environments (von Wintersdorff et al. 2016). Resistome is a pool of the existing antimicrobial resistance genes, including the regularly expressed genes, silent genes, and gene precursors, which exhibit different expression levels, and resistance genes from nonpathogenic strains (Nesme and Simonet 2015). The microbiological studies are focused on antimicrobial resistance phenotypes that are already present in the environment. Discussing this problem, we want to demonstrate that studies on a huge pool of genes can reveal the origin of antibiotic resistance genes that appear and spread within bacteria.

Concept of silent genes

Silent genes, also called cryptic ones, are DNA sequences that are not normally expressed or expressed at a very low level. It is natural that not every bacterial gene is expressed at once, but silent genes are silent even when they should be expressed. For example, antibiotic resistance genes should be expressed in the presence of an antibiotic, and lack of their expression leads to a lack of protection. Also, the genes encoding antibiotics should be expressed when other concurrent or enemy bacterial species are in the vicinity. It leads to the conclusion that silent genes are unneeded residues and do not play an essential role in the life cycle of bacteria.

A fact that makes silent genes intriguing is that they may become active after mutation (e.g., insertion) or recombination. Like every normal gene, they can also spread through HGT (Hall et al. 1983). It was proved that silent genes could become active after being transferred to a new host; for example, the silent *aadA* gene found in Shiga toxin-producing *Escherichia coli* (STEC) was expressed fully only after its transfer to *Hafnia alvei* (Zhao et al. 2001). Several data on the prevalence of antimicrobial silent genes can be found in the literature. Some publications report the relevant percent of susceptible strains carrying resistance genes; for example, 28.49% of *E. coli* strains susceptible to streptomycin were found to carry the *aadA* gene (Lanz et al. 2003), 40% of *Salmonella* spp. strains susceptible to chloramphenicol carried the *catA1* gene (Deekshit et al. 2012), and 25% of *Klebsiella pneumoniae* strains susceptible to carbapenems carried the IMP-type genes (Walsh 2005). Cantón (2009) even claimed that most of the antibiotic resistance determinants are cryptic in the natural environment. Combining all this information gives a picture that silent genes are a common phenomenon and may significantly impact bacteria's adaptive potential and evolution.

Some researchers have undermined the existing silent genes and claimed that many are silent only in laboratory conditions but are normally expressed in the natural environment. Tamburini and Mastromei (2000) proposed that silent genes should not be treated as genes with a unique regulation pattern but rather as those encoding the unusual function. Laboratory conditions are only an approximation of the natural environment that can influence bacterial phenotype. However, using the argument of "laboratory conditions" to explain the silent genes phenomenon may be too simplistic.

In contrast to Tamburini and Mastromei, some other publications supported the existence of silent genes. Lanz et al. (2003) claimed that silent genes could be a source of new resistance phenotypes. Their study suggested that silent forms of genes are not so rare, and therefore, in future studies on antimicrobial resistance, not only phenotypic resistance strains but also susceptible ones should be taken into account. Enne et al. (2008) postulated not to ignore the potential of the reservoir of silent genes because they can spread among bacteria belonging to different genera and can become active. There is a possibility that the studies not taking the silent genes into account could underestimate the antimicrobial resistance potential of the bacterial population.

In 2016, Fernandes et al. reported the results of their study on colistin-resistant *Enterobacteriaceae* in Brazil. They retrospectively tracked the plasmid-mediated colistin resistance gene (*mcr-1*) from China through Europe to Brazil. The authors concluded that the identifying *E. coli* strain carrying the *mcr-1* gene and susceptible to colistin might be the evidence of insufficient testing of strains that are only phenotypically resistant (Fernandes et al. 2016). Picão et al. (2012) claimed that silent antimicrobial resistance genes could be a real threat, and strains harboring these genes, for example, metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* susceptible to meropenem, can carry the risk of therapeutic mistakes and failure.

Several mechanisms associated with the lack of gene expression have been identified. In general, genes are silent because of three main reasons: (1) mutations, (2) adverse side effects of normal gene regulation, acquisition and manage systems, (3) simplified laboratory conditions and limitations or mistake in bacteria handling.

(1) Mutations. Even a single-nucleotide mutation can turn a fully expressing gene into nonfunctional. This loss of a function can be compensated or rarely reversed (Andersson 2003).

(2) Adverse side effects of normal gene regulation, acquisition, and management systems.

    (a) Gene expression regulator errors. Positive and negative regulators modulate gene expression. Sometimes, genes remain silent due to a strong negative transcriptional regulator or a defective promoter or regulatory gene (Sánchez and Demain 2015).

visible on the *aadA* gene, encoding the streptomycin-modifying enzyme. The strains of *E. coli* are considered susceptible when the MIC value is more than 16 mg/l for streptomycin. Some *E. coli* strains with the MIC value of 16 mg/l or even far below this breakpoint (8 or 4 mg/l) can carry the *aadA* gene. Expression of the *aadA* gene can be at a low level, or the gene can be completely silenced. Strains harboring the *aadA* gene can produce an enzyme at a low level, spread, become more active in the new host, and enhance resistance if they carry *strA-strB* genes (Sunde and Norström 2005).

The spreading of silent resistance genes could be a potential threat, especially in the transmission of carbapenemases in a hospital environment. Carvalho et al. (2011) reported about 31 clinical *Acinetobacter baumannii* strains susceptible to imipenem, of which 16.13% carried the $bla_{\text{OXA-23}}$ silent gene (Table I). This result provides evidence that hospitals can be the reservoirs of silent genes undetected in routine laboratory work and spread imperceptibly. Carvalho et al. (2011) claimed that molecular methods might be required to identify resistant strains and control and monitor multidrug resistance molecular methods in the future.

Many different mechanisms are responsible for antimicrobial resistance. The presence of some resistance-related genes in the genome could not be enough to confer a high level of resistance. For instance, *P. aeruginosa* strains carrying MBL-encoding genes can still be carbapenem susceptible. Picão et al. (2012) reported that MBL producers without phenotypic carbapenem resistance could be a reservoir of silent spreading genes and a potential risk for therapeutic failure. There is evidence that silent genes can be transferred and activated; for example, silent *aadA* was fully expressed after being transferred from *E. coli* to *H. alvei* (Zhao et al. 2001).

Though uncontrolled hospital-associated spreading of antibiotic resistance genes is a big threat to public health, it might not be the most significant danger. Only a relatively small group of people exposed to antibiotics and resistance bacteria stay in hospitals, while on the outside, everyone contacts with microorganisms through food and the environment every day. The use of antibiotics in agriculture puts pressure on bacteria leading them to develop resistance. These microorganisms and antibiotic compounds are introduced into the soil with manure or plants and in the urban environment with aerosols. Not only pathogens but also the residues of antibiotics and resistant bacteria may be present in food. Moreover, while performing our routine activities, we are exposed to the determinants of antibiotic resistance. Complex surveillance and monitoring programs should be based on molecular methods, but a more appropriate strategy is to track genes and not bacteria (Smith et al. 2005; Heuer et al. 2011).

Data obtained from WGS and genome mining give strong evidence that many bacterial biosynthesis pathways are silent in laboratory conditions. Silent gene clusters were also identified in antibiotic-producing soil microbes. These observations may lead to the conclusion that many undiscovered antibiotics are present in the environment (Fields et al. 2017). Bacterial antibiotic producers are resistant to their metabolites, which allows assuming that if there are many silent antibiotic producers present in the environment, several silent antibiotic resistance determinants also exist (Cantón 2009; Davies and Davies 2010).

It was found that silent genes can be activated in laboratory conditions using different tactics that can be combined into three groups: pleiotropic, targeted genome-wide, and biosynthetic gene cluster (BGC) approaches. Pleiotropic ones include ribosome engineering, chromatin remodeling, global regulation of genes, use of phosphopantetheine transferases (PPTases), and "one strain many compounds" approach (OSMAC). Reporter-guided mutant selection (RGMS) and the use of elicitors are targeted genome-wide methods. BGC approaches include refactoring, heterologous expression, cluster-situated regulators, and promoter exchange (Baral et al. 2018).

Koskiniemi et al. (2011) described clinical *Salmonella enterica* strain as having a silent the *aadA* gene encoding aminoglycoside adenyl transferase. The strain showed increased resistance to two aminoglycosides, streptomycin, and spectinomycin, following a mutation leading to changes in phenotype called small colony variants or after it was cultured in glucose/glycerol minimal media. Starvation for amino acids or carbon forced the bacterial cells to produce alarmone (p)ppGpp – guanosine penta/tetraphosphate. The authors proved that ppGpp acted as a positive regulator of *aadA* expression. They found that if the environmental conditions favored the increase of ppGpp, the expression of the silent gene *aadA* also increased (Koskiniemi et al. 2011). Alarmone ppGpp is produced on ribosomes, and its binding to RNA polymerase (RNAP) activates the production of antibiotics. Reports have shown that mutations of RNAP, which "pretend" ppGpp binding, can also activate the silent genes. Based on this finding, Ochi and Hosaka (2013) developed a method named "ribosome engineering" that can activate silent genes or enhance their expression and target different ribosomal proteins (e.g., RNAP or S12).

Kime at al. (2019) showed that transient silencing of antibiotic resistance by mutation (SARM) represents a significant potential source of unexpected therapeutic failure. Among 1,470 isolates of *Staphylococcus aureus* which antibiotic resistance genotype (after whole genome sequencing) was compared with phenotypic susceptibility testing, 152 isolates (10.3%)

had silenced resistance genes, including 46 (3.1%) who showed SARMs against the antibiotics used. Silencing of antibiotic resistance by mutation was associated with various mutations (point mutations: insertions, deletions, or substitutions). The most common type of mutation identified was nucleotide deletion, which in all instances involved the loss of a single nucleotide from a poly(A), resulting in a subsequent disruption of the coding sequence (Kime et al. 2019).

Supercoil nucleoid structures, which are identical to nucleoid-associated proteins or various RNAs, can restrict access to DNA and modulate the expression of bacterial genes (Baral et al. 2018). HN-S proteins organize these structures by attaching to their promoter region and causing loop formation, which traps RNAP and represses the gene expression (Dorman and Deighan 2003). As their name implies, HN-S proteins play an analogical role like histone proteins in eukaryotic cells, but they are different in structure and at the sequence level. Histone functions are controlled by histone deacetylases (HDACs). These enzymes are members of a large, ancient enzyme family and are present in animals, plants, fungi, and bacteria. Advanced researches indicated that HDACs evolved independently from histone or HN-S proteins (Gregoretti et al. 2004), suggesting that HN-S can be modified, just like histones; however, there is no evidence that bacterial HDACs target HN-S proteins (Hamon and Cossart 2008).

The basic of the OSMAC approach involves modulation of the cultivation conditions by changing the temperature, supplementation of the medium, or co-culturing with other microorganisms, which can enhance gene expression or activate the silent gene clusters (Baral et al. 2018). It was shown that co-culturing two strains of different species or genera might induce the expression of silent genes; for example, *Streptomyces lividans* produce a red pigment in the presence of *Tsukamurella pulmonis*. Cell-to-cell interactions are particular, and sometimes these are not well understood, and it is difficult to extrapolate this information to other bacterial species (Ochi and Hosaka 2013).

According to the current state of knowledge, antibiotics or growth inhibitors can regulate the production of secondary metabolites. These molecules can be synthesized and secreted in co-culture by one microorganism and stimulate the expression of silent gene clusters in the other microorganism (Okada and Seyedsayamdost 2017). The rare earth elements (REEs) can play an essential role in the activation of silent genes. Metals from this group are defined as lanthanides plus yttrium and scandium. It was shown that scandium and lanthanum forced *Streptomyces coelicolor* and *S. lividans* to produce the antibiotic actinorhodin when added to the culture medium. In addition, REEs such as cerium, neodymium, samarium, or europium enhanced the production of this antibiotic compared to the other metals, including manganese, iron, nickel, copper, and zinc, which showed no such effect (Tanaka et al. 2010; Ochi and Hosaka 2013; Nassar et al. 2015). OSMAC approach is focused on identifying the cultivation conditions that could change the phenotype, but this effect can also be achieved at the molecular level by the overexpression or inactivation of the target genes (Baral et al. 2018).

Primary metabolism pathways are controlled by the enzyme PPTase. This protein catalyzes the posttranslational modifications that activate the fatty acid carrier protein domain or peptidyl carrier protein domain in *Bacillus subtilis* (Timmusk et al. 2015). In addition, overexpression of this enzyme also has an impact on secondary metabolism (Baral et al. 2018).

Silent genes can also be activated by adding N-acetylglucosamine (GlcNAc) to the culture medium. The transcription of the genes responsible for the transport and catabolism of GlcNAc is repressed by YvoA (NagR) in *B. subtilis* and DasR in *Streptomyces*. DasR also plays an important role in the expression of resistance genes. The pleiotropic repressor DasR reacts on GlcNAc concentration, but at the same time regulates gene expression through different pathways (Bertram et al. 2011; Ochi and Hosaka 2013).

Resistance can also be silenced by expressing an intact resistance to antibiotic gene systems (Enne et al. 2008). Moreover, the process of gene silencing is reversible. According to the results presented by Enne et al. (2008), the mechanism by which it happens is unknown. Recovery of the genes has been observed in a small part of the bacterial population analyzed. In the conducted experiment, potential silencing of the resistance genes *bla*$_{\text{OXA-2}}$, *aadA1*, *sul1*, and *tetA* carried on the plasmid pVE46 in *E. coli* isolated from pigs was investigated following oral inoculation of the strain into piglets. Despite the occurrence of the pVE46, an inconsiderable proportion of strain recovered from piglet feces did not express one or more resistance genes. In most cases, the resistance genes and their promoters, even though not expressed, were intact compared to fully wild-type sequences.

RGMS is a method developed by Yang et al. (2004), combining cloning in front of the double reporter system and genome-wide mutagenesis. This method randomly activates secondary metabolism pathways, switching on the signal that enables the selection of strains with the desired phenotype (Baral et al. 2018). Another genome-wide approach is to use small molecules that can activate silent genes – elicitors. This method is based on the release of signals from green fluorescent protein or *lacZ* reporter gene fusion, which occurs after the addition of elicitors to the culture medium incubation with elicitors. Elicitors are commercially available in the form of screening libraries

(Seyedsayamdost 2014). After identifying that etoposide and ivermectin are inducers, 14 novel products were isolated and characterized from the silent gene cluster of *Streptomyces albus* One of these new proteins exhibited an antifungal activity, while some others inhibited the cysteine protease implicated in cancer (Xu et al. 2017).

It is well known that gene expression is controlled by many factors, including activators and repressors. Overexpression of a transcriptional activator replaces a native nonfunctional promoter, while deletion or damage of a transcriptional repressor can stimulate bacteria to produce secondary metabolites (Zhang et al. 2019). In addition, the incorporation of insertion sequences, which are short pieces of DNA that encode their transcription, could alter the expression of other genes (Courvalin 2008).

Contemporary genetic tools offer the opportunity to "rewrite" a sequence of silent bacterial gene clusters. For this, noncoding fragments are removed to eliminate internal regulation, and then DNA with a sequence that is possibly far from wild type is chosen. The recoded genes are combined in a new, artificial operon under the control of synthetic ribosome-binding sites (Temme et al. 2012). Refactoring includes heterologous expression in a surrogate host; for example, a refactored gene cluster encoding taromycin, from *Saccharomonospora* sp., was successfully expressed in an *S. coelicolor* host strain (Yamanaka et al. 2014). This approach is beneficial to human medicine as it provides new antibiotics and bacterial metabolites such as cyclic sesterterpenes and atolypene A and B, which are moderately cytotoxic to human cancer cell lines (Kim et al. 2019).

To investigate the gene propagation pathways, one must put resistome and genotype on the first plan instead of phenotype. Due to HGT, genetic determinants do not belong to only one genus. A continuous flow of genes lets some pool of DNA spread freely, breaking the boundaries of species and forcing us to look at the spreading of antibiotic resistance differently.

Threats and hopes

When considering the role of silent genes, it is advisable to look at two aspects: their significance in the environment and bacterial evolution and their effect on human medicine, industry, and agriculture.

Two concepts are proposed for the development of antibiotic resistance and the spreading of antibiotic resistance genes. The first one is based on antibiotic pressure, which can result in mutations activating a dormant (or silent) resistance gene so that it can express a resistance phenotype in the emergence of new genes by mutations. The second concept is based on the transfer of antibiotic resistance determinants from natural antibiotic producers to pathogenic bacteria (Andremont 2001; Martins et al. 2013). Both these phenomena have been reported by many studies (Barlow and Hall 2002; Andersson 2003; Maisnier-Patin and Andersson 2004; Toprak et al. 2012; Safi et al. 2013; Perron et al. 2015; Holmes et al. 2016), and it is hard to imagine an evolution engine without mutations or HGT. Silent antimicrobial genes can be activated under antibiotic pressure, and it has been demonstrated that environmental conditions have an influence on gene selection and expression, and on the other hand, that microorganisms already have great genetic potential and prepared "answers" for the changing conditions (Rowe-Magnus et al. 2002). Silent genes could also be considered as residues of recent antibiotic exposure, but the available data do not support this thesis (Wright 2007).

The existence of silent antimicrobial genes might have a significant influence on the survival and evolution of bacteria. Considering the role of silent genes, we should remember that silent antimicrobial genes are not always really silent. Regardless of how advanced and proven they may be, laboratory analyses are only an approximation and simplification of the real conditions. Bacterial strains are tested one by one, without the environmental context, which undeniably influences the gene expression and microbial phenotype.

The discovery of silent genes or genes not expressed in standard laboratory conditions opens a world full of new possibilities. Current tools and advances in molecular biology give us the hope of finding new antimicrobials expressed naturally by microorganisms from silent gene clusters. The existence of "sleeping" antimicrobial genes in bacterial genomes indicates the determinants of "sleeping" antimicrobial resistance. However, the evidence available for this phenomenon is not clear (Martins et al. 2013).

Apart from the natural occurrence of silent antimicrobial resistance genes, it is suggested that methods can be developed for targeting silencing genes. Despite many concerns, these methods may allow leading or controlling the evolution of antimicrobial resistance (Wright 2007). For instance, AcrAB-TolC efflux pump is involved in the resistance of bacteria to numerous substances such as macrolides and some lactams. Silencing *acrA*, a gene encoding one of the proteins that build this pump was shown to reduce the MIC of more than 10 antibiotics up to 40-fold (Ayhan et al. 2016). In addition to the possibility of silencing genes, Salipante et al. (2003) developed a tool called GeneHunter, to seek and activate the non-expressed genes. An interesting fact about this tool is that it is a transposon that can find genes without any known homologs.

Bacterial silent gene clusters are mostly sought and studied because it is believed that they may encode secondary metabolites important for human medicine, such as antibiotics, and for industrial applications (Rigali et al. 2018). Thus, their potential seems to be enormous. It has been estimated that more than 90% of the microbial metabolites are not expressed under standard laboratory conditions (Yan et al. 2018).

Conclusions

If estimates are valid, a significant part of the bacterial genome is silent in laboratory conditions. It means that there is a vast world of secondary metabolism proteins that may have a significant influence on the adaptation and evolution of bacteria and play a significant role in human and veterinary medicine and agriculture. Exploration of silent genes, as a part of resistome, may help find new biochemical pathways and establish dependencies that are not visible in standard laboratory conditions. It can also be seen as a chance to find new antimicrobials and protect bacteria from acquiring antimicrobial resistance.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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