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Antimicrobials in protein production

Brochure

Antimicrobials

Antimicrobials include various natural or synthetic substances that inhibit growth or survival of microorganisms. Depending on their mechanism of action, affected organism, spectrum of activity, source, or chemical structure, antimicrobial agents can be classified into different groups. When classified by the type of microorganism they affect, antimicrobials are grouped into antibiotics, antifungals, antivirals and antiprotozoal agents. Among these, antibiotics are most widely used in protein production. In this brochure, first the modes of action and resistance of antibiotics will be reviewed, followed by their use in protein production as well as some alternatives to antibiotics.

Antibiotics

Modes of action

There are several modes of action of antibiotics. Namely, they can inhibit cell wall synthesis, interrupt structure and the function of the cell membrane, or inhibit of proteins synthesis, replication and transcription, or folic acid synthesis.

1. Inhibition of cell wall synthesis of Gram (+) bacteria. Penicillins and cephalosporins that interfere with enzymes required for the synthesis of the peptidoglycan layer (Džidić et al, 2008).
2. Interaction with the Gram (+) bacteria membrane or the inner membrane of Gram (-) bacteria. Polymixin binds to lipid A of LPS of Gram (-) bacteria leading to the disruption of bacterial outer membrane and cell death (Džidić et al, 2008)
3. Inhibition of protein synthesis. Macrolides (erythromycin) bind to the 50s ribosomal unit, while aminoglycosides, such as kanamycin, bind to 30s ribosomal unit (Džidić et al, 2008)
4. Replication and transcription inhibition. This includes interaction with different enzymes in these processes such as topoisomerase II (DNA gyrase) in the case of quinolones during replication (Džidić et al, 2008).
5. Inhibition of folate synthesis. Inhibition of folate synthesis, a cofactor in biosynthesis of DNA and RNA building blocks, is a mode of action of sulfonamides and trimethoprim (Džidić et al, 2008).

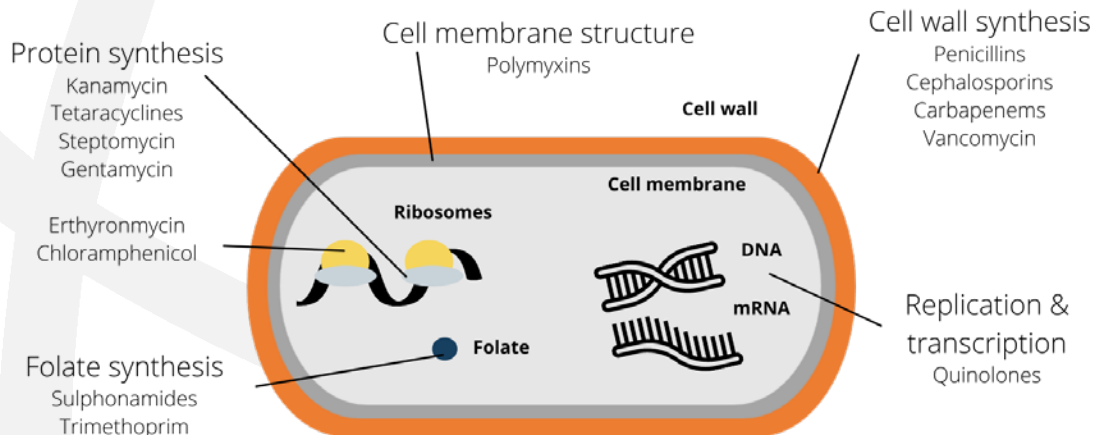

Figure 1. Modes of Action – Antibiotic target sites with examples of specific antibiotics (Based on Etebu & Arikekpar, 2016).

Mechanisms of resistance

Resistance to antibiotics generally occurs via one of 4 main mechanisms – Antibiotic inactivation, target modification, efflux of the antibiotic, and target bypass.

1. Antibiotic inactivation. Biochemically, these strategies include hydrolysis, group transfer, and redox mechanisms. Extended-spectrum β-lactamases (ESBLs) mediate resistance by hydrolysis, to all penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, ceftriaxone) and aztreonam. Transferases inactivate aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin (Džidić et al, 2008).
2. Target modification. Altering of the antibiotic target site results in antibiotic inability to bind properly to its target. Alterations among penicillin-binding proteins result in ampicillin resistance (Džidić et al, 2008).
3. Efflux of the antibiotic via efflux pumps and outer membrane permeability changes. This mechanism reduces the concentration of the antibiotics, such as, tetracyclines, macrolides, and fluoroquinolones that inhibit different aspects of protein and DNA biosynthesis, and must be inside of the cell to exert their effect (Džidić, et al, 2008).
4. Target bypass. Bypassing the inactivation of a given enzyme is a mechanism characteristic for dihydrofolate reductase and synthase involved in tetrahydrofolate biosynthesis, that are inhibited by trimethoprim and sulfonamides, respectively (Džidić, et al, 2008).

Application of antibiotics in protein production

Unlike other pharmaceuticals, proteins cannot be chemically synthesized due to their complex structure and function. Different host organisms are used to synthesize proteins that have a different origin from the host and this is why they are termed 'recombinant proteins' (Overton, 2014).

A general framework for recombinant protein production includes: cloning of the gene of interest into an expression vector, delivering the vector into the host of choice, selection of transgenic cell lines, large scale production, and purification of the protein (Rosano et al, 2014; Overton, 2014). Green fluorescent proteins (GFPs) are often used as reporter markers to screen the cells that carry the protein of interest and the fluorescence is measured as an indicator of the expression level. Similarly, antibiotics combined with their antibiotic resistance gene (ARG) are used for the efficient straightforward selection of cells that carry the gene of interest.

However, there is a difference in the selection efficiency based on which pair of antibiotic and ARG is used (Rosano et al, 2014) and each pair establishes a different threshold below, which no cell can survive. This further explains the variability of the selection process (Guo et al, 2021). Moreover, the transgene expression rapidly declines in the absence of antibiotic indicating the importance of antibiotic selection (Guo et al, 2021). In many systems, more than one antibiotic and ARG are used.

Bacterial systems

Escherichia coli remains the most popular expression system over the last 40 years, ever since its first use for human somatostatin (Itakuraetal,1977) and human insulin production (Goeddel et al,1979). *E. coli* stands for a well-established expression platform thanks to the abundance of molecular tools and protocols for high-level production, and numerous expression plasmids, engineered strains, and cultivation strategies (Rosano et al, 2014). Other physiological characteristics, such as fast growth kinetics, with a doubling time ~ 20 min (Sezonov et al, 2007) and easily achieved high-cell density qualify *E. coli* as a common choice in protein production. Moreover, the transformation process is fast and easy, and can be performed in as little as 5 min (Pope and Kent, 1996).

Recent efforts have been made to compare the suitability of different antibiotic resistance genes (ARGs) as reliable reporter markers with regards to the correlation between protein expression and resistance to the specific antibiotic. The expression of Nannobody and Affibody was demonstrated in *E. coli* K12 MG1655 with *dasher-GFP* gene as a gene of interest and six ARGs as selectable markers (Rosano et al, 2014). The tunable nature of ARGs can be achieved using different concentrations of rhamnose as an expression inducer and more interestingly, the use of antibiotics can be lowered by designing a weak coupling Shine–Dalgarno (SD) sequence.

Among several commonly used laboratory antibiotics (ampicillin/carbenicillin, chloramphenicol, spectinomycin, kanamycin, gentamicin or tetracycline), and their corresponding resistance genes (*bla*, *cat*, *aadA*, *aphA1*, *acc3*, and *tetC)*, the ampicillin resistance marker shows the largest dynamic range according to rhamnose/Minimal Inhibitory Concentration (MIC) correlation (Rosano et al, 2014). This qualifies β-lactamase for the most suitable resistance marker among the five tested selectable markers. The hypothesized reason for this is the mechanism of action of β-lactamase, which doesn't affect protein synthesis in contrast to other tested antibiotics. This implies that other ARGs that have no effects on the protein synthesis should be considered in relation to protein production efficiency, such as vancomycin and zeocin.

All markers except for gentamicin show a good correlation between rhamnose and fluorescence indicating that the expression can be tuned with rhamnose (Rosano et al, 2014). However, for chloramphenicol only concentrations up to 10 mM rhamnose show a correlation, suggesting limited tunable nature of the *cat* gene (Rosano et al, 2014).

The use of antibiotics can be lowered by enabling tunable translation of ARGs. This tunable effect can be achieved by sandwiching an alternative Translation Initiation Region (TIR) with a weak coupling SD sequence between the GFP and ARG (Rosano et al, 2014). In such way, the correlation between the fluorescence and MIC is preserved, while use of antibiotics is much lower.

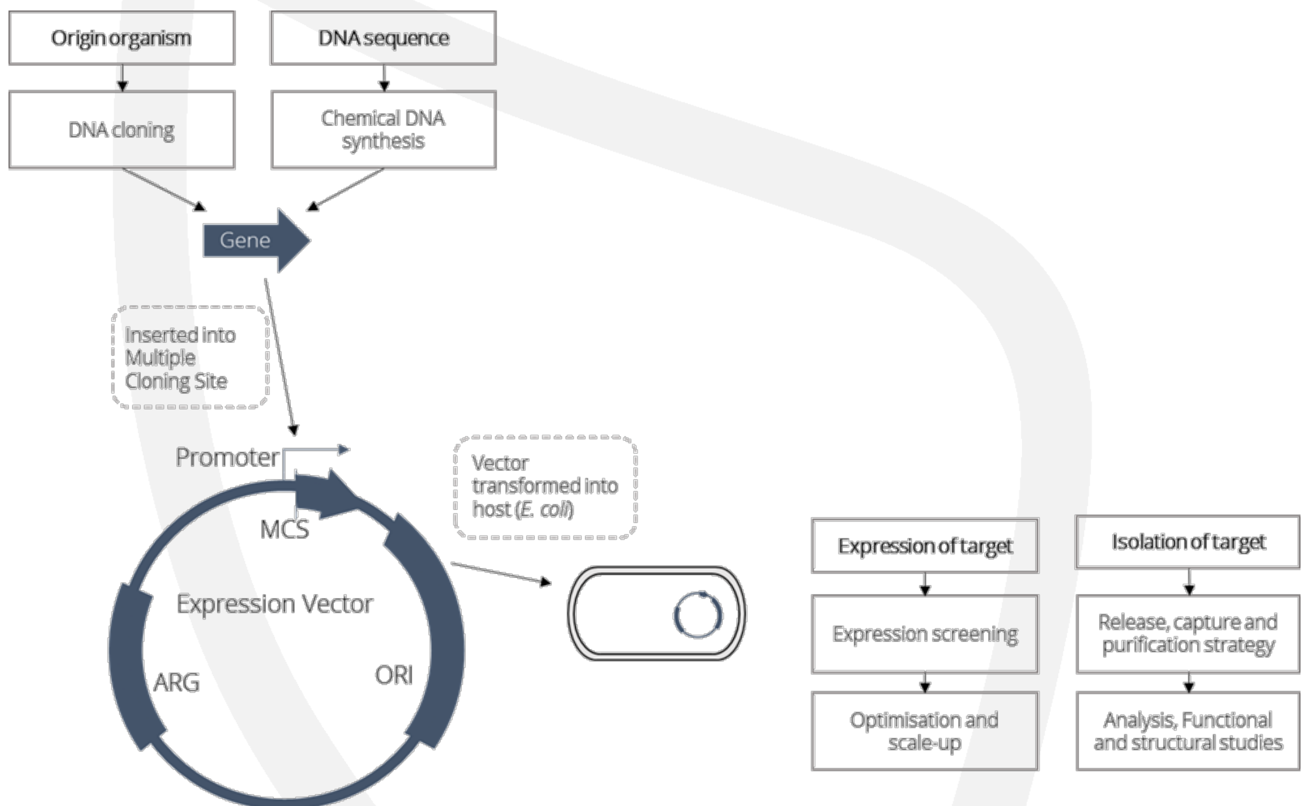

Figure 2. Recombinant protein production in bacteria. (Partially based on Overton, 2014.)

Fungal systems

Pichia pastoris is one of the most extensively applied yeast species in pharmaceutical and biotechnological industries, also known as a “biotech yeast”. *P. pastoris* has been reclassified into *Komagataella* spp. of which *K. pastoris* and *K. phaffii* represent synonyms (Valli et al, 2016). This common host for recombinant protein production is used for both academic and commercial purposes (Yang et al, 2014).

The limiting factor of the exploitation of this yeast is the low number of selectable markers. The best-known markers for expression in yeast are zeocin-, G418-, nourseothricin- and blasticidin for *K. phaffi* In addition to this, a *hph* gene for resistance to hygromycin B has also been used as a selectable marker (Yang et al, 2014). Both intra- and extracellular expression can be performed under hygromycin B resistance as demonstrated with GFP and Human Serum Albumin (HSA) protein.

The GFP expression under either constitutive or methanol-inducible promoter and the *hph* resistance gene as a selectable marker shows similar results to those previously obtained with zeocin (Yang et al, 2009). Hygromycin B is also suitable for post-transformational vector amplification (PTVA) that is commonly applied to increase plasmid copy number. This technique can be used for increasing the expression of already well-expressed proteins, as well as for poorly expressed proteins. Increasingly higher concentrations of hygromycin B (200, 700, 1500 and 4000 mg/l) can be used to perform PTVA (Yang et al, 2009). In this way, selecting the colonies with the highest level of protein of interest for each next round of PTVA results in increased protein yields after each round (Yang et al, 2009). The expression levels under hygromycin B are comparable to those obtained for vectors containing zeocin and G418 resistance (Sunga et al, 2008).

Mammalian cells

Antibiotics as selectable markers are also used in mammalian cells for recombinant protein production. Five different selectable markers *NeoR*, *BsdR*, *HygR*, *PuroR*, and *BleoR* genes, which confer resistance to the selective antibiotics G418/geneticin, blasticidin, hygromycin B, puromycin, and zeocin respectively, were compared with regards to their effects on the level of expression of the protein of interest and cell-to-cell variation of the expression levels (Guo et al, 2021).

The choice of selectable marker is important as it can have up to a 10-fold effect on the expression level (Guo et al, 2021). Among all tested antibiotics, use of zeocin results in the highest and most homogeneous expression of all; geneticin and blasticidin show the lowest average relative expression and high degrees of cell-to-cell variation, while use of hygromycin B and puromycin result in intermediate expression levels and variation (Guo et al, 2021).

The correlation between high cell-to-cell variation with the low transgene expression indicates that each selectable marker–antibiotic pair establishes a threshold of transgene expression below which no cell can survive (Guo et al, 2021). This can be explained by the specific resistance mechanism of each resistance gene. Namely, highly efficient and long-lived selectable marker proteins inactivate their antibiotic at very low levels of expression resulting in the survival of the cells expressing even the lowest level of recombinant protein, which further leads to high cell-to-cell variation (Guo et al, 2021).

NeoR and *BsdR* selectable marker enzymes may be highly active, stable, or both, thus resulting in low expression of transgene and high variation, while *BleoR* marker yields cell lines with the highest and least heterogeneous levels of transgene expression indicating that it has the lowest activity of all

selectable markers. This implies that zeocin might be the most suitable for high-yield production (Guo et al, 2021). Conversely, for difficult-to-express proteins, puromycin or hygromycin B are recommended as they allow survival at lower levels of transgene expression (Guo et al, 2021).

Other antimicrobials

In some application fields of biotechnology such as gene therapy and production of therapeutic recombinant proteins, the presence of ARGs and antibiotics is not acceptable (Vandermeulen et al, 2011). This inspired the development of antibiotic marker-free selection approaches. However, such systems are not adopted commonly due to requirements of specialized strains and/or reagents.

Triclosan is a Food and Drug Administration (FDA)-approved, non-antibiotic biocide agent that affects a broad spectrum of microorganisms, including bacteria, fungi, viruses, and protozoa ("What is the biocide triclosan?", 2021). Triclosan inhibits bacterial enoyl-acyl carrier protein reductase enzyme (ENR) encoded by *fabI* gene (Heath et al, 1999). When overexpressed, ENR results in triclosan reistance in *E. coli* (Heath et al, 1999). *FabV*, a functional homologue of *fabI* can be used efficiently to express heterologous recombinant proteins in *E. coli* and shows similar or better results than expression vectors containing β-lactamase (Ali et al, 2015). Some of the advantages of triclosan as a selection marker are: enhanced plasmid stability, applicability in diverse culture media, and compatibility with other selection systems (Ali & Chew, 2015). Namely, *FabV*-Triclosan system is compatible with use of other antibiotics, such as, chloramphenicol, kanamycin, or tetracycline that are required for certain bacterial strains either for verification purposes or to maintain additional extra-chromosomal genetic elements (Ali et al, 2015).

Name	Product Code	Cas No.	Uses
Ampicillin	BA166034	69-53-4	Ampicillin is one of the most commonly used selective agents for screening transformed bacterial cells that carry *bla*-encoded resistance and the gene of interest.
Carbenicillin	AC09915	4800-94-6	Carbenicilin is an analogue of ampicillin. Due to its greater stability compared to ampicillin, in certain cases it might be more suitable for the selection of transgene cells.
Chloramphenicol	AC09460	56-75-7	Chloramphenicol resistance gene *cat* is a well-known and commonly used selectable marker. It can also be used for common bacterial hosts exploited in protein production and food protein fermentation, such as, *B. subtilis* and *L. lactis*.
Spectinomycin	AD29664	22193-75-5	Spectinomyin is used to select transformants carrying aadA resistance gene. It is most often used for plant host cells and bacterial hosts that are used in protein production, such as, *E. coli* and *B. subtilis*.
Kanamycin	AK31431	8063-07-8	Kanamycin is a selection agent used to screen bacteria, such as, *E. coli* and yeast species, such as, *P. pastoris*, both of which represent common hosts for protein production.
Gentamicin	AG29671	1405-41-0	Gentamicin and its corresponding resistance genes are frequently applied for the selection of transgene cells, including bacterial hosts, such as, *E. coli*, but also for recombinant protein production in plants.

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Name	Product Code	Cas No.	Uses
Tetracycline	BT166207	60-54-8	Tetracycline and its resistance genes are regularly used for the selection of bacterial cells, such as, *E. coli*. Tetracycline resistance genes also find application as selectable markers in the recombinant protein production in algae, such as, *Chlamydomonas reinhardtii* and other related microalgae, as well as other tetracycline-susceptible aerobic microorganisms.
Zeocin/phleomycin	AP106412	11006-33-0	Zeocin resistance gene *Sh ble* is a common choice for eukaryotic cells including different yeast species of which *P. pastoris* and *S. cerevisiae* are most often applied for recombinant protein production. Zeocin is also marked as the most suitable for human cells, such as, HT1080 and HEK293 compared to hygromycin B, neomycin, puromycin.
G418/geneticin	G-2400	108321-42-2	G418 resistance genes are selection markers used in the selection process of all cell types including yeasts, such as, *P. pastoris* and *S. cerevisiae*, other eukaryotic cell lines, such as, CHO or HEK293, but also plant cells and bacterial cells.
Nourseothricin	NN11350	96736-11-7	Nourseothricin resistance gene (*nat*) is used as a selection marker for a wide variety of cell types including yeasts, bacteria, plants and mammalian cells.
Blasticidin	FB33855	3513-03-9	Blasticidin resistance genes, bsd and bsr are mostly used for eukaryotic cells such as yeasts like *P. pastoris*, mammalian HEK293 or HeLa cells or algae such as *Chlamydomonas reinhardtii*; but it is also suitable for bacterial cells.
Hygromycin B	AH11348	31282-04-9	Hygromycin B is efficient against bacteria, fungi and other eukaryotic cells, but is more often used for the selection of eukaryotic cells including yeasts, such as, *P. pastoris* and *S. cerevisiae* and mammalian cell lines like CHO.
Puromycin	NP09203	58-58-2	Puromycin resistance gene (pac) is a common choice for the selection of mammalian cells since it first use in 1988, especially for the production of therapeutic proteins in mammalian cells where it can be used for high-level production of protein of interest.
Rhamnose	DAA61541	3615-41-6	Rhamnose is often used as an inducer of protein expression with its corresponding rhaBAD promoter of *E. coli*.
Methanol	FM165905	1455-13-6	Methanol is an inducer of protein expression and AOX1, a methanol inducible promoter, is one of the most commonly used promoters.

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