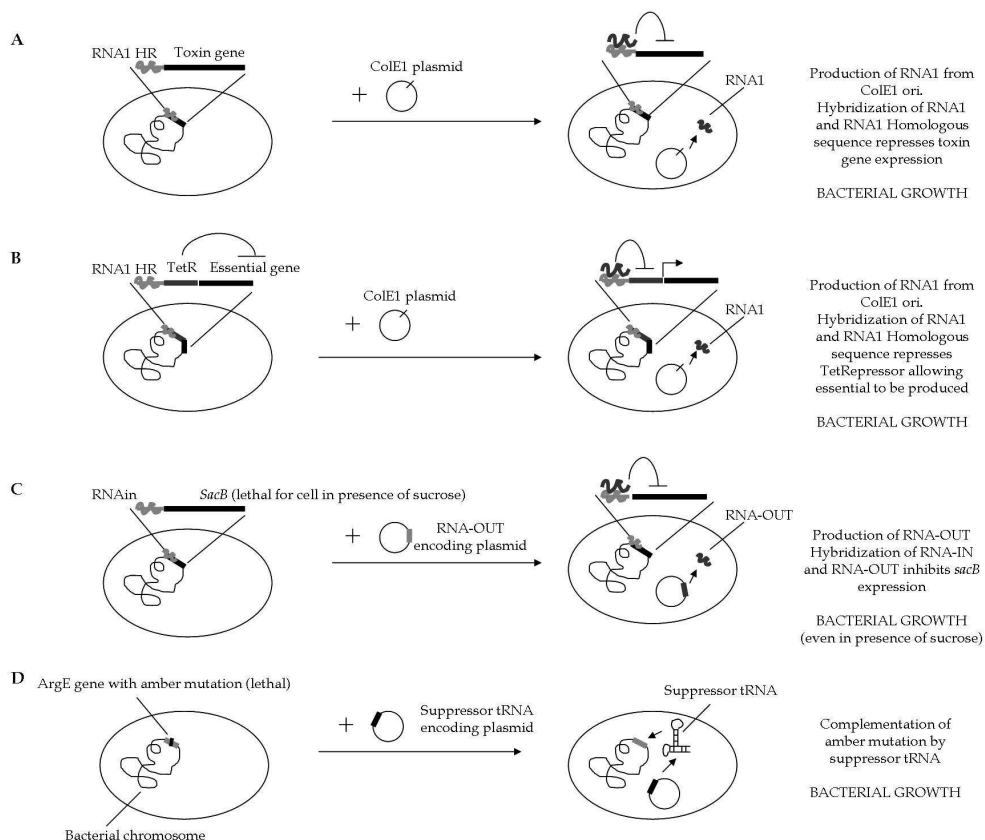


Fig. 4. RNA-based antibiotic-free systems. Illustration adapted from Pfaffenzeller et al., 2006; Luke et al., 2009 & Soubrier et al., 1999.

5.5 Post-segregational killing

Post-segregational killing is a mechanism by which plasmids are stably maintained by expressing a gene product that would be toxic to cells becoming plasmid-free upon division (see Figure 5). This mechanism, discovered on natural plasmid, has been used as selection system devoid of antibiotic.

One of these systems, based on *ccdA/ccdB* genes has been proposed by Szpirer & Milinkovitch, 2005, and is commercialized by the Delphigenetics Company. *ccdB* gene is inserted into the bacterial genome of the *E. coli* strain BL21 (DE3) and encodes a stable protein (100 aa), binding gyrase, essential for cell division. Upon binding gyrase, the *ccdB* gene product impairs DNA replication and induces cell death. *ccdA* gene, plasmid-born, encodes an instable protein (90 aa) under control of the *mob* promoter, acting as a natural inhibitor of *ccdB*. It has been shown that after 20 generations on a non-selective medium 100% of the bacteria still contain the plasmid. Two hours after induction, the plasmid is still present into all bacteria, which is not the case with a standard pET/BL21λDE3 system.

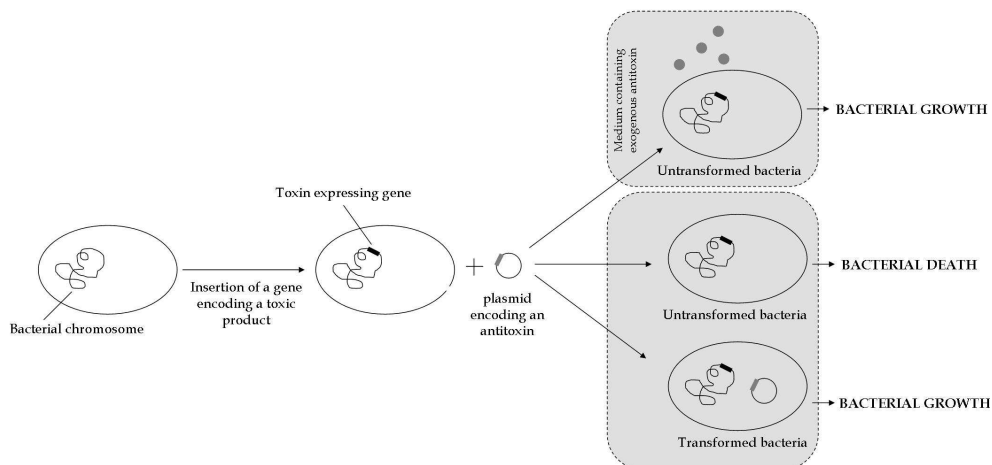


Fig. 5. Antibiotic-free selection system based on Toxin/antitoxin post-segregational killing. Illustration adapted from Szpirer & Milinkovitch, 2005.

To go further, Peubez et al. proposed in 2010 a system combining the *ccdA/ccdB* selection marker to the *cer* fragment to increase plasmid stability among long-term fermentation.

Different toxin/antitoxin (TA) systems have been described and could allow the generation of novel antibiotic-free systems. In order to detect putative TA systems, Milinkovitch's team had developed an algorithm based on predefined similarities and TA-specific structural constraints (Guglielmini et al., 2008).

Interestingly, this TA-based antibiotic-free selection system is starting to be adapted to mammalian cells selection. The toxin Kid (Killing determinant) and its antidote Kis (killing suppressor) have been used to control cell proliferation during expression of a recombinant protein in CHO-K1 cells (Nehlsen et al., 2010). If antibiotics are still used in this study, authors show that the TA strategy can significantly increase the recombinant protein expression level and could be a benefit for “difficult” to produce proteins.

6. Additional benefits

6.1 Recombinant protein production

In many cases, especially for “difficult proteins” the yield of protein production has proven to be higher with antibiotic-free system compared to a conventional one. The presence of an antibiotic resistance gene can indirectly reduce the amount of expressed protein, since even in absence of selection pressure the gene would be transcribed and account for an additional stress for the host during the fermentation process. However, even if the yield of protein is not superior to a conventional system, the antibiotic-free systems remain interesting due to their biosafety.

6.1.1 Plasmid stabilisation *cer* locus

Most commonly used multicopy plasmids are unstable and are lost during culture. Plasmid stabilization and increased maintenance during a fermentation process can be considered as

a first step towards antibiotic starvation. Genetic elements allowing plasmid maintenance during cell division and limiting the probability of plasmid loss over generations should be considered.

To stabilize the plasmid, the well-studied mechanism of site specific recombination of *E.Coli* plasmid ColE1 can be used. The 380 bp *Cer* fragment was inserted into the plasmid carrying the gene of interest and the multimers are resolved to monomers by the Xer recombinase. (Described in 5.1, see Figure 1)

Tables 1 and 2 show the contribution of the *cer* fragment in increasing the plasmid maintenance over time is clearly established. The plasmid loss is dependant on the antigen expressed.

Culture time	Plasmid without <i>cer</i>	With <i>cer</i>
2h(IPTG added)	97%	100%
4h	25%	100%
6h	20%	75%
23h	3%	79%

Table 1. Example of *Helicobacter pylori* AlpA protein produced in erlen flask in absence of Kanamycin.

Culture time	Plasmid without <i>cer</i>	With <i>cer</i>
1h	87%	100%
2h	IPTG addition	
3h	67%	100%
5h	1%	50%
25h	0%	9%

Table 2. Example of *Helicobacter pylori* Urease produced in erlen flask in absence of kanamycin.

6.1.2 *ccdA/ccdB*

The combination of different genetic elements can allow an increased stability and antibiotic-free selection. In this case, the kanamycin resistant gene present on the vector backbone was eliminated by a restriction enzyme digestion and self-relegation.

The selection system based on the couple poison (gene *ccdB*)/antidote:(gene *ccdA*), proposed by Szpirer & Milinkovitch, 2005, combined with the stabilizing element, the *cer* locus, was tested to express different recombinant proteins.

- the poison gene (*ccdB*), inserted into the bacterial genome, encodes a stable protein (100 aa), which is an inhibitor of the DNA replication capable to bind to the gyrase (an essential protein for cell division). This interaction induces the cell death.

- the antidote gene (*ccdA*), localized on the plasmid under the control of a constitutive promoter, encodes for a small unstable protein (90aa) which neutralizes the effect of *ccdB* protein action.

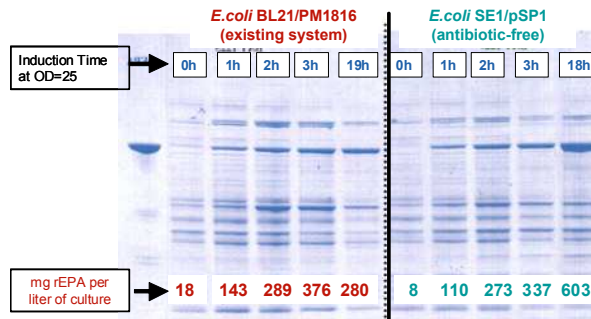


Fig. 6. Protein production evaluation in 1 liter fermenter.

Figure 6 shows a comparison of expression of the same protein with the kanamycin resistant gene (*E. coli* BL21/pM1816) and the antibiotic-free system (*E. coli* SE1/pSP1) without the kanamycin resistant gene and with the *ccdA* gene. Both have the *cer* element. Upon induction, the behavior of both systems is comparable but a clear increase in protein production is observed with the antibiotic-free system at the end of fermentation.

6.1.3 Antibiotic resistance gene elimination

Antibiotic-based selection, convenient for cloning steps, must be removed for production.

Even if antibiotic selection pressure is not used during the fermentation process, removal of this antibiotic resistance marker is of major importance to prevent horizontal transfer in the environment. This is particularly true for vectors to be used in gene therapy or DNA vaccination protocols

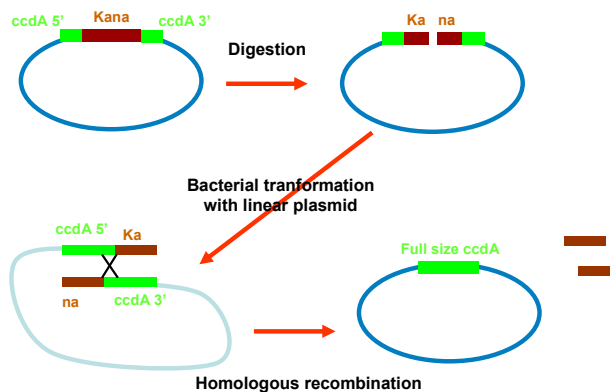


Fig. 7. Homologous recombination process allowing assembly of a functional *ccdA* encoding gene.

In order to overcome the problem of positive pressure of selection, a new approach has been developed ensuring the elimination of the antibiotic-resistance gene through homologous recombination. In this model the *ccdA* locus is split into 2 parts, containing a common sequence, and cloned at the 5' and 3' regions flanking the antibiotic resistance gene (figure 7). After digestion at a unique restriction site located inside the antibiotic selection marker and transformation of *ccdB* expressing cells with linear DNA, a fully functional *ccdA* would assemble through homologous recombination. Only bacteria containing a recombinant plasmid with a functional *ccdA* can grow upon transformation.

6.1.4 Summary of different antibiotic-free systems

System developed	Mode of action	Protein expressed	Comments/ potential drawbacks	Ref article
Plasmid-free system	Chromosome based expression system	GFP Human superoxide dismutase(SOD)	Modified <i>E coli</i> strain, required	Striedner et al., 2010
FabI-triclosan	Endogenous essential gene	None	Chemical Biocid utilisation	Goh & Good, 2008
<i>E. coli</i> strain Δ QAPRTase gene	Complementation	EGFP	Modified <i>E coli</i> strain, required	Dong et al., 2010
Pro BA	Complementation	Fab fragment	Modified <i>E coli</i> strain, required Presence of antibiotic	Fiedler & Skerra, 2001
<i>E coli</i> strain Δ glyA	Glycine auxotrophy	RhuA	Modified <i>E coli</i> strain, required. Comparable to the conventional system	Vidal et al., 2008
RNA/RNA interference	RNA/RNA interaction	EGFP	Modified <i>E coli</i> strain, required	Pfaffenzeller et al., 2006
RNA out	RNA/RNA interaction	EGFP HA vaccine candidate		Luke et al., 2009
pCOR	Complementation of amber mutation tRNA suppressor	Luciferase	Modified <i>E coli</i> strain, and minimum medium required	Soubrier et al., 1999
<i>ccdA/ccdB</i>	Toxin/antitoxin	AlpA/rEPA vaccine candidates	Modified <i>E coli</i> strain required	Peubez et al., 2010
Kid/Kis	Toxin/antitoxin	EGFP	Presence of antibiotic	Nehlsen et al., 2010

6.2 DNA immunization and gene therapy

Requirement can also be variable according to the nature of the therapeutic product, the presence of an antibiotic resistance gene, tolerated on a vector expressing a recombinant biopharmaceutical, will be totally undesirable on a gene therapy plasmid.

Among systems described in paragraph 5, some have been developed especially for DNA vaccine production such as pCOR (Soubrier et al., 1989), RNA/RNA interference (Pfaffenzeller et al., 2006) and RNA out (Luke et al., 2009).

To go further, Carnes et al., 2009, have proposed a combination of antibiotic-free selection system and an autolytic *E.coli* strain to improve both upstream and downstream processes. The antibiotic-free selection is based on RNA selectable marker described by Luke et al., 2009 and the autolytic plasmid DNA extraction method uses integrated bacteriophage endolysin gene (λR), encoding a peptidoglycan hydrolase (lysozyme) enzyme to permeabilize the bacterial cell wall, and to selectively extract the plasmid DNA from the cells in an acetate buffer. Authors found that their autolytic strain allowed efficient plasmid DNA recovery, similar to alkaline lysis plasmid DNA purification.

7. Conclusion and perspectives

Antibiotic-free selection is a general and ultimate goal that can be reached by the implementation of various and combined approaches. An increasing knowledge of bacterial physiology will give access to comprehensive information on essential genes or pathways that would be an unlimited source of inspiration for the design of novel selection means.

The major driver for the definition of antibiotic-free systems is an anticipation of fulfilling future recommendations from health authorities to overcome safety concerns. It is easy to imagine that, upon availability and functional validation, these alternative selection means will progressively gain the status of “preferred”, “strongly recommended” and finally “mandatory”.

In addition to their safety profile, some antibiotic-free systems can give access to unexpected properties such as a marked increase in recombinant protein production or plasmid recovery.

The complete elimination of any antibiotic resistance gene is, for different reasons, of critical importance for recombinant protein production, DNA immunization and gene therapy vectors.

It is likely to think that upon validation at industrial scale, antibiotic-free selection might be an added value for biotherapeutics in terms of safety profile of the product and become an important element of the marketing strategy as well. A direct consequence would be the emergence of a new “gold standard”.

8. References

- Carnes, A.E.; Hodgson, C.P.; Luke, J.M.; Vincent, J.M. & Williams, J.A. (2009). Plasmid DNA production combining antibiotic-free selection, inducible high yield fermentation, and novel autolytic purification. *Biotechnol Bioeng.*, Vol.104, No.3, (Oct 2009), pp.505-515

- Cranenburgh, R.M.; Hanak, J.A.; Williams, S.G. & Sherratt, D.J. (2001). Escherichia coli strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. *Nucleic Acids Res.*, Vol. 29, No.5, (Mar 2001), E26
- Cranenburgh, R.M.; Lewis, K.S. & Hanak, J.A. (2004). Effect of plasmid copy number and lac operator sequence on antibiotic-free plasmid selection by operator-repressor titration in Escherichia coli. *J Mol Microbiol Biotechnol.*, Vol.7, No.4, (2004), pp.197-203
- Datsenko, K.A. & Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A.*, Vol.97, No.12, (Jun 2000), pp.6640-6645
- Datta, N. & Kontomichalou, P.(1965). Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. *Nature*. Vol.208, No.5007, (Oct 1965), pp.239-241
- Degryse, E. (1985). Stability of a host-vector system based on complementation of an essential gene in Escherichia coli. *J Biotechnol.*, Vol.18, No.1-2, (Apr 1991), pp.29-39
- Dong, W.R.; Xiang, L.X. & Shao, J.Z. (2010). Novel antibiotic-free plasmid selection system based on complementation of host auxotrophy in the NAD de novo synthesis pathway. *Appl Environ Microbiol.*, Vol.76, No.7, (Apr 2010), pp.2295-2303. Epub 2010 Jan 29
- Durany, O.; Bassett, P.; Weiss, A.M.; Cranenburgh, R.M.; Ferrer, P.; López-Santín, J.; de Mas, C. & Hanak, J.A. (2005). Production of fuculose-1-phosphate aldolase using operator-repressor titration for plasmid maintenance in high cell density Escherichia coli fermentations. *Biotechnol Bioeng.*, Vol. 91, No.4, (Aug 2005), pp.460-467
- Fiedler, M. & Skerra, A. (2001). proBA complementation of an auxotrophic E. coli strain improves plasmid stability and expression yield during fermenter production of a recombinant antibody fragment. *Gene*, Vol.274, No.1-2, (Aug 2001), pp.111-118
- Gerdes, K.; Larsen, J.E. & Molin, S. (1985). Stable inheritance of plasmid R1 requires two different loci. *J Bacteriol.* Vol.161, No.1, (Jan 1985), pp.292-298
- Goh, S. & Good, L. (2008). Plasmid selection in Escherichia coli using an endogenous essential gene marker. *BMC Biotechnol.*, Vol.11, No. 8, (Aug 2008), pp.61
- Guglielmini, J.; Szpirer, C.Y. & Milinkovitch, M.C. (2008). Automated discovery and phylogenetic analysis of new toxin-antitoxin systems. *BMC Microbiol.* Vol.25; No.8, (Jun 2008), pp.104
- Hägg, P.; de Pohl, J.W.; Abdulkarim, F. & Isaksson, L.A. (2004). A host/plasmid system that is not dependent on antibiotics and antibiotic resistance genes for stable plasmid maintenance in Escherichia coli. *J Biotechnol.*, Vol.111, No.1, (Jul 2004), pp.17-30
- Jaffé, A.; Ogura, T. & Hiraga, S. (1985). Effects of the ccd function of the F plasmid on bacterial growth. *J Bacteriol.*, Vol.163, No.3, (Sep 1985), pp.841-849
- Lemuth, K.; Steuer, K. & Albermann, C. (2011). Engineering of a plasmid-free Escherichia coli strain for improved in vivo biosynthesis of astaxanthin. *Microb Cell Fact.*, Vol.26, No.10, (Apr 2011), pp.29
- Luke, J.; Carnes, A.E.; Hodgson, C.P. & Williams, J.A. (2009). Improved antibiotic-free DNA vaccine vectors utilizing a novel RNA based plasmid selection system. *Vaccine.*, Vol.27, No.46, (Oct 2009), pp.6454-6459. Epub 2009 Jun 24

- Mairhofer, J.; Pfaffenzeller, I.; Merz, D. & Grabherr, R. (2008). A novel antibiotic free plasmid selection system: advances in safe and efficient DNA therapy. *Biotechnol J.*, Vol.3, No.1, (Jan 2008), pp.83-89
- Nehlsen, K.; Herrmann, S.; Zauers, J.; Hauser, H. & Wirth, D. (2010). Toxin-antitoxin based transgene expression in mammalian cells. *Nucleic Acids Res.*, Vol.38, No.5, (Mar 2010), e32.
- Ogura, T. & Hiraga, S. (1983). Partition mechanism of F plasmid: two plasmid gene-encoded products and a cis-acting region are involved in partition. *Cell.* Vol.32, No.2, (Feb 1983), pp.351-360
- Ogura, T. & Hiraga, S. (1983). Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci U S A*, Vol.80, No.15, (Aug 1983), pp.4784-4788
- Peubez, I.; Chaudet, N.; Mignon, C.; Hild, G.; Husson, S.; Courtois, V.; De Luca, K.; Speck, D. & Sodoier, R. (2010). Antibiotic-free selection in *E. coli*: new considerations for optimal design and improved production. *Microb Cell Fact.*, Vol.7, No.9, (Sep 2010), pp.65.
- Pfaffenzeller, I.; Mairhofer, J.; Striedner, G.; Bayer, K. & Grabherr, R. (2006). Using ColE1-derived RNA I for suppression of a bacterially encoded gene: implication for a novel plasmid addiction system. *Biotechnol J.*, Vol. 1, No.6, (Jun 2006), pp.675-681.
- Rahal, J.J. Jr. & Simberkoff, M.S. (1979). Bactericidal and bacteriostatic action of chloramphenicol against meningeal pathogens. *Antimicrob Agents Chemother.* Vol.16, No.1, (Jul 1979), pp.13-18
- Soubrier, F.; Cameron, B.; Manse, B.; Somarriba, S.; Dubertret, C.; Jaslin, G.; Jung, G.; Caer, C.L.; Dang, D.; Mouvault, J.M.; Scherman, D.; Mayaux, J.F. & Crouzet, J. (1999). pCOR: a new design of plasmid vectors for nonviral gene therapy. *Gene Ther.*, Vol.6, No.8, (Aug 1999), pp.1482-1488
- Summers, D.K. & Sherratt, D.J. (1984). Multimerization of high copy number plasmids causes instability: ColE1 encodes a determinant essential for plasmid monomerization and stability., *Cell.*, Vol. 36, No.4, (Apr 1984), pp.1097-1103
- Striedner, G.; Pfaffenzeller, I.; Markus, L.; Nemecek, S.; Grabherr, R. & Bayer, K. (2010). Plasmid-free T7-based *Escherichia coli* expression systems. *Biotechnol Bioeng.*, Vol. 105, No.4, (Mar 2010), pp.786-794
- Szpirer, C.Y. & Milinkovitch, M.C. (2005). Separate-component-stabilization system for protein and DNA production without the use of antibiotics. *Biotechniques.*, Vol.38, No.5, (May 2005), pp.775-781
- Tuller, T.; Girshovich, Y.; Sella, Y.; Kreimer, A.; Freilich, S.; Kupiec, M.; Gophna, U. & Ruppin, E. (2011). Association between translation efficiency and horizontal gene transfer within microbial communities. *Nucleic Acids Res.* Vol.39, No.11, (Jun 2011), pp.4743-4755
- Vidal, L.; Pinsach, J.; Striedner, G.; Caminal, G. & Ferrer, P. (2008). Development of an antibiotic-free plasmid selection system based on glycine auxotrophy for recombinant protein overproduction in *Escherichia coli*. *J Biotechnol*, Vol.134, No.1-2, (Mar 2008), pp.127-136
- Wiedenbeck, J. & Cohan, F.M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol Rev.* doi: 10.1111/j.1574-6976.2011.00292.x. (Jun 2011), [Ahead of print]

Williams, J.A.; Carnes, A.E. & Hodgson, C.P. (2009). Plasmid DNA vaccine vector design: impact on efficacy, safety and upstream production. *Biotechnol Adv.* Vol.27, No.4, (Jul-Aug 2009), pp.353-370



Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium

Edited by Dr. Marina Pana

ISBN 978-953-51-0472-8

Hard cover, 576 pages

Publisher InTech

Published online 04, April, 2012

Published in print edition April, 2012

Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Régis Sodoyer, Virginie Courtois, Isabelle Peubez and Charlotte Mignon (2012). Antibiotic-Free Selection for Bio-Production: Moving Towards a New "Gold Standard", Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium, Dr. Marina Pana (Ed.), ISBN: 978-953-51-0472-8, InTech, Available from: <http://www.intechopen.com/books/antibiotic-resistant-bacteria-a-continuous-challenge-in-the-new-millennium/antibiotic-free-selection-for-bio-production-moving-towards-a-new-gold-standard>

INTECH

open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.