VchM - a DNA cytosine methyltransferase modulates the response of Vibrio cholerae to proteotoxic stress

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VchM - a DNA cytosine methyltransferase modulates the response of *Vibrio cholerae* to proteotoxic stress

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ABSTRACT

Antibiotic resistance has become a major global crisis. Understanding the molecular mechanisms underlying microbial adaptation to antibiotics is thus of keen importance to fight Antimicrobial Resistance (AMR). Aminoglycosides are a class of antibiotics that constitute an important source of proteotoxic stress by targeting the bacterial ribosome and causing mistranslation of proteins. Here we investigated the role of VchM, a DNA methyltransferase, in modulating the response of the human pathogen *Vibrio cholerae* to aminoglycosides. We show that deletion of *vchM* gene confers *V. cholerae* cells a form of tolerance to both sub-inhibitory and lethal concentrations of aminoglycosides, as well as to increased temperatures. Through transcriptomic and genetic approaches, we show that cells lacking *vchM* have an elevated expression of *groESL*-2 genes, which is essential for the tolerance of *vchM* mutant to lethal concentrations of aminoglycosides but not to the form of tolerance to low doses of these antibiotics and high temperatures. For the last two, the ribosome promoting hibernation factor HPF seems to play an important role, although the mechanism remains to be elucidated. We thus suggest that modulation of VchM-mediated DNA cytosine methylation affects mechanisms linked to both translation and protein quality control, resulting in a higher tolerance to proteotoxic stress.

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TABLE OF CONTENTS

INTRODUCTION **9**

I. Stress in bacteria **9**

1.1. The general stress response 9

1.2. Protein stress and Proteostasis 10

1.2.1. Proteostasis during *de novo* peptide synthesis 11

1.2.1.1. The ribosome-bound Trigger Factor (TF) 12

1.2.1.2. The DnaK/DnaJ/GrpE chaperone machine 13

1.2.1.3. The GroES – GroEL chaperonin system 14

1.2.2. Proteostasis during specific stresses 15

1.2.2.1 Increased temperature 16

1.2.2.2 Oxidative stress 16

1.2.2.3 Mistranslation 16

1.2.2.3.1 Mistranslation as a natural process 17

1.2.2.3.2 Mistranslation caused by aminoglycosides 18

1.2.3 Heat-shock response 20

1.2.3.1 Proteases and small heat shock proteins 20

1.2.3.2 Regulation of heat-shock response 21

1.2.4. The σE stress response 23

II. Antibiotics and Stress **24**

2.1 SubMIC concentrations of antibiotics as a source of stress 26

2.1.1 Effects of subMIC concentrations of antibiotics on bacteria 27

2.1.1.1 Enrichment of pre-existing mutants and selection of de novo mutations 27

2.1.1.2 Antibiotics at sub-inhibitory concentrations accelerate the rate of Horizontal Gene Transfer and mutagenesis through the SOS response 28

2.1.1.3 Antibiotics at sub-inhibitory concentrations induce a plethora of different transcriptional responses 29

2.1.1.4 Antibiotics at sub-inhibitory concentrations and antibiotic tolerance 30

2.1.1.5 Antibiotics at sub-inhibitory concentrations – final considerations 31

III. DNA methylation in bacteria **33**

3.1 DNA Orphan methyltransferases in γ-proteobacteria 34

3.1.1 Orphan DNA adenine methyltransferase Dam 34

3.1.1.2 The role of Dam in chromosomal replication 35

3.1.1.3 The role of Dam in methyl-directed mismatch repair (MMR) system 35
3.1.1.4 The role of Dam in transposition 36
3.1.1.5 The role of Dam in bistability and phase variation 36
3.1.1.6 The role of Dam in bacterial virulence 38
3.1.2 Orphan DNA cytosine methyltransferase Dcm 39
3.1.3 Orphan DNA methyltransferases in *Vibrio cholerae* 40
3.1.3.1 VchM – a DNA Orphan m5C methyltransferase 40
3.1.3.2 Vca0447 – a putative orphan DNA methyltransferase 42
3.2 DNA methylation, stress and antibiotic resistance 42
IV. Thesis project 44
RESULTS 46
Part I - Research article in preparation: "VchM, a DNA cytosine methyltransferase, modulates tolerance to aminoglycosides and heat stress in *Vibrio cholerae"* **..... 46**
Part II – "How lack of VchM favors *V. cholerae* under subMIC doses of aminoglycosides – a link between DNA methylation and translation?" **..... 69**
Part III - "vca0199-vca0200-vca0201 – new players in VchM – mediated phenotypes" **..... 75**
Part IV – "Vca0447 – A putative DNA methyltransferase induced by aminoglycosides" **..... 83**
DISCUSSION 91
CONCLUSION AND PERSPECTIVES 96
MATERIALS AND METHODS 97
SUPPLEMENTARY DATA 106
REFERENCES 113

INTRODUCTION

I. Stress in bacteria

Adaptation is a key process in Evolution. Prokaryotes are fascinating single cell organisms with a remarkable ability to rapidly adapt to the most unfavorable scenarios. This allows them to thrive in a myriad of different habitats, even under the most extreme environmental conditions. Besides that, prokaryotes have to constantly cope with deviation from optimal conditions and innumerous sources of stress. Abiotic stress comprises changes in temperature, pH, osmotic pressure, oxidative stress, high levels of radiation, nutrient limitation, among others. Also, ecological competition among microorganisms, often resulting in the production of antibiotics and other chemical molecules that kill or prevent the growth of competitors, is also considered an important source of stress (1). In order to deal with such stresses, bacteria have evolved several mechanisms that allow them to sense, respond and suppress said stresses.

1.1. The general stress response

Bacteria can deploy a plethora of specific mechanisms in response to specific stresses. However, bacteria can also mount a more robust, general stress response that not only helps with the activating stress but also confers cross-protection to other stresses like oxidative stress or low pH (2). In gram-negative bacteria this general stress response is mediated by the specialized sigma factor RpoS (σS) (3).

Sigma factors are subunits of the RNA polymerase responsible for promoter recognition and initiation of transcription. RpoD is the sigma factor that associates with the RNA polymerase for expression of all housekeeping genes and it is present throughout all phases of bacterial growth. However, in stressed cells, the expression of alternative sigma factors may compete with RpoD, enhancing the binding of RNA polymerase to the promoter of genes important for management of the inducing stress (4). In non-stressed exponential phase growing cells, RpoS is maintained at low concentrations thanks to a complex regulatory network that modulates its levels at the

transcriptional, translational and at protein stability levels. However, upon entry in stationary phase, multiple stress signals lead to de-repression of RpoS, whose levels drastically increase in the cell. As a result, through the direct or indirect activation of several regulatory cascades, RpoS controls the expression of hundreds of genes conferring cells a general stress-resistance phenotype (reviewed in (5)). For example, the expression of Dps, a DNA-binding protein shown to be involved in protection from oxidative stress, is positively controlled by RpoS when cells enter stationary-phase. However, during exponential phase under oxidative stress, the response regulator OxyR is also capable of activating *dps* expression, in a RpoS-independent manner (6, 7). This shows that many of the specific stress responses in bacteria are also activated by the general stress response ensuring that cells entering stationary phase are pre-primed to deal with stresses that may arrive as consequence of nutrient starvation (oxidative stress, in this example).

However, specific stresses require an appropriate and more specific stress response, which is characterized by the expression of genes whose products will antagonize the effect of such stress. For example, growth of bacteria under high temperatures triggers the activation of the heat-shock regulon, leading to the expression of molecular chaperones that help the proper folding of proteins damaged by the high temperature (see below) (8). In this case, heat-shock response is activated by protein stress, one of the most common stresses found by bacteria during their growth.

1.2. Protein stress and Proteostasis

Proteins are biological macromolecules of unconditional importance in all domains of life. Their versatility and complexity are key to every biological process. To properly exert their function, proteins need to be correctly decoded from messenger RNA (mRNA) and acquire a three-dimensional structure. In addition, some proteins need to be trafficked to a specific location in the cell where they interact with other molecules and acquire a new conformational state. Sometimes they also need to be cleaved at specific domains or chemically modified at a specific residue. Later, at the end of their lifespan, they need to be degraded by specific quality control machineries. The state of equilibrium among all of these processes in the cell is called Proteostasis (9).

The natural process of protein synthesis may encompass several challenges to the folding of newly synthesized proteins and compromise proteostasis, even under normal cellular conditions. In addition, external sources of stress also affect proteostasis, indirectly or directly, either by targeting

translation or by interfering with the mechanical and physical properties of certain proteins. If not taken care of, these insults may result in protein aggregation and subsequent degradation with serious consequences for the cell (10).

In order to maintain proteostasis, cells encode multiple molecular chaperones and proteases that form molecular machineries capable of aiding correct protein synthesis, folding, trafficking and degradation. These proteins have been largely studied in both Eukaryotes and Prokaryotes (9, 11, 12). In mammalians, disruption of proteostasis is at the basis of cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's (13). In prokaryotes, some chaperones have been shown to be strictly essential proteins playing roles in important biological processes such as DNA replication as others are important only at specific physiological conditions. Here I will describe how proteostasis is generally maintained in bacteria, the major chaperones involved in the process and which stresses can insult it.

1.2.1. Proteostasis during *de novo* peptide synthesis

Ribosomes are ribonucleoprotein complexes that bind to and decode mRNAs. In bacteria, the translationally active 70S ribosome is composed of two functionally distinct subunits: the 30S small subunit, which is responsible for mRNA decoding, and the 50S large subunit, which contains the peptidyltransferase center and is thus involved in peptide bond formation. After translation initiation, nascent polypeptide chains with around 30-40 amino acids exit the large subunit through the 100-A˚ -long ribosomal tunnel. At this point, co-translational folding may occur (14). However, because the proper folding of a protein often lies on the interaction between physically distant structural domains in the peptide chain, the complete folding is only possible when the entire amino acid sequence is released from the ribosome (15–17). This often constitutes a problem: while the full sequence is being translated, many nascent peptide chains are left exposed in the cytosol and are more prone to physically interact with similar peptide chains from neighboring polysome complexes. The problem is even worse in macromolecular crowded environments, where unspecific inter-molecular interactions are favored (18). Hence, these nascent polypeptides tend to aggregate with harmful consequences for the cell such as titration of proteases and chaperones, formation of toxic polypeptide species, loss of function of the aggregated proteins, among others (reviewed in (10)).

Thus, even though some newly synthesized small proteins are able to naturally fold by themselves, the majority of newly synthesized proteins need assistance to reach their native state and final destination in the cell, thus preventing aggregation (19). The presence of signal peptide sequences in the nascent protein or the formation of secondary structures, dictates the binding of specific targeting factors and chaperones that assist protein allocation in the cell membrane or cytosol (20). For example, emerging new polypeptides containing signal peptide sequences are targeted by the ribosome-bound signal recognition particle (SRP) that facilitates their translocation to the Sec complex in the inner membrane (21–23). Also, pre-secretory proteins containing hydrophobic domains are also directed to this complex through a process that depends on the chaperone SecB and the ribosome-binding ATPase SecA (23).

To help with the folding of newly synthesized cytosolic proteins, bacteria harbor a chaperone network machinery composed of three major molecular chaperone complexes: Trigger factor (TF), the Hsp70/40 family DnaK/DnaJ-GrpE and the Hsp10/60 family GroES-GroEL (16, 19, 24).

1.2.1.1. The ribosome-bound Trigger Factor (TF)

The ribosome-bound Trigger Factor (TF) is the first chaperone acting on newly synthesized peptides emerging from the exit tunnel of the ribosome (Fig. 1). It is a 48 kDa protein composed by 3 domains: a ribosome-binding N-terminal domain (domain I), a PPIase activity containing central domain (domain II), and a C-terminal domain (domain III) (25–27). The latter was shown to constitute the central module of its chaperone activity (28). The different domains interact in a way so that after TF binding to the L23 protein at the large 50S subunit of the ribosome, it forms a protected folding space around the nascent polypeptides, preventing aggregation and protecting them from degradation by proteases (29). Then, after synthesis of the full polypeptide, TF is released from the ribosome in a ATP-independent manner but it may be kept bound to its substrate and even facilitate its transfer to the downstream chaperones in the chaperone network cascade (30). Moreover, it has been suggested an additional role for TF in rescuing of a large repertoire of full-length proteins from misfolding, including ribosomal proteins (31).

It is generally accepted that the co-translational action of TF is sufficient for the correct folding of the majority of cytosolic proteins in *E. coli* without the need of further assistance by the downstream chaperones DnaK/DnaJ and GroES-GroEL (19, 32). *E. coli* cells lacking *tig* (Trigger Factor encoding gene) do not present any obvious growth defect in standard lab conditions. However, *tig*

mutants are known to be more sensitive to specific stress such as growth at 4ºC or treatment with SDS/EDTA and vancomycin (33–35). This is due to the fact that TF was shown to preferentially associate with β-barrel outer membrane proteins suggesting an important role for TF in outer membrane stability (35). Interestingly, tig cannot be deleted in a *dnaK* mutant in *E. coli* under temperatures above 30ºC (32, 36). This suggests a redundancy in the functions of TF and the DnaKJE chaperone machinery (37, 38). In fact, in the absence of TF, the percentage of substrates targeted by DnaK increases substantially (32).

1.2.1.2. The DnaK/DnaJ/GrpE chaperone machine

As said above, although TF is sufficient for the majority of newly synthesized proteins reach their native state, there is still a considerable percentage of proteins that need additional assistance. After interaction with TF, partially folded proteins are then transferred to the Hsp70/40 class chaperone machine (Fig.1) In *E. coli,* this chaperone system is constituted by DnaK, and the co-chaperones DnaJ and GrpE (39). DnaK is one of the most abundant cytosolic chaperones in *E. coli* and is composed by an N-terminal domain with ATPase activity and a C-terminal peptide-binding domain (40). The latter shows higher affinity to substrates when the N-terminal domain is bound to ADP. Thus, substrate binding by DnaK is strongly influenced by the hydrolysis of ATP. DnaJ is a 41 kDa co-chaperone that associates with DnaK and stimulates ATP hydrolysis and consequent substrate capture (41). Moreover, DnaJ's C-terminal domain recognizes hydrophobic peptides recruiting then DnaK to this type of substrates. Finally, the nucleotide exchange factor GrpE induces the dissociation of ADP from DnaK which in turn is released from the peptide, allowing for re-start of the cycle (42).

DnaK is considered of central importance for proteostasis. It was shown to interact with six hundred proteins in *E. coli* under standard lab conditions with a preference for low solubility substrates (16). In addition, DnaK has been to shown to control the levels of the alternative sigma factor RpoH, thus controlling Heat-shock response (43, 44) (detailed below). DnaK is not essential, but its deletion is associated with a multitude of temperature sensitive phenotypes, probably resulting from the higher levels of protein aggregation observed in *dnaK* mutants (19).

1.2.1.3. The GroES – GroEL chaperonin system

The chaperone GroEL form together with its co-chaperone GroES the only essential chaperone system in bacteria in all tested conditions (45). GroEL is a large barrel-like protein that has two heptameric rings of 57 kDa stacked back-to-back. Each one of the two ring subunits has three different domains that mediate intra and inter molecular interactions, as well as nucleotide and substrate binding. The cavity formed by the two rings of GroEL is where partially folded proteins bind through hydrophobic interactions. GroES is a homoheptameric ring of 10 kDa subunits that binds GroEL in an ATP-dependent manner (46). Together, the GroES-GroEL system works as a protected environment where, inside GroEL, the non-native polypeptides fold through an energetically expensive process that depends on the hydrolysis of seven ATP molecules (47). Finally, at the end of the cycle, the properly folded native protein is released from the complex, whereas proteins that still remain in a non-native state are re-captured for another cycle (48).

Typically, GroES-GroEL chaperonin machine can accommodate substrates varying from 20-60 kDa in size and some studies have shown that around 10-20% of cytosolic proteins interact with GroEL for assisted folding (Fig. 1) (16). However, accordingly to one of this studies, only around 57 proteins were strictly dependend on GroESL for proper folding and among these, 6 were essential proteins (45). This may be the reason for the GroESL essentiality observed in most bacteria.

Even though GroESL cannot be deleted in almost all bacteria tested, functional mutations in these genes are associated with temperature sensitive phenotypes. On the other hand, overexpression of GroESL has been shown to confer tolerance to various stresses affecting protein homeostasis such as extreme heat or treatment with drugs causing protein misfolding (49, 50). Moreover, overexpression of these chaperonins compensate for the simultaneous loss of TF and DnaK which, once again, reflects the functional overlap between TF, DnaKJE and GroESL in maintaining protein homeostasis (38, 51). Interestingly, several bacterial species are known to harbor multiple copies of GroEL with slightly different protein sequences, which raises the question whether these extra copies of chaperonins are functionally redundant or have a more specialized role in the cell (52, 53). According to the latter hypothesis, it was shown in different bacterial species that the presence of these “alternative” versions of GroESL play a determinant role in response to specific stresses (54).

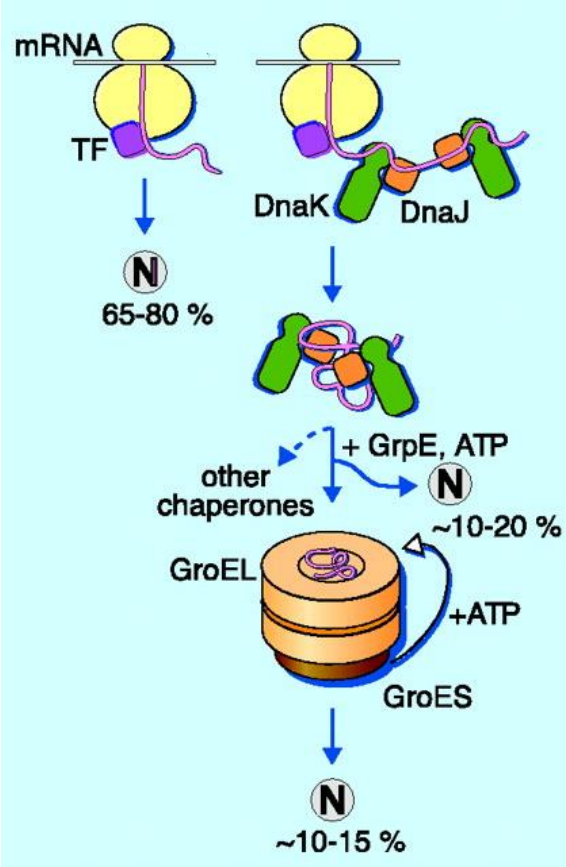


FIG 1. **Chaperone network and folding of newly synthesized proteins**. TF, trigger factor; N, native protein. Upon exit of the ribosome the nascent polypeptide first interact with TF and fold efficiently without further assistance. The majority (65-80%) of these are small proteins that achieve their native state without further assisting. Longer peptides (10 to 20%) are thought to need further assisting by DnaK and DnaJ chaperones. About 10 to 15% of the produced peptides additionally require GroEL and GroES for proper folding. Taken from (16).

1.2.2. Proteostasis during specific stresses

The ability of a protein to fold is tightly associated with its physical and chemical properties, which in turn influence the molecular interactions between its different domains. As such, environmental stresses that interfere with such interactions may cause unfolding of native proteins or impair the biogenesis of the newly synthesized ones (Fig.2).

1.2.2.1 Increased temperature

Increased temperature strongly affects protein structure and induce protein denaturation, unfolding and aggregation (55, 56). This happens due to the disruption of the weak intramolecular forces responsible for protein structure under elevated temperatures (55, 57). A recent study in *E. coli* showed that the extent of protein aggregation *in vivo* upon heat-shock treatment increased with increasing temperatures of the treatment showing that some proteins are more sensitive than others to heat denaturation (58).

1.2.2.2 Oxidative stress

Besides elevated temperatures, the presence of reactive oxygen species (ROS) also lead to protein aggregation. ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) or superoxide (O_2 ·−) appear as by-products of respiration in aerobic organisms and are mainly produced during electron transfer (59). Excessive levels of these reactive oxidative species cause post-translational modification of specific amino acids and side chains which in turn affects folding chemistry and collapse of protein structure (60, 61). Interestingly, it was recently proposed that the main targets of oxidative damage are unfolded proteins (more than misfolded ones) and that chaperone-mediated protection to oxidative damage relies on the chaperone system DnaKJE which are able to protect proteins in their unfolded state (62).

1.2.2.3 Mistranslation

Given that the amino acid sequence of a protein is crucial for its folding and native structure, it is expected that any mechanism leading to the corruption of the amino acid sequence can also lead to protein misfolding. In fact, many neurodegenerative diseases in humans are caused by genetic mutations that alter amino acid sequence and thus folding capacity of essential proteins (63). However, during translation, corruption of the translational machinery can also result in incorporation of a wrong amino acid in the translating peptide sequences. These mistranslated proteins are more prone to misfolding thus depending on the assistance of molecular chaperones.

1.2.2.3.1 Mistranslation as a natural process

When compared to important processes in cell's replicative cycle, such as DNA replication or transcription, translation is the one that accumulates more errors throughout its different steps. The average translational error rate is around 10^{-4} to 10^{-3} per codon in most microorganisms (64, 65). The result, is the occurrence of at least one mis-incorporated amino acid in 10 to 15% of all proteins in an actively growing *E. coli* cells (66, 67). Such a high error rate is likely explained by the fact that translation is a complex and multifactorial process which creates an opportunity for errors at the different steps of the process (67). For example, key processes such as the pairing of amino acids and tRNAs by aminoacyl-tRNA synthetases (aaRSs) and faithful decoding of mRNA codons by the corresponding aminoacyl-tRNAs (aa-tRNAs) on the ribosome are often corrupted, leading to a loss in translational fidelity. The high diversity of the different amino acids may constitute a challenge for aaRSs, which need to proper discriminate between cognate or near-cognate amino acids (68).

Several mechanisms of quality control exist to make sure that correct aminoacylation and decoding of mRNA molecule occurs. For example, aaRSs are able to recognize and hydrolyze misactivated amino acids and misacylated tRNAs (69). However, mutations that affect this recognition are known to lead to misacylated aaRSs, which ultimately results in the misincorporation of certain amino acids in the peptide chain (70). Moreover, the correct activation of cognate amino acids by aaRSs is further affected by chemical modifications of such amino acids that can occur as a result of oxidation by reactive oxygen species (ROS) (71).

Despite having to correctly activate cognate amino acids, cells should also guarantee a correct selection of tRNA among the pool of tRNAs existent in the cell. This selection is often based on the specific sequences on the tRNA molecule, as well as modification of certain residues (69, 72, 73). Thus, modification enzymes and conditions that can alter the modification state of tRNAs will likely result in wrong selection of tRNAs and subsequent mistranslation. For example, it was discovered in *E. coli* that the lack of a specific modification in the adenine at position 37 of tRNA-Leu leads to its incorrect aminoacylation and difficulties in decoding transcripts enriched in rare leucine codons (74). Moreover, the relative abundance of tRNA and aaRSs is also known to affect correct aminoacylation (75).

Amino acid starvation is equally an important factor that can lead to mistranslation. In fact, the balance between the cellular tRNA pool and the availability of aa-tRNAs have been shown to

affect translational fidelity. This happens because upon specific amino acid limitation, incorporation of near-cognate aa-tRNAs may be favored, in order to prevent ribosome stalling caused by incorporation of deacylated tRNAs (67).

Stress conditions that directly or indirectly perturb any of the previous mechanisms can thus lead to mistranslation and thus affect proteostasis. Depending on the level of mistranslation, such stress conditions may lead to cell death. That is the case of aminoglycosides, a well-known class of antibiotics that target the ribosome and increase the mistranslation rate of proteins (76).

1.2.2.3.2 Mistranslation caused by aminoglycosides

Aminoglycosides (namely kanamycin, tobramycin, gentamicin, neomycin, amikacin, and streptomycin) are a group of bactericidal antibiotics that target the ribosome to induce amino acid misincorporation (77). The primary target of aminoglycosides is the 30S small ribosomal subunit. Aminoglycoside binds to the 16S rRNA, at the tRNA acceptor A site (aminoacyl site) and affects a proofreading mechanism responsible for correctly distinguish between cognate and non-cognate tRNAs at the ribosome. This proofreading mechanism involves two conserved nucleotides at the mRNA decoding site. Upon faithful binding of cognate tRNAs, the correct base pairing between the anticodon and the mRNA codon induces a structural change that leads to retention of the tRNA and incorporation of the respective amino acid in the peptide chain. On the other hand, the binding of non-cognate tRNAs fails to induce this structural change and the tRNA is not accepted (67, 78, 79). Studies on the crystal structure of ribosome-bound aminoglycosides showed that these antibiotics are able to indiscriminately induce this structural change, which leads to retention of both cognate and non-cognate tRNAs and increased incorporation of wrong amino acids (76, 80). Moreover, additional studies on the interaction of aminoglycosides with the ribosome unveiled a second binding site for aminoglycosides on the ribosome: a site an overlapping binding site that of ribosome recycling factor (RRF). The results showed that when the primary binding site (at the 30S ribosomal subunit) is already saturated with the antibiotics, binding of these to the 50S subunit suppresses the action of RRF and recycling of ribosomes for another round of translation (81).

According to the current model, it is generally accepted that aminoglycosides first enter the cell by an energy-dependent process that dependents on membrane potential. The immediate

consequence is the binding of aminoglycosides to 16S rRNA at the 30S ribosomal subunit which interfere with proofreading mechanisms occurring at the A site. As a result, mistranslation increases and so does the levels of misfolded proteins in the cell. Many of these misfolded proteins are inserted in the membrane and cause changes in membrane potential and permeability leading to further aminoglycoside uptake. This results in the accumulation of high concentrations of aminoglycosides in the cytosol and lead to massive mistranslation and/or translation arrest due to mistranslation of ribosomal proteins and inhibition of ribosome recycling (77, 81, 82).

In agreement with a higher level of misfolding, several studies have shown the induction of heat-shock genes and the importance of several chaperones and proteases in bacteria treated with aminoglycosides (49, 50, 83–86)

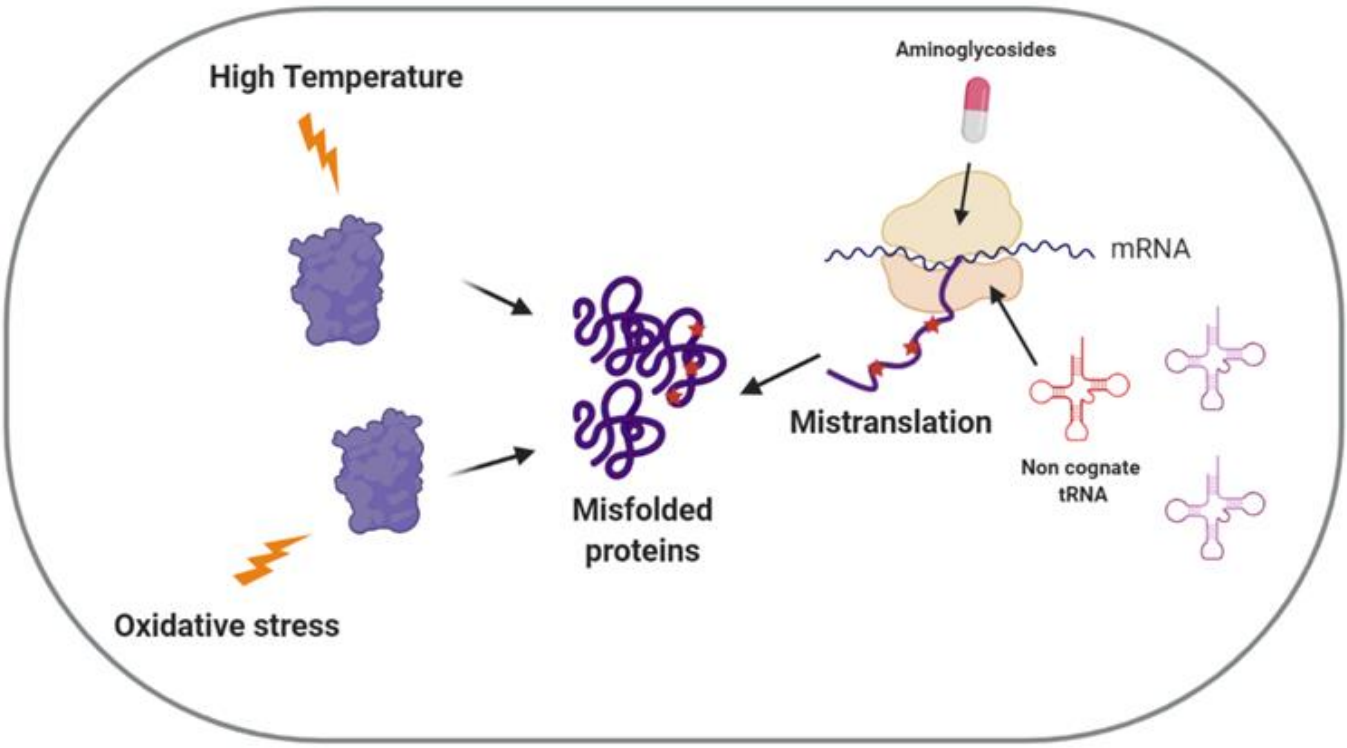


FIG 2. **Stresses affecting proteostasis.** Environmental and external stresses such as increased temperatures and oxidative stress lead to unfolding of proteins. Additionally, aminoglycosides and conditions that perturb translation quality control mechanisms lead to mistranslation of newly synthesized proteins. As a consequence, misfolded proteins accumulate disturbing proteostasis. Created with BioRender.com.

1.2.3 Heat-shock response

All of the above-mentioned stresses are serious threats to proteostasis and sometimes, depending on the type and intensity of the stress, the constitutively expressed housekeeping chaperones do not keep up with the level of protein damage in the cell. Thus, in response to this stresses, bacteria deploy a specific stress response that leads to the transcription of specific chaperones and proteases, as well as the upregulation of the housekeeping TF, DnaKJE and GroESL. This stress response is known as the heat-shock response (87).

1.2.3.1 Proteases and small heat shock proteins

In addition to chaperones that help folding of proteins, activation of the heat-shock response in bacteria elevates the expression of an arsenal of proteases that are able to degrade damaged proteins that no longer can be re-folded (88). Most proteases are from the AAA+ family, i.e. associated with ATPase domains that oligomerize to form a barrel-shape complex composed of several multimeric rings. Substrate proteins are captured in this complex which can unfold and subsequently degrade them, in a process dependent on ATP hydrolysis (10, 88). Some proteolytic machines are able to unfold and degrade substrates using a single protein, which is the case of highly conserved proteases such as FtsH, Lon and the periplasmic serine protease DegP. Others, act by the combined action of different components with separate unfoldase and peptidase functions. This is the case of the two-component systems such as HslUV and ClpA, ClpC, ClpE and ClpX unfoldases that associate with the ClpP peptidase. Thus, these proteins are essentially responsible for the removal of damaged polypeptides from stressed cells. ClpB is another protease playing an important role in protein quality control by coupling the energy from ATP hydrolysis to protein disaggregation in collaboration with the DnaKJE chaperone system (89).

Moreover, in addition to the proteases and the housekeeping chaperone network already mentioned here, induction of heat-shock response also results in the production of a specific class of effectors, called small heat-shock proteins (sHSPs) (90). The main role of sHSPs is to bind and protect unfolded proteins, preventing its aggregation and subsequent degradation by proteases until ATP-driven chaperones complete their folding (91, 92). In fact, sHSPs cannot rescue already unfolded and aggregated substrates and thus should be present when the substrate in unfolding. Interestingly, a study that looked for specific substrate recognition by sHSPs observed a preference for translation

related proteins such as ribosomal proteins, translation factors and aaRSs (93). In *E. coli*, the sHSPs inclusion body protein A (IbpA) and Inclusion body protein B (IbpB) are the most well studied proteins from this class. IbpA/IbpB have been found to be associated with aggregated proteins after heat stress and function by protecting the aggregates from the action of proteases while facilitating substrate transfer to DnaKJE and ClpB systems for further processing (94).

Many of these proteases, sHSPs and the housekeeping chaperones DnaKJE are known to function in collaboration with each other (89, 95), providing the cell with an efficient protein quality control network, to face proteotoxic stress.

1.2.3.2 Regulation of heat-shock response

In *E. coli* and other Gram negative bacteria the regulation of the heat shock response is mediated by the alternative sigma factor σ^{32} (RpoH). Regulation of RpoH itself is elegant but complex and occurs at multiple levels: the rapid and transient expression of heat shock genes upon temperature stress is primarily linked to the translational regulation of RpoH, which will affect the amount of active σ^{32} in the cell (96, 97). Thus, while in normal conditions active RpoH is present at low concentrations, under heat-shock stress there is a fast but transient increase of its active form, mainly due to changes in RpoH stability and synthesis. One of the reasons why RpoH synthesis is affected by high-temperature is related with its mRNA structure: tt is known that under optimal temperature conditions rpoH mRNA assumes a complex secondary structure that impedes the binding of ribosomes. However, increase of temperature partially melts this structure allowing for proper translation initiation (98).

On the other hand, stability and activity of RpoH are also controlled by both chaperones and proteases that are part of the heat-shock regulon themselves. In fact, it was shown that 1) mutations in the genes encoding the DnaK/DnaJ/GrpE family chaperones led to the induction of the heat-shock response at low temperatures and 2) after temperature upshift, the levels of RpoH failed to go back to basal levels (43). Later, another study showed evidence of direct protein-protein interactions between RpoH and DnaK/DnaJ/GrpE chaperones (44). In addition, it was shown that GroEL is also capable of binding to RpoH and that GroEL overexpression resulted in decreased activity of this sigma

factor *in vivo* (99). Interestingly, this effect of GroEL on the activity of RpoH could be prevented by the co-expression of a GroEL-specific substrate (99). Additionally, an extra layer of regulation occurs through the FtsH protease-mediated degradation of RpoH (100).

These studies suggest that activation of heat-shock response is tightly regulated, but what essentially dictates its activation is the balance between misfolded proteins and chaperone occupancy in the cell: when the levels of misfolded proteins are low, free DnaK and GroEL bind and sequester RpoH. However, when misfolded proteins start to accumulate, these chaperones become busy and leave RpoH available for heat-shock response activation (Fig. 3) (99, 101).

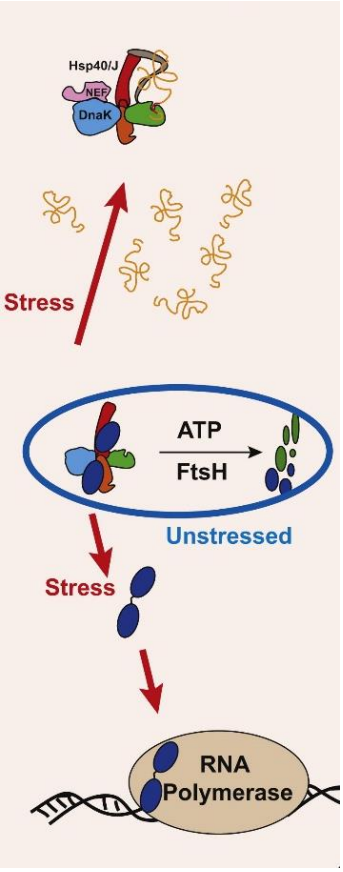


FIG 3. Heat-shock response regulation in Gram-negative bacteria. In absence of stress, DnaKJE homologs bind to RpoH (dark blue) and handle it to FtsH protease for degradation. Upon proteotoxic stress, chaperones become overwhelmed with unfolded proteins and leave RpoH free. RpoH can then associate with RNA polymerase for activation of heat shock genes. Taken from (87).

1.2.4. The σE stress response

In addition to the heat-shock response, bacteria can deploy a more specialized response against the incorporation of misfolded proteins in the outer membrane or general envelope instability. This is generally called “envelope stress response” and involves the participation of several two-component systems to fight aberrant proteins incorporation at perturbation of the periplasm and inner and outer membranes (102). These stress response systems include the CpxRA (103), the Rcs (regulator of capsule synthesis) (104), the Bae (bacterial adaptive response) (105) and the Psp (phage shock protein) (106) systems, as well as the σE (RpoE)-mediated stress response system.

The presence of misfolded outer membrane proteins (OMPs) in the outer membrane of gram-negative bacteria is the major factor that modulates RpoE envelope stress response activation (107–109). Moreover, OMPs overproduction and antibiotics or drugs that affect lipopolysaccharide (LPS) structure are also potent inducers of this response.

In absence of stress, RpoE is sequestered by the anti-sigma factor RseA at the inner membrane (102). Hence, upon aberrant cell envelope structure, the intramembrane protease DegS is activated and cleaves the periplasmic domain of the membrane-bound anti sigma factor RseA. This further induces proteolysis of the cytosolic portion of RseA by DegP. These cleavages result in release of RpoE in the cytosol which is now able to control the expression of a specific set of genes to help with membrane stress (102, 110). Part of the RpoE regulon are small RNAs that reduce transcription of OMPs and lipopoliproteins, and several proteins that degrade aberrant membrane proteins (111–113). Interestingly, RpoE also increases expression of the sigma factor RpoH, highlighting a response to unfolded proteins (114). RpoE is an essential protein in *E. coli* and *V. cholerae* and can only be deleted in presence of secondary suppressor mutations that increase envelope stability (109, 110, 115).

Thus, the concerted activation of RpoE and RpoH – mediated stress responses by protein misfolding is crucial to fight against proteotoxic stress that occur both inside and outside of the cytoplasm.

II. Antibiotics and Stress

From the Greek ἀντι anti, and βίος bios, "life", the word *antibiotic* literally means “against live”. In 1928, the british biologist Alexander Fleming (accidently) observed that a “mould juice”, produced by a mould from the genus *Penicillu*m, had important antimicrobial properties against a culture of Staphylococci. That was the moment the first antibiotic was discovered (116). Since then, it became clear that many microorganisms produce these chemical molecules in order to kill or inhibit the growth of others, and that the isolation and purification of these molecules was an important tool to prevent and treat bacterial infections. The subsequent discovery of new classes of antibiotics, together with synthetic biochemical modifications, completely revolutionized medicine in the 20th century. Moreover, for many years, antibiotics were used in agriculture as promoters of animal growth, a practice that was banned in Europe after 2006 (117, 118).

This generalized use of antibiotics in modern medicine and agriculture led to an incredible selective pressure upon bacteria and it was just a matter of time until the first cases of antibiotic resistance emerged (Fig.4). Since then, development of antibiotic resistance by bacteria has been considered one of the biggest threats to global health. It is estimated that by 2050, 10 million people per year will be killed by antimicrobial resistance (AMR) related diseases (119).

As said in the beginning of this Introduction, bacteria are extremely good at adapting to unfavorable environments. Part of such remarkable plasticity is related to their capacity of generate genetic variation, which is key for natural selection to act upon. Genetic variation in bacteria can be achieved through two main ways: mutation and horizontal gene transfer (HGT) (120). In addition, epigenetic mechanisms can also lead to phenotypic heterogeneity, another source of variation that is crucial for persistence of bacterial populations under transient selective pressure (121). Interestingly, the same mechanisms that lead to antibiotic resistance in bacteria, are also modulated by stress caused by low doses of antibiotics themselves.

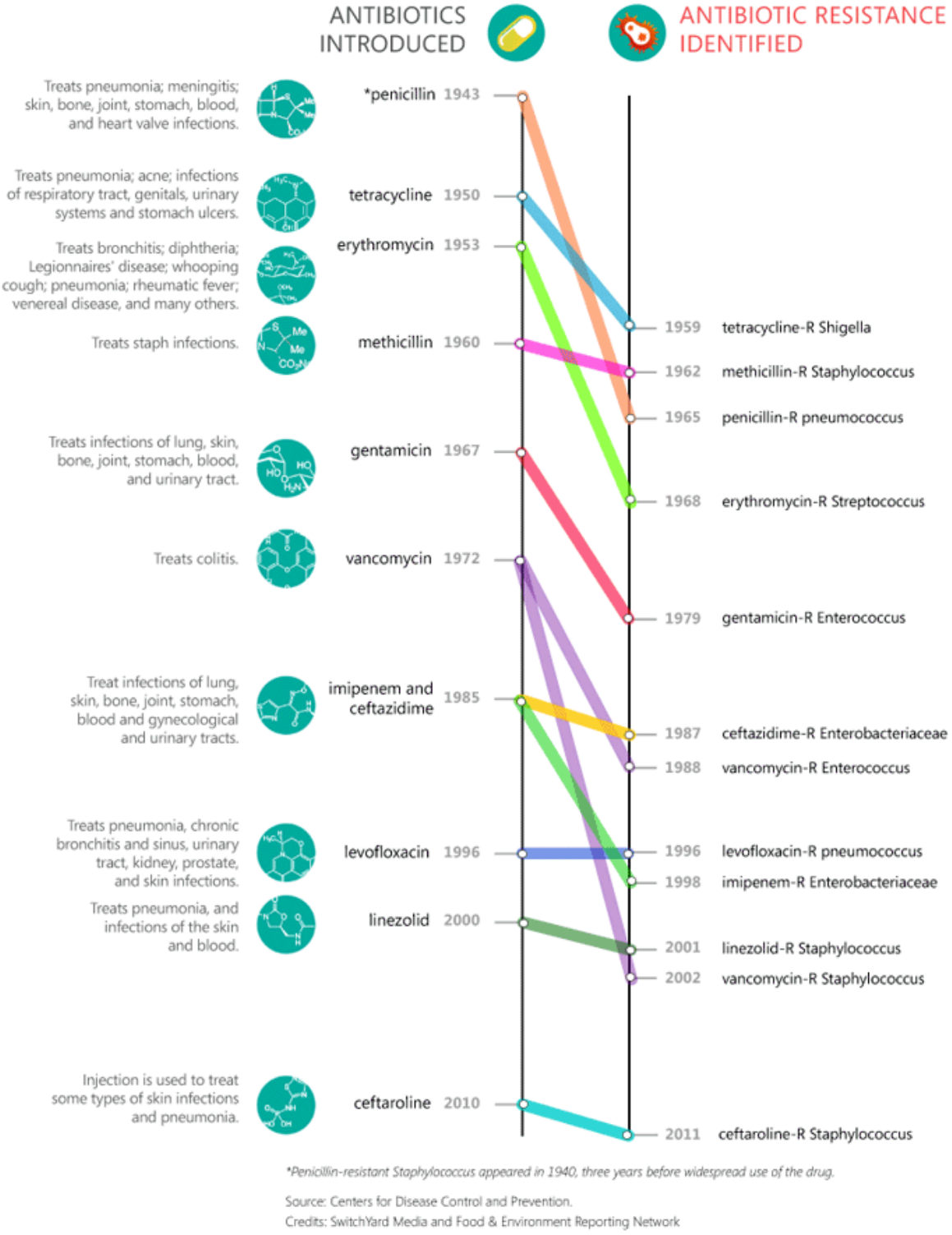


FIG 4. Antibiotic discovery and antibiotic resistance timeline. Adapted from (122).

2.1 SubMIC concentrations of antibiotics as a source of stress

For the past decades, the over/misuse and large scale production of antibiotics by pharmaceutical industries has created a serious ecological problem: a large proportion of the antibiotics ingested are released intact in the environment (123, 124) and found at trace levels or as gradients in various environments (Fig.5) (125, 126). This is particularly relevant in the aquatic environment (127) and in the mammalian hosts of pathogenic and commensal bacteria, where antibiotics can play a very important role in the selection of resistant bacteria (128). Hence, in these environments, one can find the presence of very low doses of antibiotics that, although not enough to kill or prevent the growth of bacterial populations, are still able to trigger important stress mechanisms that often result in development of antibiotic resistance (126). How do sub-inhibitory concentrations of antibiotics contribute to the emergence of antibiotic resistance?

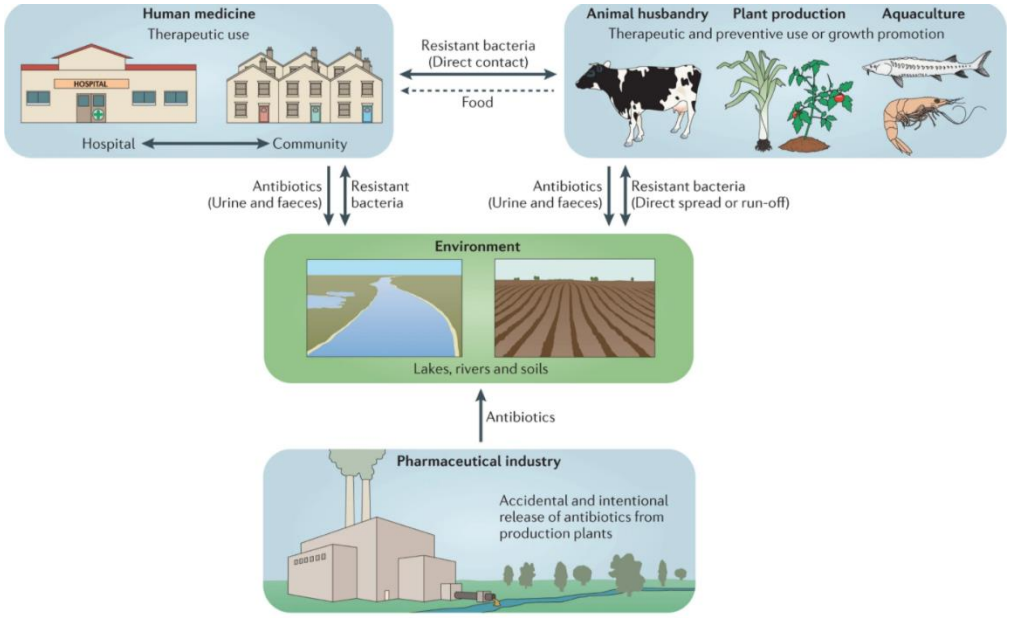


FIG 5. Antibiotic mis/overused in different anthropogenic activities end up cycling between different environments. Taken from (126).

The molecular mechanisms involved in antibiotic resistance have been extensively studied in the context of selective pressure under lethal concentrations of antibiotic, i.e. concentrations of antibiotic above the MIC (Minimal Inhibitory Concentration). The MIC can be defined as the lowest concentration of a drug needed to inhibit visible growth of a bacterial population under certain experimental conditions (129). However, growing evidence suggests that selection of antibiotic

resistant bacteria can occur at concentrations below the MIC, (subMIC), i.e. at concentrations that do not cause growth inhibition (130–133). For clarity, hereinafter I will use the terms “subMIC”, “sub-inhibitory” and “sub-lethal” as synonyms.

2.1.1 Effects of subMIC concentrations of antibiotics on bacteria

2.1.1.1 Enrichment of pre-existing mutants and selection of *de novo* mutations

The presence of antibiotics at sub-inhibitory concentrations allows for the enrichment of pre-existing mutants in bacterial populations. In fact, while resistance mutations confer a fitness cost in the absence of selective pressure (i.e. in the absence of antibiotic), they can be selective advantageous even at very low doses of antibiotic, outcompeting the sensitive bacteria (133). This was demonstrated *in vivo* in mixed cultures of susceptible and resistant strains of *Staphylococcus aureus* in a murine infection model (134). Moreover, the presence of antibiotics at sub-inhibitory concentrations select for *de novo* resistance in initially susceptible populations as it was demonstrated by Gullberg and colleagues in 2011 (133). In this study, multiple independent lineages of susceptible *E. coli and S.* Typhimurium were evolved *in vitro* in presence of subMIC levels of ciprofloxacin or streptomycin for 600 generations. After evolution, the authors observed the presence of subpopulations that developed high level resistance to the antibiotics used in the study.

To better understand these observations one should have in mind that the dynamics of selection under low doses of antibiotics are different from those at lethal doses. In the latter, the selective pressure is very high and only rare mutants with high level resistance are selected, while the rest of the population dies. On the other hand, under low doses of antibiotics, the weak selective pressure mostly selects for more frequent, small effect mutations with low fitness-cost and a slight fitness advantage over the rest of the population (128). Moreover, survival of the susceptible population allows for a larger “mutational window”, giving the opportunity for these mutations to appear and accumulate, resulting in a build-up for high level resistance (130).

2.1.1.2 Antibiotics at sub-inhibitory concentrations accelerate the rate of Horizontal Gene Transfer and mutagenesis through the SOS response

Horizontal gene transfer (HGT), mutagenesis and recombination events are strong generators of genetic variation. These mechanisms can be triggered by antibiotics, mainly through the induction of SOS response (133, 135–139). SOS response is triggered by bacteria in response to DNA damage. When single strand DNA (ssDNA) is generated in the cell (for example through DNA damage), it binds to RecA protein forming a nucleofilament that results in the auto-cleavage of the SOS response repressor LexA. This leads to de-repression of the SOS regulon, a well-defined set of genes whose functions are related to mutagenesis and DNA repair pathways (140, 141). Thus, antibiotics that directly or indirectly cause DNA damage, are able to induce the SOS response and induce mutagenesis.

SOS-mediated mutagenesis is mostly the result of the action of error-prone DNA polymerases such as DNA pol V (UmuD) or DNA pol IV (DinB), that (mis)introduce nucleotides opposite of DNA lesions and are both members of the SOS regulon (142–144). Furthermore, recombination events such as integron cassette reshuffling also constitute an important factor that contribute to genetic change (145). These events were also shown to be induced by SOS (138, 146). For example, integron cassette rearrangements in *V. cholerae* and *E. coli* were found to be induced by subMIC doses of ciprofloxacin and mitomycin C. Such induction occurred via de-repression of the integrase gene *intA*, which contains a LexA-box (138).

SOS response is also known to contribute to horizontal gene transfer, both in Gram-negative and Gram-positive bacteria (147). In fact, it was shown that ciprofloxacin-induced SOS response is able to contribute to genetic change through the dissemination of SXT, an integrating conjugative element (ICE) containing multiple antibiotic resistance genes in *Vibrio cholerae* (135). Moreover, a different study demonstrated that SOS-dependent prophage induction by sub-lethal concentrations of mitomycin C, led to the high-frequency transfer of a co-resident pathogenicity island in *S. aureus* (136).

In *E. coli,* SOS response is induced by antibiotics that somehow target the DNA or DNA-dependent processes (fluoroquinolones, trimethoprim, Mitomycin C), while antibiotics that target different molecules in the cell do not have any effect on SOS induction. However, this seems to be an exception rather than the rule, as it was shown in 2010 by Baharoglu *et al*. (139) that subMIC doses of antibiotics

that do not target DNA (such as aminoglycosides, chloramphenicol and tetracycline) are also able to induce SOS in three different species such as *V. cholerae, Klebsiella pneumoniae and Photorhabdus luminescens* (139).

These examples clearly show how subMIC doses of antibiotics, through induction of SOS response, are able to generate and disseminate antibiotic resistance.

2.1.1.3 Antibiotics at sub-inhibitory concentrations induce a plethora of different transcriptional responses

Besides altering selection dynamics and promoting mutagenesis and HGT through DNA damage and activation of SOS response, antibiotics at sub-inhibitory concentrations also work as signaling molecules, thus having the remarkable capacity of induce profound adaptive responses through modulation of gene expression (148, 149).

For example, sub-lethal concentrations of the β-lactam ampicillin were able to induce RpoS (responsible for the activation of the general stress response) in *E. coli, V. cholerae* and *Pseudomonas aeruginosa*, by increasing RpoS stability at the post-transcriptional level (150). This lead to the upregulation of the RpoS-controlled small RNA *sdsR*, that prevented translation of MutS, an important component of the methyl-directed mismatch repair system (MMR) that helps correct postreplication errors (151). Moreover, induction of RpoS also lead to the expression of DinB, the DNA pol IV that induce mutagenesis. Thus, subMIC-induced depletion of MutS in a high mutagenesis context led to a huge increase in the mutation frequency of these species (150).

Several studies have also considered the impact of low doses of antibiotics on virulence traits and biofilm formation, essential for successful host infection. Interestingly, the literature suggests that the effect of low doses of drugs on the expression of virulence factors is mainly antibiotic- and virulence factor-dependent. For example, in community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), the use of sub-inhibitory concentrations of clindamycin and linezolid reduced mRNA levels of Panton–Valentine leucocidin (PVL) and protein A (SpA), both staphylococcal specific virulence factors. However, only clindamycin (but not linezolid) was able to reduce expression of α-haemolysin (Hla), a different virulence factor (152). In a different study,

subMIC concentrations of clindamycin (and other protein synthesis inhibitors) promoted increased expression of the virulence regulator *agr*, in the same species (153).

In *Listeria monocytogenes* sub-inhibitory concentrations of ampicillin and tetracycline caused up- and down-regulation of stress response and virulence genes, respectively, and both antibiotics caused increased sensitivity to acid stress. However, *L. monocytogenes* virulence was not affected (154).

In the multi-resistant *Salmonella enterica* subsp. enterica serovar Typhimurium DT104, tetracycline was shown to induce the expression of several virulence factors, including motility genes and positive regulators of a pathogenicity island. In this case, this transcriptomic changes were associated with an increased ability to invade epithelial cells *in vitro* (155).

Finally, a recent study showed that subMIC aminoglycosides cause dramatic phenotypic changes in *P. aeruginosa*, affecting biofilm formation capacity and attenuating several virulence properties. Further investigation showed that these effects occur through direct binding and inhibition of LasR (a known *quorum sensing* regulator) by aminoglycosides (156).

All of these examples (a vast number of other examples can be found in the literature), show that there is not a common mechanism underlying virulence modulation by subMIC in bacteria which reflects the huge phenotypic variation associated to the effects of low doses of antibiotics.

2.1.1.4 Antibiotics at sub-inhibitory concentrations and antibiotic tolerance

As initially stated, non-genetic phenotypic variation is an important factor that can contribute to the emergence of antibiotic tolerance, persistence and resistance. Although I have been focusing on the latter, antibiotic tolerance and persistence are of major interest in the context of this thesis. Moreover, both have been shown to be modulated by subMIC doses of antibiotics. The concepts of Resistance, Tolerance and Persistence may be ambiguous and thus require clarification:

Antibiotic resistance describes the inherited ability of bacteria to grow at lethal concentrations of an antibiotic. Thus, “resistance” is characterized by an increase in the MIC of that antibiotic relative to the susceptible population. On the other hand, “tolerance” can be defined as the ability of bacteria to survive transient exposure to lethal doses of antibiotic and it doesn’t show any differences in the

MIC relative to the susceptible population. “Persistence” can be considered a subcategory of “Tolerance”, as it describes a sub-population formed by tolerant cells. “Tolerance and Persistence” have thus distinct survival dynamics in a time-kill curve upon treatment with lethal doses of a bactericidal antibiotic. In practical terms, for the same duration of treatment, a population of “tolerant” cells have a higher fraction of survivors relative to the susceptible population. However, “persisters” are only a small subpopulation of tolerant cells within a large subpopulation of susceptible ones. In this case, the resulting time-kill curve will be biphasic, with an initial fast drop of survivors being observed (killing of the susceptible subpopulation) followed by an attenuation of the killing rate (killing of the tolerant subpopulation) (Fig.6) (157).

The higher antibiotic tolerance of “persisters” is generally attained mainly through the reduction of the metabolic activity of the cells, which enter a physiologically dormant state with reduction of growth rate. This way, the cellular processes targeted by the bactericidal antibiotic become protected from its action (158). It is important to say that this is a transient state that reverse to its normal upon antibiotic removal.

The formation of persister cells is thought to happen by two distinct ways: 1) completely stochastic emergence of phenotypic variation, or 2) triggered by specific stress signaling pathways such as the SOS response, the general stress response or (p)ppGpp-dependent stringent response. Curiously, all of these pathways are induced by sub-inhibitory concentrations of antibiotics.

Formation of persister sub-populations is one of the reasons for the reduced rate of clearance of clinical bacterial infections (159, 160) and one study have pointed for the induction of persistent cells formation by sub-lethal doses of antibiotics (161).

2.1.1.5 Antibiotics at sub-inhibitory concentrations – final considerations

The role of subMIC antibiotics as generators of phenotypic and genetic variation couldn’t be clearer. Throughout this chapter, it was shown how low doses of antibiotics are able to activate various stress signaling pathways such as the general stress response, which confers bacteria a higher level of cross-tolerance to innumerous other stresses. It was also shown how, through damage of key macromolecules like DNA or proteins, subMIC antibiotics induce SOS response, promoting recombination events, HGT and mutagenesis. Also, it was demonstrated that subMIC can impact

expression of virulence genes, and thus modulate virulence of important human pathogens. Finally, all of the subMIC effects here described are linked to antibiotic tolerance ("persisters" or not) and are thus indirect mediators of its emergence. Hence, the impact of subMIC doses of antibiotic on bacterial physiology and stress signaling activation should be taken seriously when addressing the major problem of AMR.

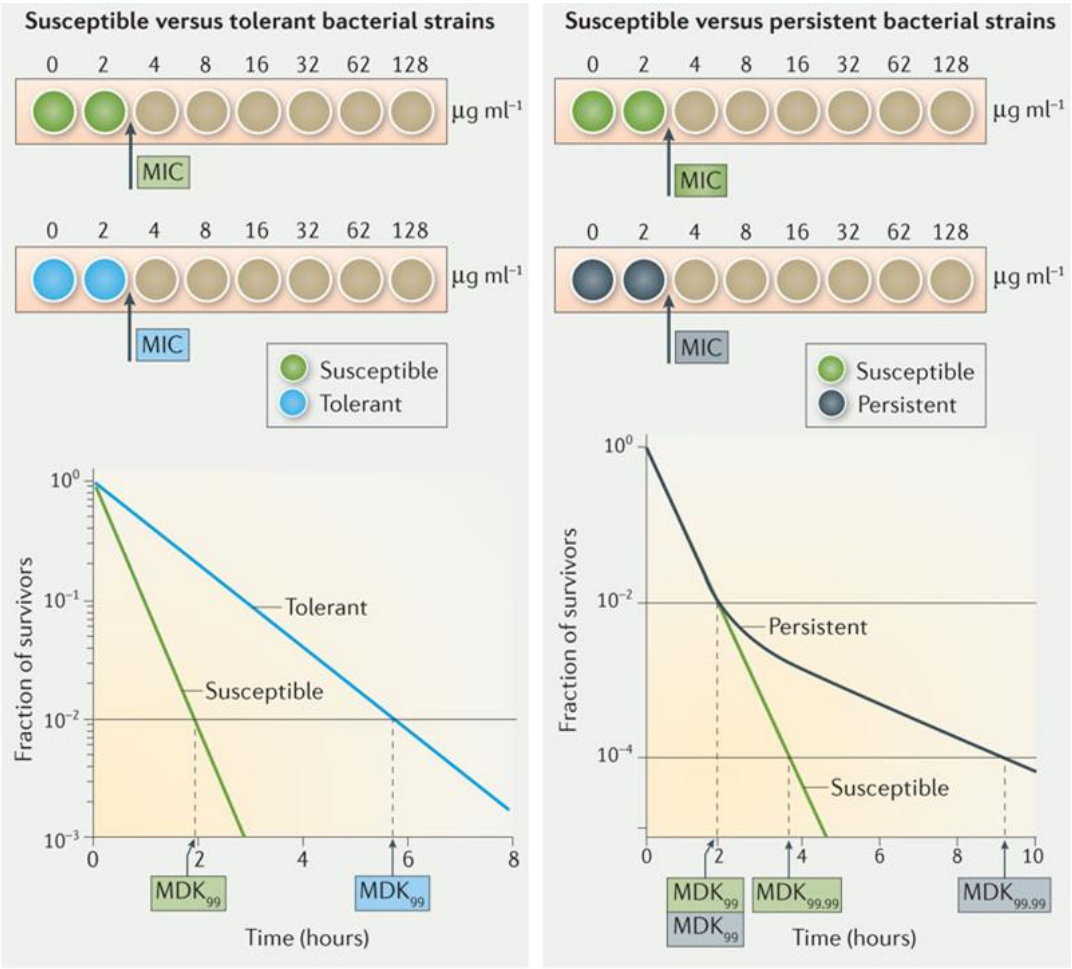


FIG 6. Killing curves dynamics of tolerant and persistent bacterial strains. The panel on the left shows that killing curves of both susceptible and tolerant populations are monophasic but with a higher Minimal Duration for Killing (MDK) in the case of tolerant strains (in the given example, MDK_{99} corresponds to the time needed to kill 99% of the population). The panel on the right shows that the killing curve of persistent bacterial strains is biphasic. In the given example, the MDK for 99% of the population of persistent strain is the same than the susceptible one, but it takes longer to kill 99.99% of the persistent strain (higher $MDK_{99.99}$) due to the presence of a sub-population of tolerant cells. Note that the MIC is the same in all cases. Concentrations and timescales chosen are for illustration purposes only. Adapted from (157).

III. DNA methylation in bacteria

The biological information contained in a DNA molecule is not only represented in its nucleotide sequence. In fact, there is an additional layer of information that goes beyond the genetic level (epigenetics). DNA modification consists on the chemical modification of nucleotides without altering its sequence. The most studied type of DNA modification throughout all kingdoms of life is DNA methylation.

DNA methylation is a biochemical process consisting on the enzymatic addition of a methyl group to adenine and cytosine in the DNA molecule. This reaction is catalyzed by enzymes called DNA methyltransferases (DNA MTases) that transfer a methyl group from S-adenosyl-L methionine (SAM) to adenines and cytosines in specific DNA motif contexts (162, 163) As a result, one can find the existence of small amounts of N6-methyl-adenine (6mA), C5-methyl-cytosine (5mC) and N4-methyl-cytosine (4mC) in the DNA of both eukaryotes and prokaryotes. In the latter, the existence of this modified DNA bases have been shown to play a role in critical processes such as in protection against invasive DNA, DNA replication and repair, cell cycle regulation and control of gene expression (163, 164).

Mechanistically, as these epigenetic marks protrude from the major groove of the double helix, methylation of specific sites may result in the alteration of DNA curvature (165), affecting its mechanical properties and binding of transcription factors or other proteins that directly interact with the DNA molecule (166, 167). DNA methylation is thus a factor that affects DNA-protein interaction. 5mC is the epigenetic mark most commonly found in eukaryotes, and it is associated to repression of gene expression through inhibition of TF binding (168). In prokaryotes the majority of the cases are 6mA although regulation by 5mC and 4mC also exist (169–172).

In bacteria, DNA methylation was initially studied in the context of Restriction-Modification systems (R-M). R-M systems are composed by a restriction endonuclease (RE) and a cognate DNA methyltransferase, which protects DNA from endonucleolytic cleavage by the endonuclease. Hence, these systems provide bacteria with a clever defense mechanism against exogenous DNA (such as bacteriophage DNA): upon invasion, the foreign and unmethylated DNA of the phage is rapidly digested by the endonuclease while that of the host remain protected (173, 174). Interestingly, besides the adaptive value of these systems in bacterial immunity, a Type I RM system was recently

found to play an additional role in regulation of gene expression through phase variation, thus contributing to phenotypic variation (175).

3.1 DNA Orphan methyltransferases in γ-proteobacteria

Besides those associated to RM systems, several species of bacteria also encode DNA methyltransferases without any associated cognate restriction endonuclease. Because these enzymes lack a RE partner, they are called Orphan (or Solitary) Methyltransferases. The reason for the existence of Orphan DNA MTases in some bacterial species is highly debatable in the field (176). One hypothesis is that, throughout evolution, the restriction endonuclease partner was simply lost. A second hypothesis suggests that Orphan DNA MTases may have been acquired through HGT and maintained under strong selective pressure (177). In fact, some orphan DNA MTases have major roles in processes like DNA replication (178–180) and MMR (181) and in some species they even have become essential proteins in standard lab conditions (180, 182).

3.1.1 Orphan DNA adenine methyltransferase Dam

In bacteria, the most well characterized orphan DNA MTase is the Dna adenine methyltransferase (Dam) (183). Dam is well conserved in γ-proteobacteria and *E. coli* is the favorite model to study this enzyme (176). Dam is a 31 kDa protein that functions as a monomer and methylates the adenine nucleotide in the palindromic motif 5'-GATC-3' (184). DNA methylation is a post-replicative process. After the passage of the replication fork, the newly synthetized strand is unmethylated for a short period of time. During this period, DNA is kept at an hemimethylated state. Shortly after, depending on the abundance and processivity of the DNA MTase, the new strand is methylated, originating a new fully methylated DNA molecule. However, some proteins with high affinity to hemimethylated DNA are able to bind it and prevent methylation by Dam – a process called methylation hindrance (169). If methylation hindrance persists, then after two rounds of replication the DNA will be unmethylated.

Interestingly, methylation hindrance depends on processivity of Dam. With a high processivity activity, Dam is able to methylate several GATC motifs within the same DNA molecule without unbinding (185). However, processivity may vary depending on genome context as it was observed

for Dam-dependent regulation of the pap operon in *E. coli* (163). Moreover, Dam's enzymatic activity is thought to be affected by GATC-flanking AT-rich sequences (186). Since its discovery, Dam-dependent methylation has been show to play a role in several relevant cell processes (163).

3.1.1.2 The role of Dam in chromosomal replication

DNA replication reinitiation in *E. coli* is modulated by the methylation of GATC sites present in *oriC* region (the origin of chromosome replication) (187, 188). As said above, DNA methylation is a post-replicative process. Thus, immediately after replication of DNA, GATC sites in *E. coli* exist in an hemimethylated stated but become fully methylated by Dam shortly after. The exceptions are the 11 GATC sites present in *oriC* as well as the 8 sites in the promotor of *dnaA* (189) (which encodes the replication initiation factor that binds *oriC* for replication initiation). These GATC sites remain in an hemimethylated state because they are bound by SeqA (a protein with high affinity for hemimethylated GATC motifs) which protects them from Dam methylation (190, 191). Thus, SeqA-mediated occlusion of *dnaA* promoter prevent expression of this gene while SeqA-mediated occlusion of *oriC* prevents further binding of DnaA to this region (192). This double control of DnaA activity avoids multiple firing of *oriC* so that reinitiation cannot occur until after cell division.

This elegant regulation of chromosome replication by Dam methylation is complex but it doesn't render Dam essential in *E. coli.* However, that is not the case for *V. cholerae* which possesses two chromosomes. In this pathogen, replication of the second chromosome is triggered by binding of the initiation factor RctB to the origin of replication of the second chromosome (*ori2*) (193, 194). This binding is only possible if the GATC motifs present in *ori2* are fully methylated, thus rendering Dam essential in *V. cholerae* (195, 196).

3.1.1.3 The role of Dam in methyl-directed mismatch repair (MMR) system

Besides its role in DNA replication, Dam was also found to play a critical role in methyl-directed mismatch repair (MMR) (151). Here, after detection of a mismatched base pair by MutS, MutL binds to MutS and then it recruits the MutH endonuclease. MutH recognizes the nearest hemimethylated GATC motifs and cleaves the phosphodiester bond of the 5' guanine in the non-methylated strand

(the strand with the wrong nucleotide). Then, exonuclease UvrD cuts this single strand and DNA polymerase III resynthesize it, hence correcting the mismatch. Thus, Dam activity and abundance allows for proper strand discrimination and repair (197). Accordingly, both deletion or overexpression of *dam* were shown to lead to higher mutation rates in *E. coli* (198, 199). Deletion of *dam* results in unmethylated GATC motifs and thus MutH cuts indiscriminately leading to a probable cut of the correct template strand and fixation of the mutation in the DNA (199). On the other hand, overexpression of *dam* results in full methylated GATC motifs which cannot be cut by MutH and, as a result, the mismatch is not repaired (198).

3.1.1.4 The role of Dam in transposition

Transposition events were also found to be controlled by the methylation state of specific GATC motifs present in the promoter of the transposases *IS10* and *IS50* in *E. coli* (200, 201). In addition, the methylation state of GATC within the end sequences of these transposons modulate binding of the transposase, further limiting transposition (201). While fully methylated GATCs block transcription and activity of this transposases, upon DNA replication the hemimethylated state of these motifs allow this regulation and induce transposition activity during cell growth.

3.1.1.5 The role of Dam in bistability and phase variation

As seen above, methylation of DNA has the potential to modulate DNA-protein interactions and thus, it is not surprising that it can affect gene expression which depends on the binding of several proteins (RNA polymerase, transcription factors, etc) to regulatory regions of genes. In fact, Dam-dependent methylation is able to create alternative methylation patters in the promoters of some genes hence controlling the expression of bistable loci. This creates a regulatory mechanism that results in the existence of subpopulations of bacteria with different levels of expression for certain genes – phase variation. Phase variation is thus able to induce phenotypic variation within a bacterial population, which has a high adaptive value in rapidly changing environments and in response to sudden stresses.

In *E. coli* one of the best studied examples of Dam-dependent phase variation expression is that of the Pyelonephritis-associated pili (Pap) of uropathogenic *E. coli*, a surface antigen that mediate adhesion to the mucosa in the urinary tract (202). Since these pili are highly immunogenic but still needed for proper host invasion, a balance between cells that express ("ON") and cells that repress ("OFF") these pili is crucial. This balance is mediated by Dam methylation which regulates phase-variation of the operon *papBA* encoding the Pap pili (Fig. 7) (164, 202). The regulatory region of this *papBA* possesses six binding sites for the leucine-responsive regulatory protein Lrp. Sites 1-3 are located immediately upstream of the operon (proximal sites) while sites 4-6 are farther (distal sites). Lrp sites 2 and 5 contain each an overlapping GATC motif (designated $GATC^{prox}$ and $GATC^{dist}$, respectively). In the OFF state, Lrp binds sites 1-3 and prevents binding of RNA polymerase and expression of *papBA*. The presence of Lrp in the proximal sites further prevents its binding to the distal sites. Due to the binding of Lrp in the proximal site, $GATC^{prox}$ becomes unmethylated after the passage of the replication fork. Given that Lrp has a high affinity for unmethylated binding sites, the OFF state is perpetuated after each replication cycle. The passage to ON state depends on the translocation of Lrp from sites 1-3 to sites 4-6. This translocation is aided by the protein PapI that binds Lrp and dramatically increases its affinity to sites 4-6. Hence, $GATC^{prox}$ becomes available for Dam methylation which prevents Lrp binding to sites 1-3 and leads to expression of *papBA.* PapB activates papI transcription, thereby creating a positive feedback loop and propagation of the ON state (164, 203)(Fig. 3).

Transition from ON to OFF is not totally full understood but it is suggested to occur during chromosome replication which leads to release of Lrp-PapI from $GATC^{dist}$. Then, a 2-fold increased preference of Lrp for the $GATC^{prox}$ dictates the binding of this regulator to the proximal sites leading to the OFF state (203).

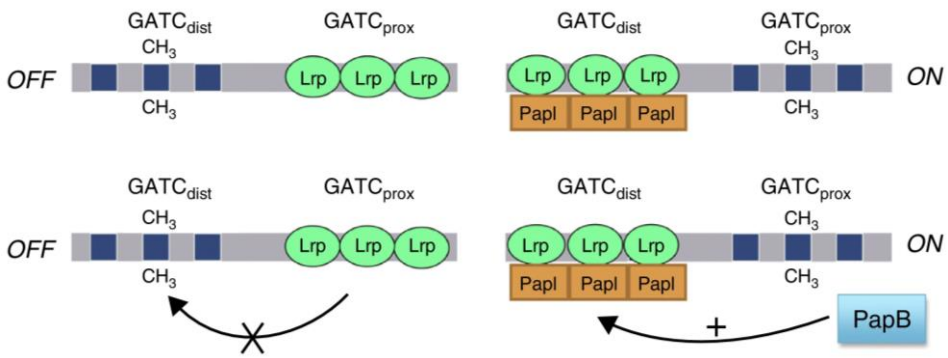


FIG. 7. Regulation of Pap operon. There are six Lrp binding motifs (in dark blue) within this region. Three of these sites are immediately upstream of the operon (proximal sites) while the other three are located farther away (distal sites). One GATC motif overlaps with one of the Lrp proximal sites ($GATC^{prox}$), while a second GATC overlaps with one of the Lrp distal sites ($GATC^{dist}$). In the OFF state, Lrp binds to the proximal sites and prevents binding of RNA polymerase and expression of *pap* operon. In the ON state, PapI binds Lrp and dramatically increases its affinity to the distal sites. The $GATC^{prox}$ becomes available for Dam methylation, which prevents further binding of Lrp to the proximal sites and allow expression of the operon. One of the products of the Pap operon is PapB, which stimulates expression of PapI thereby creating a positive feedback loop that locks the ON state. Taken from (164).

This is not the only example of Dam-dependent phase variation regulation. In fact, it has been shown that this type of regulation exists for several other genes. For example, the regulation of the agn43 gene of *E. coli*, which encodes an adhesin important for auto-aggregation and biofilm adhesion, is also modulated by the hemimethylated/methylated states of the three GATC motifs in the promoter of this gene. Differential binding affinities and abundance of certain proteins dictate the availability of these sites for methylation by Dam during DNA replication, hence controlling the ON/OFF transition of this gene.

In *S. enterica* several operons are also regulated through similar mechanisms, all dependent on Dam-dependent methylation of GATC motifs. These include *gtr*, encoding glucosyltransferases that add sugars to the *O*-antigen lipopolysaccharide (204); *opvAB,* which encodes membrane proteins that reduce the length of the same antigen (205, 206); and *std,* which encodes fimbriae that are important for proper adhesion in the large intestine (207).

In all of the previous examples, Dam methylation plays an important role in the control of phase-variation expression of genes that someway are important for bacterial virulence. The phenotypic variability generated through these mechanisms may reflect a bet-hedging strategy where heterogeneous populations are able to adapt to different future challenges.

3.1.1.6 The role of Dam in bacterial virulence

In addition to some of the previous cases, where Dam controls phase-variation expression of some virulence determinants, several transcriptomic studies have shown an important number of virulence genes affected in *dam* depleted strains of *S.enterica* (208, 209). In fact, a transcriptomic study in *S. enterica* SL1344 observed a considerable number of virulence genes downregulated in two different *dam*- strains, relative to the WT. Among those were invasive genes of pathogenicity island

SPI-1 (209). Moreover, lack of Dam results in virulence attenuation of several pathogens such as *Klebsiella pneumonia* (210), *Yersinia pseudotuberculosis* (211) and *Haemophilus influenza* (212).

3.1.2 Orphan DNA cytosine methyltransferase Dcm

Dcm is an orphan DNA cytosine methyltransferase in *E. coli* found to methylate the second cytosine in 5' – C<u>C</u>WGG – 3' motifs (where W=A or T) (183, 213). Moreover, a study suggests that ~1% of the cytosines of *E. coli* K-12 are methylated and that Dcm is the only DNA MTase responsible for cytosine methylation in this species (172). It is homologous to the methyltransferase of a plasmid encoded *EcoRII* R-M system known to induce postsegregational cell killing. Interestingly, a study showed that Dcm is able to protected CCWGG from restriction by *EcoRII* and thus participate in cell defense against selfish *EcoRII* R-M systems (214).

The biological significance of *dcm* is not totally clear. The *dcm* gene is not essential in *E. coli*, although some studies have shown its potential in controlling gene expression. Namely, it was shown that the expression of two ribosomal protein genes was repressed by Dcm in stationary phase but not in exponential phase. Interestingly, these genes have several CCWGG motifs in their coding region and only one CCWGG motif located 364 bp upstream of the initiation codon, raising the possibility that methylation control of gene expression is not only restricted to the regulatory regions of the genes (172). Moreover, a subsequent study assessed the role of Dcm in several growth phases using microarrays and discovered that the expression of a large set of stationary phase specific genes were further increased in an *E. coli dcm* mutant. Among these genes was *rpoS*, the general stress response main regulator. Thus the authors suggest Dcm modulates the expression of stationary phase genes in *E. coli* through increased *rpoS* expression, although the exact mechanism remains to be elucidated (215). Later, another study showed Dcm represses *sugE*, a RpoS- controlled gene, member of the small multidrug resistance family and shown to confer resistance to Ethidium Bromide (ETBR) in *E. coli*. The authors showed that a Dcm knockout mutant was thus more resistant to ETBR than the wild-type strain and that the effect was dependent on the upregulation of *sugE* in the *dcm* mutant (216).

In *E. coli* the *dcm* gene is part of an operon containing the *vsr* gene, which encodes the Vsr, a protein of the very short patch repair system (VSPR) (217, 218). This arrangement may be explained given the mutagenic potential of m5C in the DNA: 5-methyl cytosine is spontaneously deaminated,

giving origin to thymine (219). As a consequence, a T:G mismatch is formed. The endonuclease Vsr recognizes and, together with other proteins, repairs this mismatch, substituting thymine for cytosine (218, 220). The presence of *vsr* next to *dcm* is thus necessary to compensate for the mutagenic effect of m5C (218). However, certain species encoding m5C methyltransferases (such as *V. cholerae* or *Helicobacter pylori*) lack this or a similar kind of repair mechanism. In these bacteria m5C is considered a mutational hotspot. Accordingly, m5C methylation by a specific cytosine methyltransferase of *H. pylori* was found to contribute to the high mutation frequency in this species (171).

3.1.3 Orphan DNA methyltransferases in *Vibrio cholerae*

V. cholerae is a Gram-negative pathogen and causative agent of cholera. Similar to what happens with *E. coli,* the majority of the studies on DNA methylation in *V. cholerae* have been on the DNA adenine methyltransferase Dam and its role on chromosomal replication. As said in *section 1.3.1.1*, Dam is essential in *V. cholerae* as the binding of the replication initiator RctB to the origin of replication of chromosome 2 is dependent on the methylation of the multiple GATC motifs present in this region (195, 196). However, *V. cholerae* O1 El Tor N16961 encodes more three DNA MTases: *vc1769* – a homologue of the type I R-M system *hsdM*; *vca0447* – a putative DNA orphan methyltransferase; and *vca0198* (*vchM*) – a m5C DNA orphan methyltransferase (110, 221). The last two being the main focus of this thesis.

3.1.3.1 VchM – a DNA Orphan m5C methyltransferase

Although *V. cholerae* strain N16961 lacks homologues of the *E. coli dcm* and *vsr* genes (222), it was found to encode a putative cytosine methyltransferase without any apparent restriction enzyme associated to it (221).

Banerjee and Chowdhury, 2006, characterized this gene and demonstrated that it encodes an Orphan DNA MTase that methylates the first cytosine in 5'-RCCGGY-3' motifs in *V. cholerae* strain O395 genome and named it VchM (221). The authors also questioned the presence of *vchM* in other serogroups and biotypes of *V. cholerae* and through PCR they concluded that the *vchM* gene could be amplified in all six strains belonging to *V. cholerae* serogroup O139 and in six of the seven non-O1

non-O139 strains tested. However, lack of amplification indicated the absence of *vchM* in strains E82 and V94.

vchM is located in the chromosome 2 of *V. cholerae* N16961 (locus *vca0198*) Downstream of *vchM* lies an operon composed by three unidentified ORFs, *vca0199*, *vca0200* and *vca0201*. Interestingly, the region containing *vchM* and *vca0199-vca0201*, has a lower GC content, relative to the rest of the genome, suggesting this segment may have been acquired horizontally. This hypothesis is further supported by the presence of a IS*1004* transposase gene immediately upstream of *vca0201*.

vchM mutants were found to have a lower mutation frequency relative to the WT strain. This observation is in accordance to what is known about the mutagenic potential of m5C in genomes missing a very short patch repair-like system (see *section 1.3.1.2*). In fact, analysis of *rpoB* sequences of spontaneous rifampicin mutants of *V. cholerae* WT cells, showed the presence of C -> T mutations, occurring at the first C of at least one of three RCCGGY motifs present in this gene. Thus, VchM imposes a higher mutation frequency in *V. cholerae* (221).

Recently, a new study used several high-throughput approaches in order to better characterize *VchM* (110). By constructing deletion mutants of *vchM*, the authors observed that VchM is required for optimal growth of *V. cholerae,* both *in vitro* and during infection of mice, even though the biological processes responsible for the intrinsic growth defect observed in these mutants remains to be elucidated. In addition, bisulfite sequencing analysis of *V. cholerae* shows that all cytosines within RCCGGY motifs were methylated in *V. cholerae*, during exponential and stationary phases, with the exception of three of these sites. Curiously, these three specific motifs had been previously shown to be constantly undermethylated in *V. cholerae* (223). As all of these sites lie in intergenic regions, it is thought that their lower methylation frequency is due to blocking, through binding of transcription factors (223).

In addition, using Transposon Insertion Sequencing (TI-Seq) to assess genetic interactions between *vchM* and other genes, the authors discovered a higher number of insertions of the transposon in genes needed for the activation of the envelope stress response, including the essential sigma factor *rpoE* (110, 224). This suggests that the absence of *vchM* allows for disruption of *rpoE* in *V. cholerae*. Further investigation led the authors to conclude that methylation of the three RCCGGY motifs present within the coding region of vc2437 (a gene important for LPS structure), represses its

expression, leading to envelope instability and increased RpoE levels in the cell. On the other hand, in a *vchM* mutant, all the three RCCGGY motifs are unmethylated, expression of vc2437 increases and leads to cell envelope stability, thus allowing for disruption of *rpoE* in this context (110).

3.1.3.2 Vca0447 – a putative orphan DNA methyltransferase

The analysis of *V. cholerae* N16961 genome sequence reveals a locus, vca0447 in the chromosome II, encoding a new putative orphan DNA methyltransferase. Contrary to VchM, VCA0447 is not required for optimal *in vitro* nor *in vivo* (110). To this date, the function and targets of VCA0447 remain a mystery.

3.2 DNA methylation, stress and antibiotic resistance

As previously said, DNA methyltransferases were initially studied in the context of bacteria R-M systems but it is now obvious that their role in shaping bacterial evolution goes beyond its role in bacterial defense. With the advent of powerful methylome analysis tools, such as single-molecule real-time sequencing (SMRT sequencing) (225), the number of studies characterizing new DNA MTases and the methylome of several bacterial species have dramatically increased in the last years. Interestingly, these studies have made clear the involvement of DNA MTases in controlling gene expression and affecting processes such as virulence, sporulation and antibiotic resistance (170, 226–230).

The acquirement of Orphan DNA methyltransferases through HGT and their retention in bacterial genomes after loss of restriction endonuclease partners are indicative of their adaptive value. Indeed, their integration within the regulatory network of relevant processes and genes have made them essential proteins in some species (180, 182). In others, they are able to directly or indirectly modulate sigma factors levels thus controlling important bacterial stress responses (110, 215).

One key aspect of bacterial DNA methyltransferases is their capacity of generating heritable phenotypic variability, like it happens with Dam (202, 206, 207). The epigenetic marks added to DNA

by this enzyme can induce phase-variation in bacteria, allowing them to survive highly dynamic environments by modulation the expression levels of certain genes on an ON/OFF manner. This allows for the existence of phenotypically heterogeneous sub-populations which have been linked to the emergence of antibiotic tolerance and persistence (158, 231–234). In turn, survival of these tolerant cells during antibiotic treatment may give cells the chance to trigger stress responses and develop genetic antibiotic resistance, as it was already discussed in 2.1.1.1.

Moreover, epigenetics and genetics are intertwined in a more direct way. In fact, the biochemistry inherent to some DNA modifications (such as m5C) contribute to genetic changes in the DNA molecule, as it happens for m5C which is considered a relevant mutational hotspot in bacterial genomes deprived of an appropriate repair system (discussed in 3.1.2). Also, methylation is important for proper repair of post-replicative errors by MMR: both undermethylation or full methylation of GATC sites result in increased mutation frequency, as exemplified in 2.1.1.3.

HGT may also be epigenetically regulated as transposition and conjugation events were shown to be directly modulated by methylation (200, 201, 235).

Considering all this, it is important to acknowledge the potential of DNA methylation and DNA methyltransferases as important modulators of bacterial stress response and antibiotic resistance.

IV. Thesis project

Antibiotic resistance is currently one of the biggest threats to global health. Many of the mechanisms responsible for resistance development are associated with the overuse of antibiotics and their cycling in the ecosystems, mainly at residual concentrations. In the past decades, a substantial amount of evidence has shown us that bacteria can sense such residual concentrations of antibiotics that work as signaling molecules inducing several molecular responses and mechanisms which lead to phenomena such as antibiotic tolerance and, later, antibiotic resistance.

Our lab has been studying the response of *V. cholerae* to low doses of aminoglycosides, and have uncovered some mechanisms employed by this pathogen in respond to subMICs of this class of antibiotics. Results from these studies have shed evidence that differential DNA (or RNA) methylation is likely among those mechanisms. DNA methylation is an epigenetic mechanism that can contribute to phenotypic heterogeneity and it has been shown to modulate important stress responses in bacteria. Thus, the main objective of this thesis is to unveil how DNA methylation is linked with the response to low doses of aminoglycosides in *V. cholerae*. Specifically:

1) Understand the role of VchM, a cytosine methyltransferase whose deletion seems to be advantageous in low doses of aminoglycosides;

2) Characterize a novel putative DNA methyltransferase Vca0447 – of unknown function and part of heat-shock regulon of *V. cholerae* – that is upregulated by low doses of tobramycin.

The results obtained during my PhD will be structured in the following way:

Part I - I will describe my main findings regarding VchM, which constitute also a manuscript in preparation for publication. I will address how deletion of *vchM* affects response to aminoglycosides and heat stress, highlighting a functional overlap between the two stresses. Moreover, I will suggest a model depicting how cells lacking *vchM* use two different mechanisms to deal with low and lethal doses of aminoglycosides and how translation and protein quality control effectors are playing a role in these mechanisms.

Part II - I will present preliminary results of a recent Transposon insertion sequencing experiment in *vchM* mutant, which showed that t/rRNA processing, modification and chaperones are affected in this mutant.

Part III - contains data relative to an intriguing phenomenon observed during the course of this study and that suggests the existence of additional players controlling the main phenotypes of a *vchM* mutant.

Part IV - I will present the main data obtained from the study of Vca0447. Specifically, I will present evidence showing Vca0447 is controlled by heat-shock response activation as a consequence of subMIC tobramycin exposure. Moreover, I will show Vca0447 does not play a role in DNA cytosine methylation and likely neither in adenine methylation.

RESULTS

Part I - Research article in preparation: "VchM, a DNA cytosine methyltransferase, modulates tolerance to aminoglycosides and heat stress in *Vibrio cholerae*"

Carvalho, A. *et al.* (in preparation)

INTRODUCTION

Antimicrobial resistance (AMR) is currently one of the major menaces to global health. It is estimated that by 2050, 10 million people will be killed per year by drug-resistant infections (119). It is thus important to study and better comprehend the molecular mechanisms through which antimicrobial resistance can be attained.

For the past decades, the over/misuse and production at large scale of antibiotics by pharmaceutical industries has created a serious ecological problem. In fact, a large proportion of the antibiotics ingested are released intact in the environment (123, 124) and found at trace levels or as gradients in various environments (125, 126). Hence, in these environments, one can find the presence of very low doses of drugs commonly referred as subMIC, i.e. under the MIC (Minimal inhibitory concentration). Although not enough to kill or prevent the growth of bacterial populations, subMIC doses of antibiotics are proposed to work as signaling molecules (149) and trigger important stress mechanisms that often result in development of antibiotic resistance (126). Moreover, the responses that subMIC doses of certain antibiotics trigger in bacteria may vary depending on the species. For instance, we have previously shown that subMIC doses of antibiotics that do not target DNA (such as aminoglycosides, chloramphenicol and tetracycline) are able to induce SOS in the pathogen *Vibrio cholerae* but not in the common model *Escherichia coli* (139). Thus, species-specific factors may determine different responses to the same drug treatment.

In a previous study, in order to characterize the response of *Vibrio cholerae* to low doses of aminoglycosides, we used a powerful screening tool - Transposon Insertion Sequencing (TI-seq) - to identify the genes whose inactivation is detrimental or essential for bacterial fitness during growth

in presence or absence of low doses of the aminoglycoside Tobramycin (TOB) (236). Data obtained from this experiment suggests that transposon insertion (and thus disruption) in locus *vca0198*, encoding *vchM*, is beneficial in presence of 50% MIC of this aminoglycoside (0.6 µg/ml). *vchM* codes for an orphan m5C DNA methyltransferase that causes DNA methylation at 5'-RCCGGY-3' motifs. (221). DNA methylation is catalyzed by enzymes called DNA methyltransferases (DNA MTases) that transfer a methyl group from S-adenosyl-L methionine (SAM) to adenine and cytosine in specific DNA motif contexts (162, 163) As a result, one can find the existence of small amounts of N6-methyl-adenine (6mA), C5-methyl-cytosine (5mC) and N4-methyl-cytosine (4mC) in the DNA of both eukaryotes and prokaryotes. In the latter, the existence of this modified DNA bases have been shown to play a critical role in processes such as protection against invasive DNA, DNA replication and repair, cell cycle regulation and control of gene expression (163, 164). 6mA constitutes the most well studied epigenetic mark in bacteria but examples of regulation by 5mC and 4mC have also been emerging in the field (110, 169–172).

While it was previously shown that VchM plays a role in the cell envelope stress response of *V. cholerae* (110), no link between this DNA MTase and antibiotic stress has yet been established. Here, we show that tolerance to lethal doses of tobramycin was dramatically increased in a *vchM* deletion mutant. Transcriptome analysis of a Δ*vchM* strain revealed the upregulation of *groESL-2* chaperonin genes, which we show to be essential for the high tolerance to lethal antibiotic doses observed in Δ*vchM.* Although the presence of 4 VchM motifs in *groESL-2* operon, this study shows that DNA methylation of such sites do not seem to be directly involved in transcriptional regulation of this operon.

Moreover, we show that absence of VchM confers cells a competitive advantage for growth in presence of sub-lethal stresses affecting proteostasis. While *groESL-2* did not seem to contribute to the competitive advantage of the Δ*vchM* strain grown under sub lethal aminoglycosides or increased temperature, we showed that HPF, a ribosome hibernation promoting factor, was upregulated in Δ*vchM* cells and it is likely the reason for this phenotype. Additional work is necessary to fully comprehend VchM mediated control of *groESL-2* and *hpf* genes in *V. cholerae.* Based on our results, we propose that while Δ*vchM* displays a fitness defect in the absence of stress, it has a more robust response to proteotoxic stress, possibly through more efficient translation/ribosome protection and protein folding/homeostasis?

RESULTS

1.1. vchM mutant growth defect gradually disappears with increasing concentrations of subMIC aminoglycosides

In order to explore a possible role of *vchM* in the adaptation of *V. cholerae* to aminoglycosides, we constructed an in-frame deletion mutant of *vchM* by allelic replacement with an antibiotic cassette, and compared its growth to the isogenic wild-type (WT) strain, in rich media, with or without increasing concentrations of low doses of tobramycin (i.e. subMIC tobramycin) (FIG 8). Interestingly, we observed that growth of Δ*vchM* mutant in solid media gives origin to smaller colonies when compared to the WT strain (FIG S1). As expected (110), this mutant exhibits a reduced doubling rate when grown in monoculture in antibiotic free rich media (FIG 8A). However, the growth defect of Δ*vchM* becomes gradually more negligible with increasing concentrations of subMIC TOB (FIG 8B-D). In fact, it seems that the growth of Δ*vchM* is not affected at these concentrations. In presence of near-MIC TOB (0.9 ug/ml), Δ*vchM* even displays a clear advantage over the WT (FIG 8D). In order to test whether this response is specific for tobramycin or it extends to other aminoglycosides, we tested the growth of Δ*vchM* in subMIC doses of gentamicin (FIG 8E.) As a control, we also tested the effect of subMIC doses of chloramphenicol, an antibiotic that inhibits protein elongation (FIG 8F). Interestingly, *vchM* mutant displays the same tendency in gentamicin, i.e. increasing concentrations of this aminoglycoside cancels the growth disadvantage relative to the WT. However, in presence of subMIC doses of chloramphenicol, the fitness defect of Δ*vchM* was maintained across all the subMIC tested which suggests that the advantage phenotype observed in this mutant requires correct protein elongation.

Altogether, these results show that absence of *vchM* in *V. cholerae* impacts colony morphology and growth in the absence of antibiotics. However, in presence of sub lethal doses of aminoglycosides (but not chloramphenicol), lack of *vchM* confers cells a more tolerant phenotype to the concentrations tested. It is important to note that this phenotype does not reflect genetic resistance to these drugs, as the MIC values for these drugs are the same in Δ*vchM* and WT strains (FIG S2).

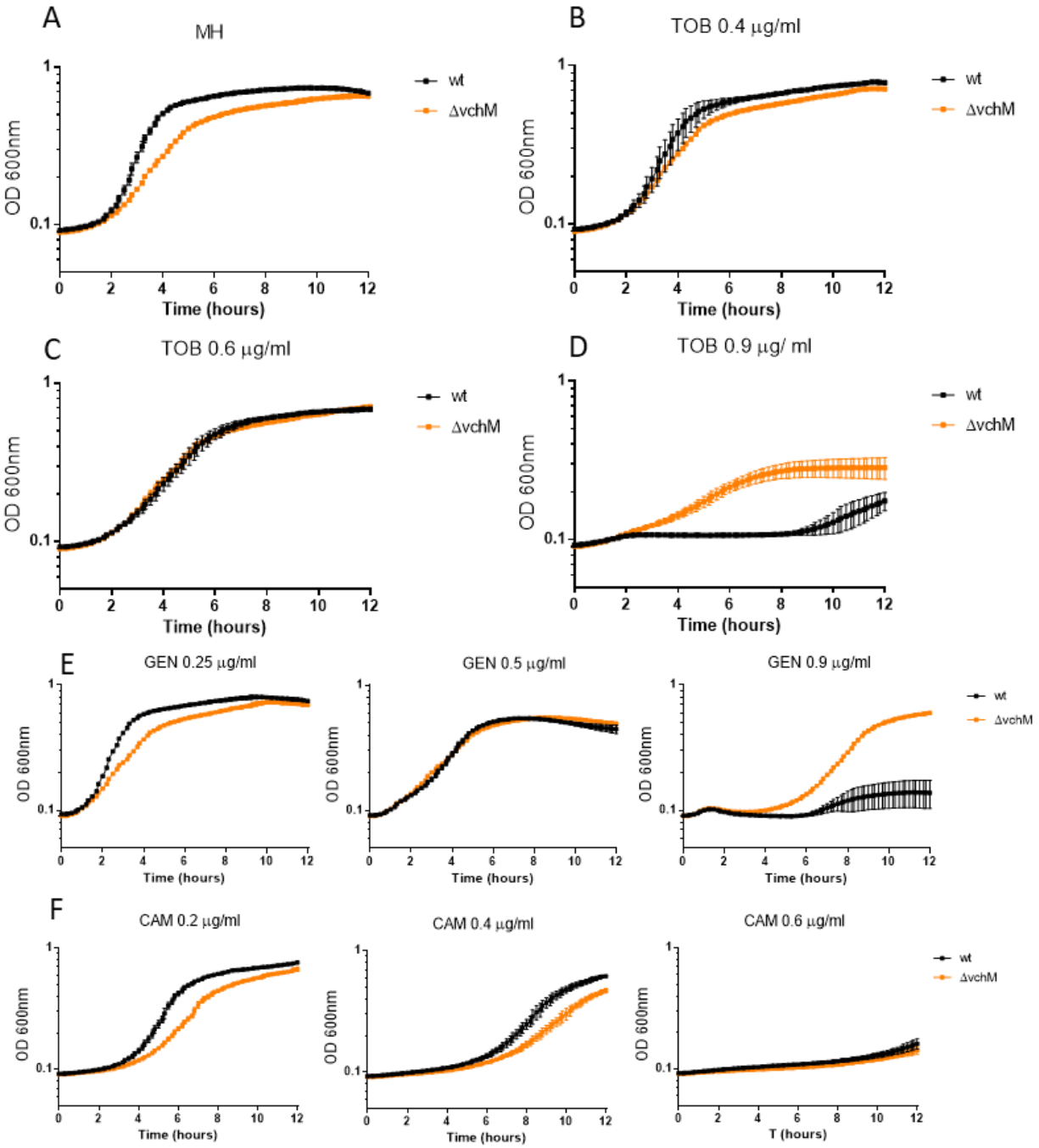


FIG 8. Growth of *V. cholerae* WT and *vchM* mutant in the presence of subMIC concentrations of tobramycin (TOB) **(A-D)**; gentamicin (GEN) **(E)** and chloramphenicol (CAM) **(F)**. Growth was measured with the Tecan Infinite plate reader. MH is rich medium without antibiotic. Standard deviations are represented. n=5. This experiment was repeated at least 3 times.

1.2. ΔvchM has a higher relative fitness in the presence of subMIC TOB

Next we asked whether the growth phenotype observed in monocultures was translatable to a higher relative fitness in co-cultures in the presence of subMIC doses of tobramycin. For that, we competed both WT and Δ*vchM* strains with an isogenic *lacZ*- mutant, with an initial ratio of 1:1, in MH or MH supplemented with 0.6 ug/ml TOB. We then assessed relative fitness by plating cultures after 20 hours of growth. Competition of WT against the *lacZ*- mutant served as a control to exclude any effect of *lacZ*- deletion on growth. As expected, WT competes equally with *lacZ*- mutant in MH and at subMIC doses of tobramycin (Fig. 9). However, accordingly to the previous results, Δ*vchM* is outcompeted by the *lacZ*- mutant in MH (around 10-fold difference). More importantly, in the presence of subMIC TOB, Δ*vchM* displays a clear growth advantage, outcompeting the reference strain by 10-fold (Fig. 9). These results suggest that lack of *vchM* confers a selective advantage to *V. cholerae* in the presence of sub-lethal doses of tobramycin.

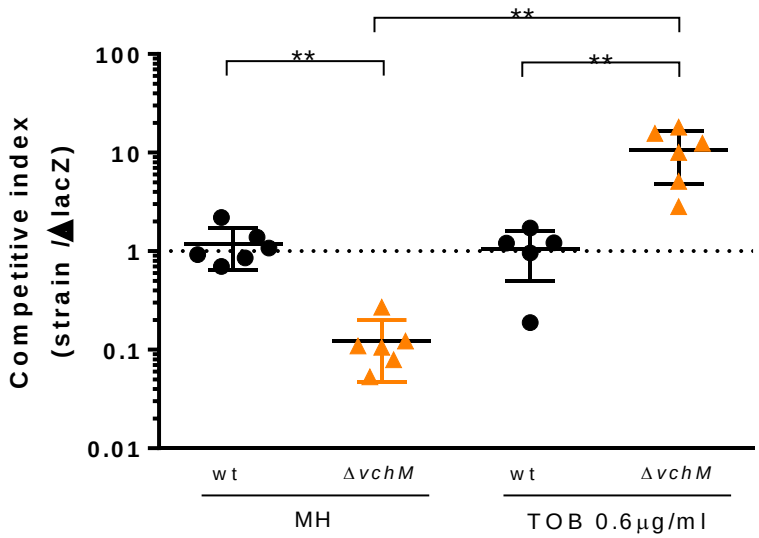


FIG 9. Competitions of *V. cholerae* WT and Δ*vchM* against a *lacZ*- mutant in the presence or absence of 0.6 µg/ml (TOB). After 20 hours in co-culture, cells were diluted and plated in MH agar supplemented with X-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain. ** p-value < 0.01 Mann-Whitney test; n ≥ 5.

1.3. vchM deficiency leads to higher tolerance to lethal doses of aminoglycosides

The growth advantage displayed by the Δ*vchM* mutant under subMIC concentrations of tobramycin and gentamicin is indicative of some form of tolerance to these aminoglycosides. However, we wondered whether the tolerance observed so far in Δ*vchM* under sub-lethal antibiotic treatment could also be observed upon treatment with lethal doses of aminoglycosides. Thus, we assessed the survival rate of stationary phase cells of *V. cholerae* WT and Δ*vchM* strains during treatment with lethal doses of tobramycin and gentamicin at 20X and 10X the MIC, respectively (Fig.10). The reason why we chose to test stationary phase cells was that expression of *vchM* in *V. cholerae* was shown to be higher at this growth phase (221). Thus, its deletion could have a major impact in cell physiology at this phase.

Strikingly, survival to both antibiotics was increased several fold in the Δ*vchM* mutant, suggesting that the absence of *vchM* somehow allows *V. cholerae* to transiently withstand lethal doses of this aminoglycosides (Fig.10).

One crucial aspect that determines the efficacy of aminoglycoside treatment is the uptake of these drugs by bacteria. This process is energy dependent and requires a threshold membrane potential (237). However, preliminary results indicate no difference in membrane potential in Δ*vchM* relative to the WT (not shown). Thus, differential uptake of aminoglycosides is unlikely the reason for the increased tolerance to these drugs in Δ*vchM*.

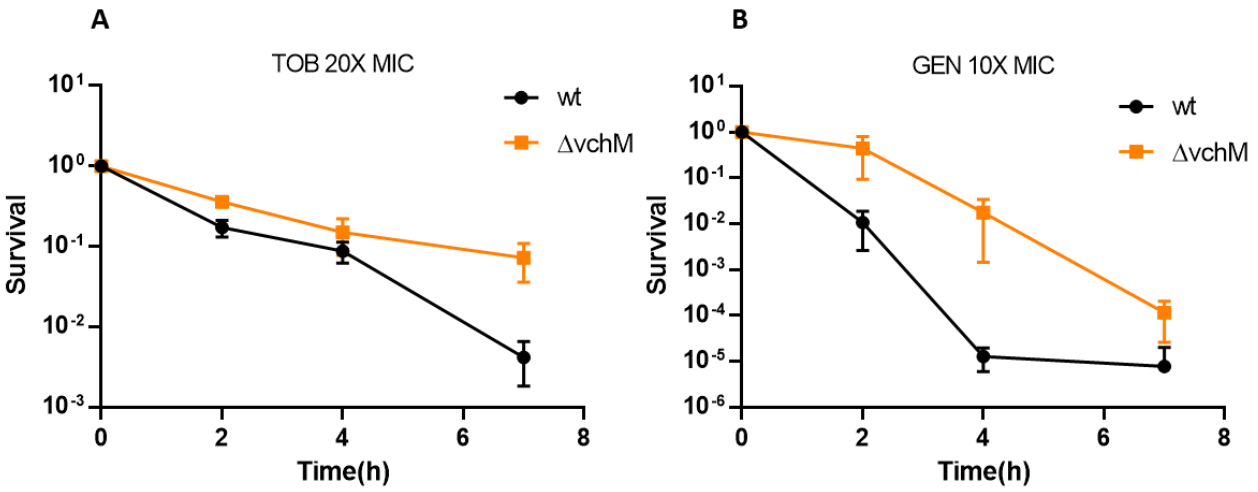


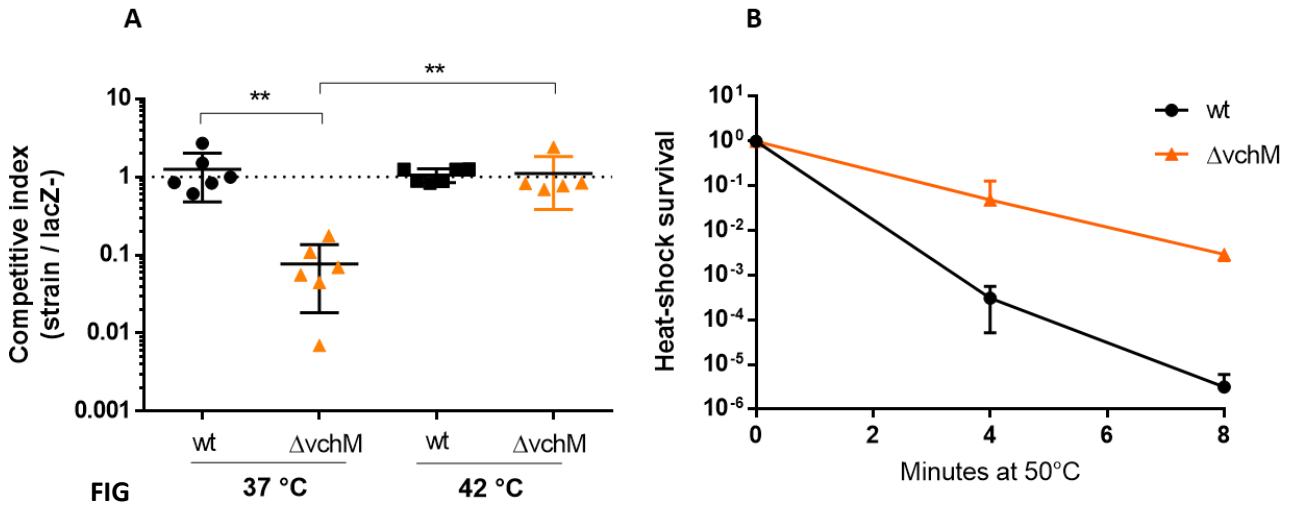
FIG 10. Survival of stationary-phase *V. cholerae* WT and Δ*vchM* cells exposed to lethal doses of TOB (A), and GEN (B). Survival is determined by dividing the cfu/ml at each time point by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. This is a representative experiment of at least three independent experiments.

1.4. ΔvchM relative fitness increases with temperature and is more tolerant to heat-shock

Aminoglycosides are a class of antibiotics that bind directly to rRNA of the 30S subunit of the ribosome causing mistranslation of the genetic code. A direct consequence of mistranslation is the misincorporation of wrong amino acids during peptide synthesis, which leads to accumulation of misfolded proteins, disturbing proteostasis (86, 238, 239). Besides aminoglycosides, other stresses, like increased temperature, can affect protein structure and lead to accumulation of misfolded proteins in the cell (56, 58). To test whether the increased tolerance to aminoglycosides in Δ*vchM* was a consequence of a more robust tolerance to stresses affecting proteostasis we competed WT and Δ*vchM* strains at 42°C, a temperature known to disturb proteostasis in several bacteria, including *V. cholerae* (58, 240, 241). Figure 11A shows that during growth at 42°C, Δ*vchM* is no longer outcompeted by the WT. Moreover, and contrary to what was shown in competitions with 0.6 ug/ml TOB (Fig. 9), the relative fitness of Δ*vchM* did not surpass that of the WT (compare the relative fitness of Δ*vchM* in the presence of 0.6 ug/ml TOB (Fig.9) with that of Δ*vchM* at 42°C (Fig.11A)). One possible explanation for this result is that the proteotoxic stress caused by subMIC TOB is stronger than that caused by growth at 42°C. Thus, the selective advantage of Δ*vchM* would be higher in the first

situation, explaining the higher relative fitness of Δ*vchM.* Independently, in both cases the results indicate that Δ*vchM* mutant has a higher relative fitness in presence of proteotoxic stress than in its absence.

To further evaluate tolerance to proteotoxic stress in Δ*vchM*, we tested survival of *V. cholerae* to heat-shock by incubating WT and mutant strains under lethal temperature. (Fig 11B). Deletion of *vchM* significantly increases survival to 50ºC lethal heat treatment, confirming a higher tolerance to heat stress.



11. **A**. Competitions of *V. cholerae* WT and Δ*vchM* against a *lacZ*- mutant at 37°C or 42°C. After 6 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain. ** p-value < 0.01 Mann-Whitney test; n ≥ 5. **B.** Survival of stationary-phase *V. cholerae* WT and Δ*vchM* cells exposed to lethal temperature (50ºC). determined by dividing the cfu/ml at each time point by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. This is a representative experiment of two independent experiments.

1.5. Chaperonin genes are upregulated in *ΔvchM*

To better understand the high tolerance to aminoglycosides and heat stress observed in Δ*vchM*, we performed RNA-seq on stationary phase cells of WT and mutant strains grown in rich media. The analysis of the transcriptome of Δ*vchM* reveals the significant upregulation (fold change ≥ 2) and downregulation (fold change ≤ -2) of 68 and 53 genes, respectively (Table S1 and S2). Given

the effect of aminoglycosides and heat stress in disturbing proteostasis, we expected genes involved in protein quality control to be upregulated in *ΔvchM*. In fact, four genes belonging to this category were upregulated (Table 1). Those are the molecular chaperones GroEL and co-chaperonins GroES, both members of the heat-shock regulon of *V. cholerae* (242). In many bacterial species, GroEL and its co-chaperonin GroES form a molecular machine essential for folding of large newly synthetized proteins also helping re-folding of proteins damaged by proteotoxic stress (16). Interestingly, overexpression of GroES and GroEL proteins was found to promote short-term tolerance to aminoglycoside-induced protein misfolding in *E. coli* (49). *V. cholerae* harbors two copies of *groES – groEL* (*groESL*) bicistronic operons. *groESL-1* is encoded in chromosome 1 (*vc2664-vc2665*), while *groESL-2* is located in chromosome 2 (*vca0819-0820*). GroES-1 protein shares 66% amino acid identity with GroES-2, while GroEL-1 shares a 76% amino acid identity with GroEL-2 of chromosome 2 (Fig. 12A). In order to confirm differential expression of these genes in Δ*vchM,* we measured *groES-1* and *groES-2* relative gene expression in exponential and stationary phase cells of WT and mutant strains (Fig. 12B). Digital q-RT PCR confirms a higher relative abundance of *groES-2* transcripts in both exponential and stationary phase Δ*vchM* cells with a fold change of 10X and 5X, respectively. However, relative expression of *groES-1* is hardly noticeable. This suggests a different regulation mechanism of *groESL-1* and *groESL-2* operons in Δ*vchM* mutant.

Table 1. Protein quality control genes upregulated (>2 fold) in Δ*vchM*

Locus	Name	Fold change (Δ*vchM*/WT)	Predicted function
vc2665	*groEL-1*	2.24	molecular chaperone GroEL
vca0820	*groEL-2*	3.54	molecular chaperone GroEL
vc2664	*groES -1*	2.60	co-chaperonin GroES
vca0819	*groES-2*	6.78	co-chaperonin GroES

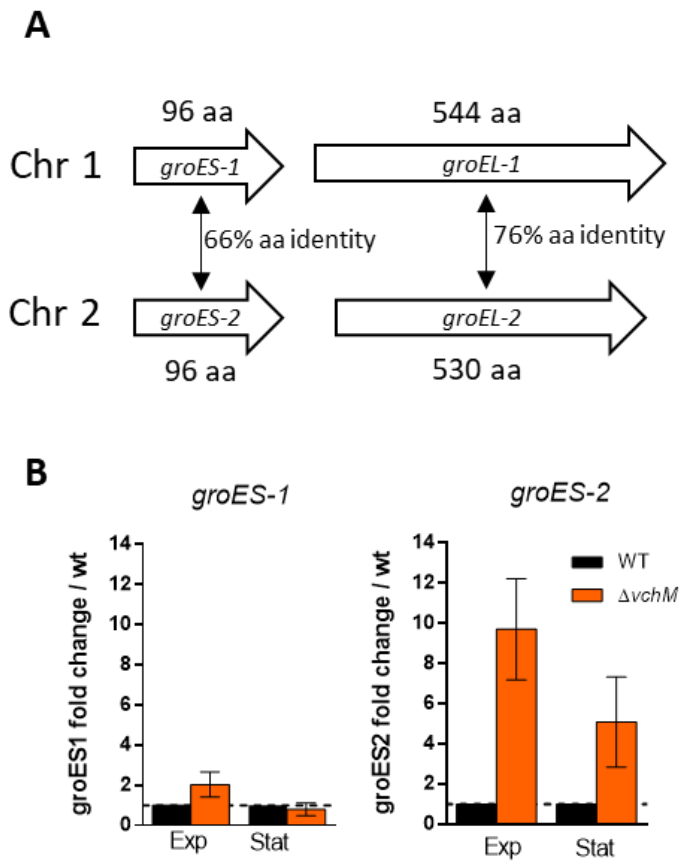


FIG 12. **A**. Representation of the two operons encoding GroES and GroEL proteins in *V. cholerae*. "aa" stands for "amino acid". **B.** Relative expression of *groES-1* and *groES-2* measured by digital qRT- PCR in exponential (Exp) and stationary phase (Stat) cells of WT and Δ*vchM* strains of *V. cholerae*. Standard deviations are represented, n=3.

1.6. Deletion of *groESL-2* operon abolishes *ΔvchM* high tolerance to lethal doses of tobramycin

The previous results led us to hypothesize that the upregulation of chaperonin genes in Δ*vchM* could be the reason for the high tolerance to aminoglycosides and heat stress observed in this mutant. Thus, we tried to delete *groESL-1* and *groESL-2* from *V. cholerae* WT and Δ*vchM* strains. While Δ*groESL-2* and Δ*vchM groESL-2* strains were easily obtained, we could not manage to delete *groESL-1* in either strain after several attempts. This shows that GroESL-1 (but not GroESL-2) is essential for *V. cholerae* viability under our lab conditions. Moreover, it suggests that GroESL-1 is probably the main house-keeping chaperonin system while alternatively, GroESL-2 could act synergistically in response to high levels of misfolding or having specific substrates upon protein

damaged caused by specific stresses. In order to assess the role of *groESL-2* in the high tolerance to aminoglycosides we cloned this operon under a constitutive promoter in a multi-copy plasmid and overexpressed it in *V. cholerae* WT strain. As expected, overexpression of *groESL-2* increases survival of *V. cholerae* subjected to lethal doses of tobramycin. (Fig. S3).

We then compared tolerance of Δ*vchM* to that of a Δ*vchM groESL-2* double mutant and we found that the absence of *groESL-2* abolishes high tolerance to tobramycin in Δ*vchM* (Fig. 13A). Moreover, complementation of the double mutant with a low copy plasmid expressing *groESL-2* from a constitutive promoter partially restored the high tolerance phenotype (Fig. 13B). It is possible that ectopic expression from a constitutive promoter results in unbalanced levels of GroESL-2 protein, needed for full phenotype rescue. Accordingly, it is known that too much GroESL in *E. coli* cells limits RpoH activity and induction of heat-shock response whose regulon may play a synergistic role in response to lethal doses of aminoglycosides (99). Notwithstanding, these results show *groESL-2* plays an important role in Δ*vchM* tolerance to lethal tobramycin.

Interestingly, survival of a Δ*groESL-2* single mutant is comparable to that of the WT strain (Fig. 13A), meaning that in WT cells, GroESL-2 is dispensable in these conditions. We thus suggest that the high level expression of *groESL-2* in Δ*vchM* cells (Fig 12B) is key for its high survival, priming the cells for higher tolerance to future aminoglycoside stress. Intriguingly, although showing a lower tolerance to lethal doses aminoglycosides, the Δ*vchM groESL-2* mutant still outcompetes a WT lacZ- strain in competitions under subMIC TOB or at 42°C (Fig. S4). Thus, the increased expression of *groESL-2* in Δ*vchM* does not explain the fitness advantage of this mutant in these conditions. It is tempting to assume that the level of proteotoxic stress generated by lethal doses of aminoglycosides is much higher from that generated by subMIC stress or increased temperatures. The former would require a more dramatic response and an increased expression of *groESL-2* chaperonins could be an appropriate solution. The advantage of Δ*vchM* in presence of proteotoxic stress caused by subMIC aminoglycosides or growth at increased temperatures may thus involve other factors.

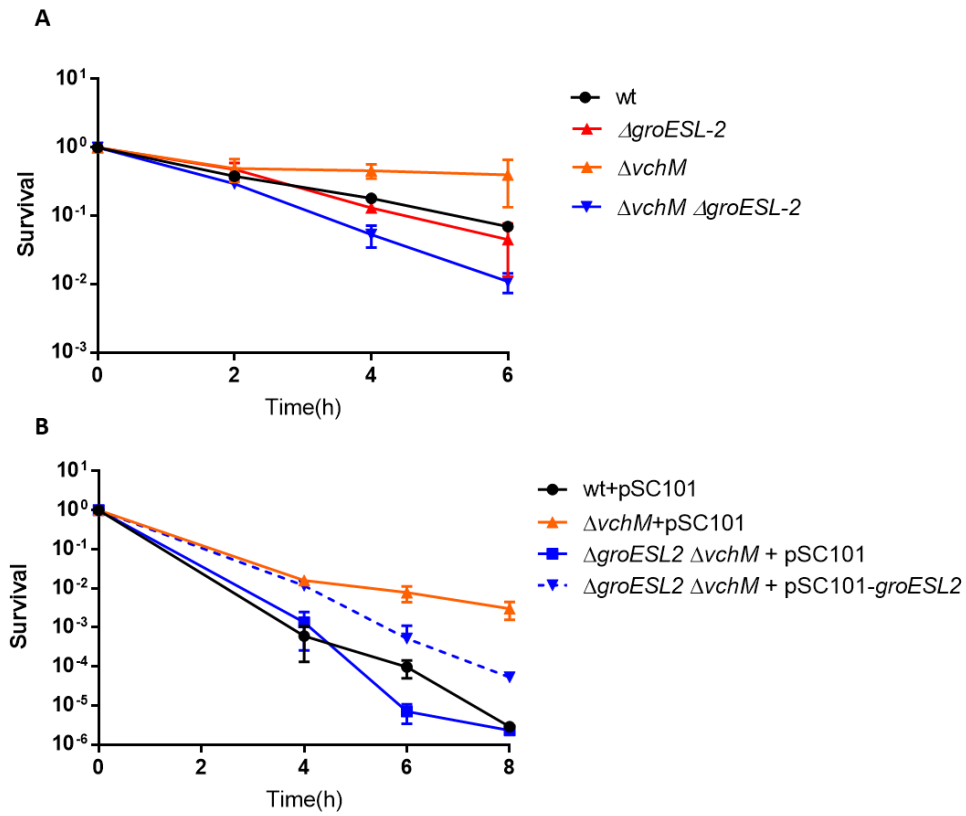


FIG 13. **A**. Survival of stationary-phase of WT, ΔvchM, ΔgroESL-2 and ΔvchM groESL-2 *V. cholerae* cells exposed to lethal doses of TOB 20X MIC (20µg/ml). **B**. Survival of complemented ΔvchM ΔgroESL-2 strain. Survival is determined by dividing the cfu/ml at each time point by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. A and B are representative experiments of at least three independent experiments.

1.7. Mechanism of regulation of *groESL-2* through VchM cytosine methylation

Our digital qRT-PCR data pointed for an elevated expression of *groESL-2* but not *groESL-1*. Moreover, no other heat-shock genes were significantly affected in our RNA-seq data, suggesting that the upregulation of *groESL-2* in Δ*vchM* mutant was not part of a general activation of the heat-shock response. Knowing the role of VchM in regulating gene expression in *V. cholerae* (110), we asked whether VchM controls *groESL-2* expression directly through DNA methylation. VchM is an orphan DNA m5C methyltransferase that methylates the first cytosine in 5'-RCCGGY-3' motifs (221). This prompted us to look for such motifs in *groESL-2* operon. We found a total of four VchM motifs in *groESL-2* region: #1 is within the 5' UTR of the operon, 47 bp away from the initiation codon; motif #2 is within the coding region of *groES-2* and motifs #3 and 4 are located within the coding region of *groEL-2* (Fig. 14A). We speculated that the methylation state of these motifs could modulate the

transcription of these genes. To investigate this, we tried to replace RCCGGY motifs #1-3 with non-consensus sites without altering the protein sequence, in *V. cholerae* WT cells. However, despite multiple attempts, we could not obtain such mutants. We thus decided to clone the *groESL-2* operon with the non-cognate VchM sites in a low copy plasmid (under the control of a constitutive promoter) and compare its expression with that of a WT version of *groESL-2* operon cloned in a similar plasmid. To exclude the endogenous expression of these genes, we transformed these plasmids in a *ΔgroESL-2* mutant. However, we could not observe any difference in expression between the two versions of the operon (Fig. 14B). It is possible that the methylation of these sites affect the transcriptional process more upstream of this operon, at the level of the regulatory region, which also contains a VchM motif itself (RCCGGY #1) (Fig. 14A). If that is the case, we would not see it in our experiment, given that we did not clone *groESL-2* with its native promoter. We are currently addressing this possibility.

We also investigated a possible role of RCCGGY motif #1 to control expression of *groESL*-2. This site is located within the 5' UTR region of the operon and thus is unlikely to directly affect RNA polymerase binding more upstream of the transcriptional starting site (TSS) (Fig. 7A). Yet, it is possible that it affects binding of a DNA binding protein which in turn could affect DNA structure and correct transcription. To explore this possibility, we replaced RCCGGY #1 in the WT strain with R**A**CGGY, which can no longer be targeted and methylated by VchM methyltransferase. We then compared expression levels of this mutant and WT. The results show no differences in *groES-2* expression, independently of the methylation state of the 5' UTR VchM motif (Fig 14C). The mechanism through which VchM controls expression of *groESL-2* remains to be elucidated.

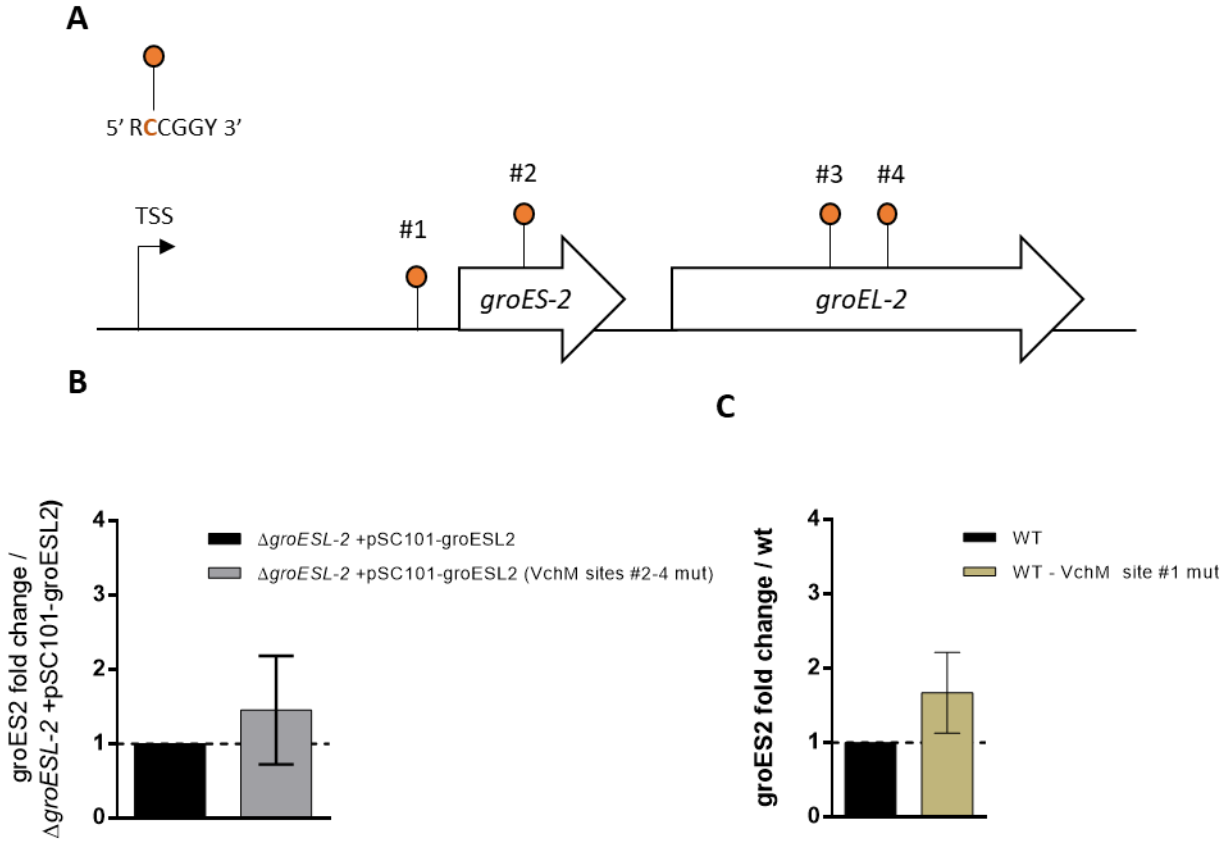


FIG 14. **A**. Representation of the 4 VchM motifs present at *groESL-2* operon region. **B.** Relative expression of *groES-2* measured in strains overexpressing *groESL-2* with VchM sites #2-4 mutated. **C**. Relative expression of *groES-2* measured in strains *groESL-2* with VchM site #1 mutated. Standard deviations are represented, n=3.

1.8. Hpf is needed for ΔvchM's higher relative fitness in low doses of tobramycin

In Δ*vchM* mutant, *hpf* transcript levels are 3X higher than in WT (Table S1). In *E. coli*, *hpf* codes for a ribosome hibernation factor which was recently shown to facilitate tolerance of bacteria to aminoglycosides (243). We thus assessed the role of *hpf* in Δ*vchM* tolerance to aminoglycosides and heat stress by constructing a Δ*vchM Δhpf* double mutant.

Just like the Δ*vchM* single mutant, a Δ*vchM* Δhpf double mutant also formed small colonies on agar plates (not shown) and displayed a growth disadvantage in competitions with a WT strain, in absence of stress (Fig. 15A). Note that a Δ*hpf* single mutant displays a lower relative fitness either in absence or presence of antibiotic, even though it forms normal WT-like colonies in solid media (not

shown). This is likely due to a slight loss of viability at stationary phase caused by the lack of 100S ribosome dimer formation in Δ*hpf* mutants, as previously suggested (244, 245). Importantly, low doses of tobramycin did not aggravate this phenotype. However, while deletion of *vchM* is advantageous in low doses of tobramycin (as previously shown in Fig. 8 and 9), deletion of both *vchM* and *hpf* not only prevents this competitive advantage but it further decreases the relative fitness of cells, comparing to a single Δ*vchM* mutant (Fig. 15A). This highlights HPF requirement for Δ*vchM* strain's tolerance to subMIC tobramycin. On the other hand, unlike for what we observed for *groESL-2*, absence of HPF in a Δ*vchM* strain does not affect its tolerance to lethal concentration of tobramycin as can be seen in (Fig. 15B). This once again reflects the involvement of different factors in response to different levels of aminoglycoside stress.

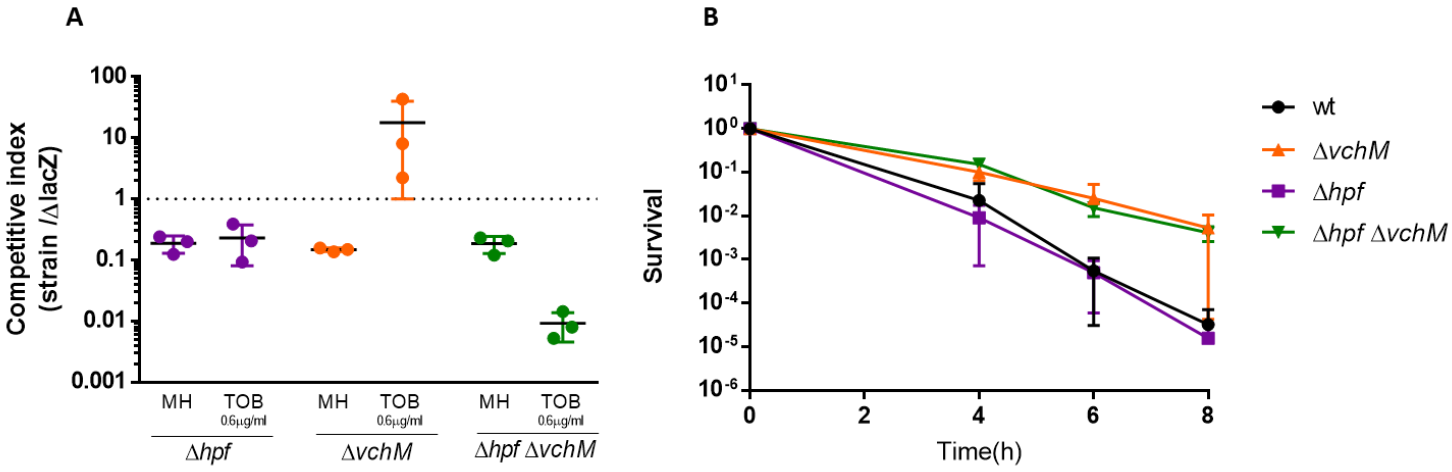


FIG 15. **A**. Competitions of *V. cholerae* indicated strains against a *lacZ*- mutant in the presence or absence of TOB 0.6 µg/ml. After 20 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain, n=3. **B**. Survival of of *V. cholerae* indicated strains to lethal doses of TOB 20X MIC (20µg/ml). Survival is determined by dividing the cfu/ml at each time point by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. A and B are representative experiments of at least two independent experiments.

1.9. Hpf is needed for ΔvchM's higher relative fitness in competitions at 42°C

Given the involvement of HPF in ΔvchM's advantage in subMIC TOB, we asked whether this ribosome-associated factor also determines relative fitness of *V. cholerae* in competitions at 42°C, where an increase in relative fitness was observed for the Δ*vchM* strain (Fig. 11). Interestingly, HPF

deficiency prevents Δ*vchM* cells from increasing their relative fitness at 42°C (Fig. 16). Notice that in this case, where competitions occur for 6 hours, deletion of *hpf* in a WT background doesn't seem to affect relative fitness at any temperature, contrary to what happens in subMIC tobramycin competitions, where cells are grown during 20 hours. We hypothesized that, in the latter, deletion of *hpf* may influence *V. cholerae'*s viability in longer stationary phase, which is reflected in relative fitness at 20 hours.

These results show that HPF is involved in growth advantage of Δ*vchM* mutant at 42°C *vs* 37°C.

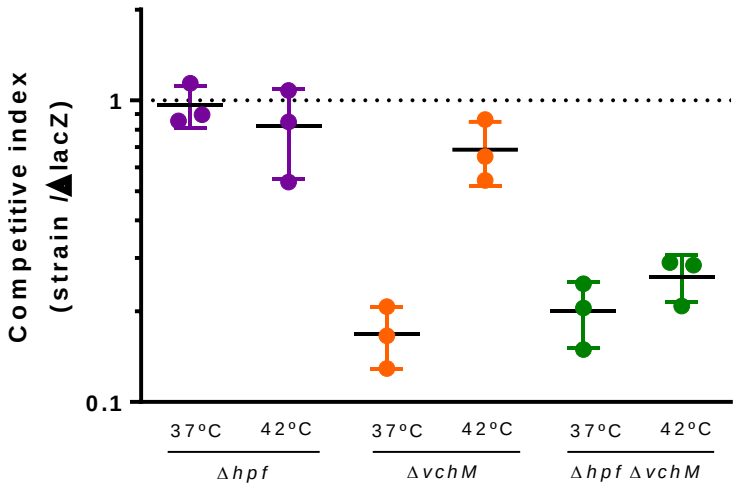


FIG. 16. Competitions of *V. cholerae* indicated strains against a *lacZ*- mutant at 37°C or 42°C. After 6 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain, n=3.

Together, these results suggest that the high expression of the ribosome hibernation promoting factor in Δ*vchM* is crucial for its higher competitive advantage under subMIC TOB and high temperature. However, HPF does not seem to be involved on tolerance to lethal doses of aminoglycosides, where other factors, like high expression of GroESL-2 (see above), have a stronger role.

DISCUSSION

It is widely accepted that the presence of low doses of antibiotics (at subMIC concentrations) in the environment constitute a source of bacterial stress and induce in bacteria a plethora of mechanisms involved in antibiotic resistance (126, 128, 130, 246). Thus, the study of the molecular mechanisms that lead to bacterial adaption in presence of subMIC doses of antibiotics is key to understand antimicrobial resistance.

In this study, we assessed the role of VchM, an Orphan DNA m5C methyltransferase, in *V. cholerae*'s adaptation to both sub-lethal and lethal doses of aminoglycosides, a well-known class of antimicrobial drugs that cause disruption of the translation process and cause protein misfolding (76, 86). We found that deletion of VchM in *V. cholerae* El Tor N16961 strain leads to both an alteration of colony morphology (with Δ*vchM* cells producing smaller colonies than the WT isogenic strain (Fig. S1)), and decreased growth rate in liquid cultures in rich media. While the latter had already been observed in Δ*vchM* mutants of *V. cholerae* strains El Tor C6706 and O395, no reference to the small colony phenotype following deletion of *vchM* can be found (110). More interestingly, we found that Δ*vchM* cells seem to be more tolerant to the negative effects on growth generated by increasing subMIC doses of aminoglycosides (Fig. 8A and B). In other words, subMIC doses of aminoglycosides seem to affect WT cells more effectively than it affects cells lacking VchM methyltransferase. Thus, due to intrinsic growth defect, Δ*vchM* loses *in vitro* competitions with the WT strain in absence of antibiotics, but it strikingly outcompetes WT in presence of subMIC doses of tobramycin (Fig. 9). However, low doses of chloramphenicol (an antibiotic that blocks protein elongation) seem to affect the growth of WT and Δ*vchM* at the same extent which suggests that Δ*vchM*'s competitive advantage in subMIC aminoglycosides is specific of this class of antibiotics. Moreover, we also show that a Δ*vchM* mutant is not only a better competitor in subMIC doses of aminoglycosides but is also more tolerant to killing by lethal doses of tobramycin, a member of this class of antibiotics (Fig. 10).

Aminoglycosides promote mistranslation by increasing decoding of messenger RNA codons by noncognate tRNAs on the ribosome (76, 239). In turn, mistranslation leads to misfolding and aggregation of proteins, which if not taken care of may become harmful to the cell (84, 86, 247). This led us to hypothesize that other stress conditions where protein misfolding is likely to occur, could constitute a selective environment for Δ*vchM*. Indeed, the lower relative fitness of Δ*vchM* cells observed in absence of stress, is cancelled when competitions occur at 42°C (Fig. 11A) a temperature

known to induce protein misfolding (56, 58). Similar to what happened with lethal doses of tobramycin, a higher tolerance to high lethal temperature was also observed for the Δ*vchM* mutant.

Both aminoglycosides and heat-shock stress are known to induce similar responses in bacteria, which makes sense given that both stresses generate a common outcome (83, 248). In line with this idea, a recent study suggests that temperature stress responses have been co-opted to deal with antibiotic stress affecting the same targets (249).

One way to deal with the accumulation of misfolded proteins in the cytosol is to use molecular chaperones that help with the folding and/or re-folding of damaged proteins. Interestingly, there is evidence in the literature suggesting that the overexpression of the well-known chaperonin system GroES-GroEL can promote short-term tolerance to aminoglycoside-induced protein misfolding and help prevent protein aggregation in *E. coli* (49, 250). Interestingly, we found that both *groES-groEL* operons of *V. cholerae* were upregulated in Δ*vchM*, with *groESL-2* genes showing a greater induction (Fig. 12). *V. cholerae* harbors two copies of GroES and GroEL thus belonging to the group of 30 % of bacterial species that harbors multiple copies of these chaperonins (53). Deletion of *groESL-2* abolished Δ*vchM* high tolerance to aminoglycosides showing that its overexpression is crucial for this phenotype (Fig. 13). At this point we don't know if overexpression of *groESL-2* confers aminoglycoside tolerance due to a synergistic effect with *groESL-1* or if it has a different set of substrates and a specialized function in the cell as previously described (52, 251). In support of the latter, deletion of *groESL-2* did not cause any obvious growth defect or increased sensitivity to lethal doses of tobramycin in WT cells, differently from *groESL-1* which seems to be essential in *V. cholerae*. Moreover, *groESL-1* and *groESL-2* have slightly different amino acid sequences, which may indicate their association with different substrates as it happens for example in *Myxococcus xanthus* (252). It would be interesting to test whether the overexpression *groESL-1* would equally contribute to a higher tolerance to aminoglycosides in *V. cholerae*.

VchM is a *V. cholerae* DNA orphan m5C methyltransferase that methylates the first cytosine in 5'-RCCGGY-3' motifs (221). It was shown VchM modulates the envelope stress response of *V. cholerae* by repressing a gene involved in LPS biogenesis. This repression was caused by methylation of three VchM motifs present within the body of that gene, although the exact mechanism of regulation is unknown (110). Our discovery of four VchM motifs spread across the regulatory and coding regions of *groESL-2* genes was a strong indication that methylation of such sites could control its expression. However, despite our preliminary experiments, we could not observe any link

between *groES-2* expression and methylation state of these sites. Alternatively, we cannot exclude the possibility that upregulation of *groESL-2* is a result of the action of an unknown transcription factor whose expression is controlled by VchM.

One intriguing observation during our study was that deletion of *groESL-2* in Δ*vchM* background did not affect this mutant's selective advantage in presence of subMIC doses of tobramycin or during growth at increased temperatures. Thus, high expression of *groESL-2* seems to play a protective role only against lethal doses of aminoglycosides, while at non-lethal aminoglycoside stress or high temperature, other factors must be involved. In fact, the damage caused by lethal doses of a drug is likely very different from that caused by sub-lethal concentrations of the same drug, and thus originate different molecular responses (83, 253).

Interestingly, our analysis of RNA-seq performed in Δ*vchM* strain, also revealed the upregulation of *vc2530*, a locus encoding the ribosome hibernation promoting factor HPF. HPF is a well-conserved ribosome-associated protein involved in ribosome dimerization and formation of 100S dimers. In most γ-proteobacteria, 100S ribosome dimers are formed through the sequential action of ribosome modulation factor (RMF) and HPF: RMF first forms a 90S dimer and then HPF binds to it and further stabilizes into the mature 100S form. On the other hand, in gram-positive bacteria, ribosome dimerization is exclusively modulated by a long form of HPF (lHPF). In these bacteria, two monomers of lHPF bind two 70S ribosomes and, through their C terminal-domain, they interact and form 100S ribosome dimers (254).

The biological significance of ribosome dimerization in bacteria is yet not totally clear, but it has been shown to affect bacterial survival to prolonged stationary phase (244, 245), virulence (255), survival under heat shock (256) and sensitivity to antibiotics, as it was shown ribosome hibernation facilitates tolerance of stationary phase bacteria to aminoglycosides (243).

The link between HPF and tolerance to aminoglycosides can be explained by their binding sites at the ribosome. In fact, the crystal structure of 100S ribosomes shows that HPF binding sites overlap with those of the mRNA, transfer RNA, and initiation factors, which are also known to be the target of aminoglycosides (257–259). Thus, two models explaining how ribosome dimerization affect susceptibility to aminoglycosides have been debated in the field: 1) ribosome dimerization by RMF and HPF result in reduction of overall protein production, thus slowing growth, metabolism and energy production and subsequently diminish aminoglycoside uptake; and 2) Binding of HPF directly

competes with aminoglycoside binding, protecting the ribosome from aminoglycoside action and translational corruption. The latter has been shown not to be the case in *E. coli*, where ribosome inactivation, rather than blocking of aminoglycoside binding, is thought to protect cells from lethal doses of gentamicin (243). However, the role of HPF in counteracting aminoglycoside-ribosome binding was never explored under low concentrations of these antibiotics, where the dynamics of competitive binding may have a more important contribution.

In this work, we showed that HPF is necessary for *vchM*'s mutant selective advantage under low doses of tobramycin, but it does not contribute to its higher tolerance to lethal doses of this aminoglycoside. Since *hpf* is upregulated in this mutant, it is tempting to hypothesize that the high levels of HPF contribute to aminoglycoside tolerance at low doses, by directly competing for the binding to the ribosome. It is also theoretically possible that a higher expression of HPF and consequently, higher amounts of 100S ribosomes at stationary phase provide cells with a robust pool of ribosomes that are protected from degradation (260). Then, upon transfer to fresh media, a higher dissociation of 100S ribosomes would result in higher levels of translationally active 70S ribosomes, which could maintain a more efficient translation in presence of aminoglycosides or heat stress. In fact, rRNA maturation and ribosome biogenesis have been shown to be affected by increased temperatures in a process that depends on chaperones such as DnaK/DnaJ (261–263). Upon stresses causing misfolding of proteins, these chaperones are overwhelmed with misfolded proteins and cannot participate in ribosome biogenesis, thus causing a delay in this process. The involvement of HPF in promoting higher relative fitness of Δ*vchM* during growth at 42°C may indicate a role of HPF in helping ribosome biogenesis in heat stress conditions.

Additionally, heat stress have been shown to be linked with ribosome degradation in bacteria (264) and stabilization of 100S ribosomes by HPF can be important in preventing ribosome degradation. How? In fact, HPF shares the ribosome binding site with translation initiation factors, such as IF1, IF3 and EF-G (259, 265), and likely, due to structural similarities, EF4 (LepA) (266, 267). Some of these factors lead to ribosome dissociation (268, 269) and it was shown HPF-mediated ribosome 100S dimers were harder to dissociate by IF3 *in vitro* assays (270). Thus, through 100S ribosome stabilization, HPF can prevent the binding of some of these factors to the ribosome and inhibit its dissociation into free subunits and consequently inhibits its degradation (271, 272).

Further work is necessary to fully understand the role of HPF in Δ*vchM* cells and several questions remain to be answered: do these cells accumulate a higher fraction of 100S ribosomes

during stationary phase? Are they more protected to ribosome degradation upon stress? Does this putative protection require 100S dimerization or can HPF directly compete with aminoglycosides or translation initiation factors for 70S ribosome binding? Moreover, how is VchM controlling expression of *hpf*? No RCCGGY motifs can be found in the vicinity of the DNA sequence of *hpf*, excluding direct regulation through DNA methylation. Thus, other mechanisms controlled by VchM should be responsible for *hpf* regulation. In *E. coli* and *Synechococcus elongatus*, *hpf* is induced by (p)ppGpp, an alarmone produced upon nutritional starvation (273, 274). Although (p)ppGpp levels were not assessed in Δ*vchM* cells to date, our transcriptomic data reveal the downregulation of *guaA* (-2.9 fold change) and *guaB* (-2.3 fold change) (Table S2), two genes involved in *de novo* GTP biosynthesis pathway and that are negatively controlled by (p)ppGpp (275, 276). Thus, it is possible that GTP homeostasis is disturbed in Δ*vchM* cells and controls *hpf* expression.

Considering the overall of our results, we suggest that the loss of VchM modulates *V. cholerae* tolerance to proteotoxic stress in two ways: 1) loss of VchM leads to increased expression of *groESL-2* chaperonin system which confers tolerance to lethal aminoglycoside stress by fighting protein misfolding (Fig. 17); 2) loss of VchM increases relative fitness under sub lethal proteotoxic stress through the action of HPF. As none of this mechanisms seems to be directly regulated by VchM-dependent DNA methylation, we cannot exclude they are a consequence of a pleotropic effect of VchM deletion.

But how has this modulation of proteotoxic stress tolerance by VchM evolved in *V. cholerae*? In other words, which are the conditions leading to reduced levels of VchM and VchM-dependent DNA methylation in the cells? Very little is known about regulation of VchM. While it was previously suggested expression of *vchM* was slightly increased in stationary phase (221), here we could not confirm such observation as the transcript levels of *vchM* expression in exponential phase grown WT cells are identical to those measured in stationary phase (Fig. S5). Interestingly, it was recently shown the *V. cholerae* quorum sensing low density transcriptional regulator AphA binds the *vchM* region but its effect on *vchM* expression remained to be assessed (277). We wonder whether *vchM* is part of QS network of *V. cholerae* where different cells densities would dictate different *vchM* expression levels. On another note, how would different levels of *vchM* expression impact the levels of cytosine methylation in the DNA? In fact, Bisulfite Sequencing analysis of Δ*vchM* methylome (i.e. the methylation profile of the entire genome of Δ*vchM* cells) has revealed a total absence of cytosine methylation in RCCGGY motifs in this mutant (110) but nothing is known about how different levels

of VchM expression would impact cytosine methylation of these motifs. In addition, bisulfite sequencing and other high-throughput approaches to study DNA methylation are done at the whole population level thus possibly masking differential methylation in single cells or small sub-populations. It would be equally interesting to assess *vchM* expression and RCCGGY methylation in *V. cholerae* cells under different stress conditions (including proteotoxic stress) or in mutants for known stress response regulators, and see whether stress can affect either *vchM* expression or cytosine methylation in DNA of *V. cholerae*.

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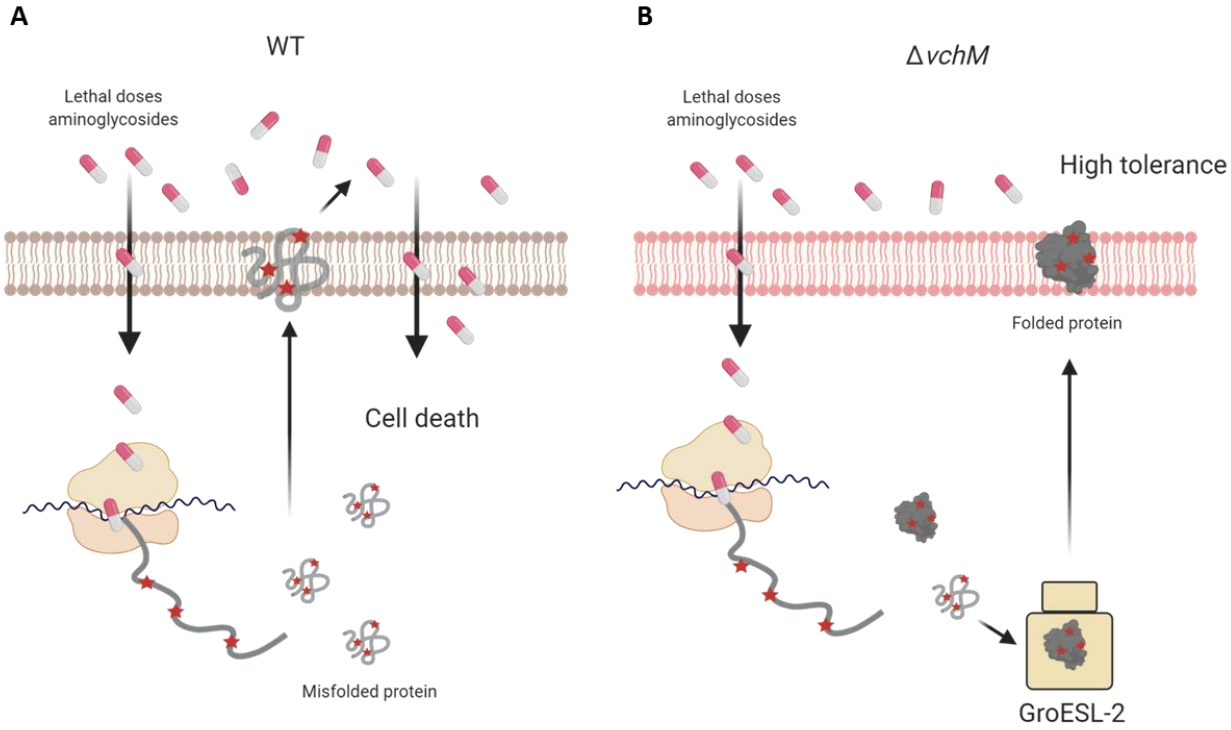

FIG 17. Model depicting the mechanisms of high tolerance to lethal doses of aminoglycosides in a *ΔvchM* mutant. **A**. In WT cells, a first round of aminoglycoside uptake leads to mistranslation of the mRNA molecule and production of peptides with wrong amino acids (red stars) that cannot properly fold. Misfolded cytosolic proteins accumulate inside the cell and incorporation of misfolded proteins in the membrane lead to further uptake of aminoglycoside and aggravated translation problems, leading to cell death. **B.** Loss of VchM leads to increased expression of groESL-2 chaperonin system, which aids the folding of mistranslated proteins counteracting the effects of the antibiotic. Less misfolded proteins accumulate in the cell, which confers a higher tolerance to lethal aminoglycoside stress. Created with BioRender.com

In order to identify additional processes affected by loss of VchM we conducted Transposon Insertion Sequencing experiment in *V. cholerae* cells, in absence or presence of subMIC tobramycin. The preliminary analysis of these studies will be the subject of the Results – part II of this thesis.

Part II – **"How lack of VchM favors *V. cholerae* under subMIC doses of aminoglycosides – a link between DNA methylation and translation?"**

In the previous section we showed that lack of VchM is able to modulate tolerance of *V. cholerae* to lethal doses of aminoglycosides and heat stress, known to affect proteostasis. Moreover, our data shows that loss of VchM causes a growth defect in absence of stress, but it is advantageous in presence of sub-lethal doses of aminoglycosides or growth at 42°C. While the upregulation of the chaperonins *groESL-2* was shown to be essential for tolerance to lethal doses of aminoglycosides, the upregulation of *hpf*, a gene encoding a ribosome-associated protein known to promote ribosome dimerization, seems to be responsible for the higher relative fitness of a Δ*vchM* under sub-lethal stress.

In order to identify proteins, genes and cellular processes that could explain the tolerance phenotypes associated with deletion of VchM in *V. cholerae*, we conducted a Transposon Insertion Sequencing (TI-Seq) study in WT and Δ*vchM* strains, in absence or presence of subMIC doses of tobramycin. The preliminary analysis of these results shows us that rRNA metabolism, tRNA modification and protein chaperoning are affected in Δ*vchM*, and may be behind the tolerance to aminoglycoside and heat stress observed in this mutant.

2.1. TI-Seq and the identification of important cellular processes in Δ*vchM*

Transposon insertion sequence has become recognized as a powerful tool to identify new gene functions in a high-throughput way. By combining large-scale transposon mutagenesis with next-generation sequencing, this approach estimates the essentiality and/or fitness contribution of each genetic feature in a bacterial genome (278, 279).

Here, we used this approach to identify partners and molecular mechanisms involving VchM, in absence or presence of subMIC TOB after short-term evolution. In other words, this approach will reveal the relative contribution of genes and cellular processes in each strain and conditions tested, as previously showed in our lab (236).

We thus constructed large transposon inactivation libraries in *V. cholerae* WT and Δ*vchM* strains and we subjected them to growth for 16 generations in media without and with subMIC TOB at 0.6 µg/ml (50% of the MIC). After sequencing of the regions flanking the transposon, insertion detection, mapping and counts, we identify genes where detected insertions increase or decrease. Loss of detected insertions in a specific gene means that the inactivation of this gene is detrimental in the tested condition. On the contrary, enrichment of detected insertions means that the inactivation of the gene is beneficial. Then, we searched for such loss or enrichment of insertions and compared detected insertions in Δ*vchM* strain versus in the WT at time 0 (T0), after 16 generations (T16), and in the absence and presence of the antibiotic. These results are being analyzed at the moment.

Table 2 contains the genes with differential insertions at T0 in Δ*vchM* strain, i.e., it contains the genes where the number of transposon insertions was higher or lower in Δ*vchM* compared to WT. No transposon sequences were mapped in *vchM* region in the mutant strain, confirming its deletion. Although this is a preliminary analysis, some hits caught our attention because they are directly or indirectly involved in processes related to the phenotypes observed in Δ*vchM* mutant, and I would like to discuss them briefly.

Table 2. Genes with differential insertions at T0 in Δ*vchM* strain

Change in no. of insertions in ΔvchM mutant at T0 (compared to WT) and gene type	Locus	Name	Normalized reads (no. of sequenced insertions) of strain at T0		Fold change (Δ*vchM*/WT)	P-value	Predicted function
			WT	*ΔvchM*			
Decreased							
Translation							
	VC2599	rnr*	215	19	**-11,2**	9E-80	RNase R
	VC_r013	16Se	2179	272	**-8,0**	1E-09	16S ribosomal RNA
	VC2716	yhgF	3738	588	**-6,4**	3E-246	RNA-binding transcriptional accessory protein
	VCr011	23Sd	3253	683	**-4,8**	2E-05	23S ribosomal RNA
	VC1407	rhlE*	113	35	**-3,2**	3E-06	ATP-dependent RNA helicase RhlE
Proteases							
	VC1143	*clpS*	530	101	**-5,2**	3E-09	ATP-dependent Clp protease adapter ClpS
	VC1920	*lon**	135	44	**-3,0**	2E-12	la endopeptidase
Other							
	VCA0198	*vchM*	40	0	**NA**	2E-09	Orphan DNA methyltransferase VchM
Increased							
Translation							
	VC_t032		8	497	**62,9**	0E+00	tRNA-Thr-5
	VC_t030		11	524	**47,3**	0E+00	tRNA-Thr-4
	VC2280		50	2302	**45,8**	0E+00	ribosomal protein S6 modification protein (ATP-dependent zinc protease)
	VC_t014		11	480	**43,6**	0E+00	tRNA-Thr-2
	VC0443	*rsmA*	14	149	**10,9**	2E-11	16S rRNA(adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase RsmA
	VC2536	*yjgA*	132	1281	**9,7**	0E+00	(YjgA) ribosome-associated protein
	VC0671	*rppH*	23	174	**7,7**	2E-10	RNA pyrophosphohydrolase
	VC2775	*mnmG*	66	462	**7,0**	0E+00	tRNA uridine-5-carboxymethylaminomethyl(34)synthesis enzyme MnmG
	VC0346	*miaA*	104	665	**6,4**	0E+00	tRNA(adenosine(37)-N6)-dimethylallyltransferase MiaA
	VCr001	*16Sa*	129	821	**6,4**	0E+00	16S ribosomal RNA

	VCr021	*16Sg*	130	804	**6,2**	0E+00	16S ribosomal RNA
	VC0003	*mnmE*	51	286	**5,6**	1E-14	tRNA uridine-5-carboxymethylaminomethyl(34)synthesis GTPase MnmE
	VC1432	*ttcA*	266	1412	**5,3**	0E+00	tRNA 2-thiocytidine(32) synthetase TtcA
	VC0291	*dusB*	95	424	**4,5**	0E+00	tRNA dihydrouridine synthase DusB
	VC0046	*def*	220	837	**3,8**	0E+00	peptide deformylase
Chaperones							
	VC0856	*dnaJ*	6	207	**33,8**	0E+00	molecular chaperone DnaJ
	VC1923	*tig*	98	840	**8,6**	0E+00	Trigger factor
	VC0985	*htpG*	221	1399	**6,3**	0E+00	molecular chaperone HtpG
Proteases							
	VC2675	*hslV*	295	2282	**7,7**	0E+00	ATP-dependent protease subunit HslV
Others							
	VCA0885	*tdh*	197	651	**3,3**	0E+00	L-threonine 3-dehydrogenase
	VC2363	*thrB*	127	697	**5,5**	0E+00	homoserine kinase (threonine metabolism)
	VCA0201		45	3195	**71,3**	0E+00	hypothetical protein
	VCA0199		128	6208	**48,7**	0E+00	hypothetical protein
	VCA0200		235	11256	**47,8**	0E+00	hypothetical protein

* genes likely essential in absence of VchM.

2.1.1. RNase R becomes essential in absence of VchM

Unexpectedly, insertions of the transposon in *rnr*, encoding Ribonuclease R (RNaseR), were much less tolerated in *ΔvchM* strains than in the WT. In fact, the almost negligible number of transposon insertions detected in *rnr* in *ΔvchM* strain suggests that disruption of this gene is lethal in absence of VchM. RNases are important enzymes that participate in RNA metabolism in bacteria (280). They are involved in processes such as maturation and degradation of rRNA and thus are important players in both ribosome biogenesis and degradation. RNaseR is particularly suggested to be a key factor ensuring translation accuracy because it both contributes to rRNA quality control and degrades defective RNA's in a process called *trans*-translation (281, 282). Thus, we hypothesize that in *ΔvchM* cells, rRNA or tRNA maturation and ribosome biogenesis is affected in a way that the action

of RNaseR is indispensable. *rhlE*, a DEAD-box RNA helicase, also participates in ribosome biogenesis and was shown to interact with RNaseR (283–285). Interestingly, *rhlE* also contains much less transposon insertions in *ΔvchM* than in WT, indicating RNaseR and RhlE may play together an important role in rRNA processing and ribosome maturation in *ΔvchM* mutant.

2.1.2. Higher number of transposon insertions in several RNA modification genes in *ΔvchM* mutant

The higher number of transposon insertions detected in genes encoding several tRNA modification enzymes, suggests that the disruption of these genes is more tolerated in the *vchM* mutant. These genes, *mnmG*, *mnmE*, *miaA, ttcA* and *dusB* are known to affect translation in different ways: *mnmGE* – mediated tRNA modification was shown to be critical for translation accuracy (286); *miaA* is required for efficient binding of certain tRNAs to the ribosome (287) and is necessary for translation of proteins enriched in certain amino acids, as it happens for RpoS (288); *ttcA*'s specific role in translation is not yet totally understood but mutations in this enzyme were shown to affect to increase susceptibility to oxidative stress in *P.aeruginosa* (289)*; dusB* is a dihydrouridine synthase that causes tRNA structural changes but little is known about the consequences of *dusB* loss (290). Interestingly, loss of *dusB* in *V. cholerae* confers competitive advantage in competitions against a WT strain in subMIC of aminoglycosides (unpublished data).

Moreover, a higher number of insertions in *rsmA* (previously *ksgA*) was detected in *ΔvchM* mutant. RsmA is 16S rRNA modification enzyme that is required for the efficient processing of the rRNA *termini* during ribosome biogenesis (291) and it was recently shown to contribute to aminoglycoside tolerance in *Acinetobacter baumanni*, by securing translational fidelity during aminoglycoside treatment (292)

The fact that *ΔvchM* contained a higher number of transposon insertions in t/rRNA genes tells us these t/rRNAs modifications are dispensable in this mutant or that their absence is beneficial in a *ΔvchM* background. Changes in rRNA and tRNAs may influence translation at several levels, such as ribosome stability and translational fidelity (293, 294), which are also known targets of aminoglycoside stress. Thus, it is possible that in *ΔvchM*, these processes are somehow ensured by other mechanisms (perhaps HPF-mediated ribosome protection allows for their disruption).

2.1.3. Trigger factor and DnaJ are dispensable in *ΔvchM* mutant

Similar to t/rRNA modification genes, transposon insertions in *tig* and *dnaJ* genes were much more frequent in a *ΔvchM* mutant. In fact, insertions in these genes were barely detected in the WT strain. Thus, disruption of *tig* and *dnaJ* is highly detrimental in WT but appears to be inconsequential (or beneficial) in the mutant. Trigger factor and DnaJ (together with DnaK) are molecular chaperones that assist the folding of newly synthetized proteins and promote re-folding of misfolded proteins. Moreover, both TF and DnaJ have been shown to be involved in ribosome biogenesis (31, 262). DnaJ was also found to be important for DnaK collaboration with HtpG, a heat-shock protein 90, whose gene was also disrupted with a higher frequency in *ΔvchM* (Table 2). (295, 296). Thus, while important in WT cells to maintain proteostasis, the chaperone network TF-DnaKJ-HtpG becomes dispensable in *ΔvchM*, suggesting that in this mutant, ribosome biogenesis or folding of newly synthesized proteins are secured in another way. One possibility, would be an upregulation of chaperonins *groESL-2,* as demonstrated in the "Results - part I" section of this thesis. Another hypothesis, is that necessity of TF and DnaKJ chaperone network to secure folding of new proteins is dispensable when the synthesis rate of these proteins is reduced. In agreement with this, a study showed that degradation of the EF-Tu (elongation factor-Tu), and consequent reduced protein synthesis, allowed for growth of *E. coli* in absence of TF and DnaKJ system (297).

In conclusion, the preliminary analysis of these results highlights the role of VchM in processes affecting translation at different levels. r/tRNA processing and modification may be affected in *ΔvchM* causing downstream effects on translation and the equilibrium of folded proteins. All of the previous processes are known to be targeted by aminoglycosides and that may explain why this mutant is more tolerant to this class of antibiotics and temperature stress, although the exact mechanisms remain to be studied.

Part III - "vca0199-vca0200-vca0201 – new players in VchM – mediated phenotypes"

Deletion of *vchM* in *V. cholerae* El Tor N16961 strain originates smaller colonies (comparatively to the WT) in solid rich media (Fig S1). Similarly, these colonies have a lower growth rate when grown in liquid cultures, and lose competitions against the WT strain. However, as previously showed, Δ*vchM* is able to outcompete WT strain in presence of sub-lethal doses of aminoglycosides or increased temperatures. Whether the competitive advantage in presence of these stresses is directly associated to Δ*vchM* intrinsic growth defect or it relies on independent mechanisms, is an interesting question to ask. In fact, small colony variants (SCVs) are known to be associated with slow growth and decreased susceptibility to aminoglycosides in several pathogen bacteria, but their occurrence is mostly determined by defective electron transport or thymidine biosynthesis and happens at a sub-population level (298). However, electron transport in Δ*vchM* does not seem to be affected (preliminary data) and our RNA-seq data does not point to any transcriptional changes in thymidine biosynthesis pathway (Tables S1 and S2). So what are the mechanisms that lead to small colonies and growth defect in a *vchM* deletion mutant?

3.1. Suppressors of small colony and growth defect phenotypes arise during overnight growth of ΔvchM

Intriguingly, during the course of our study, after culture of Δ*vchM* small colonies in large volumes of media and subsequent plating, we noticed the appearance of both small and larger, WT-like colonies in petri-dishes (Fig. 18). The frequency of appearance of these larger colonies varied but in extreme cases they overtook the whole population in just 20 hours of growth (data not shown). Moreover, re-streak of these larger colonies only gives origin to larger colonies and revertants were never observed, which suggests that a phase-variation kind of regulation is unlikely. Thus, we considered the possibility that the appearance of larger colonies in Δ*vchM* cultures corresponded to genetic suppressors. Interestingly, the spontaneous mutation frequency of Δ*vchM* is lower than that of the WT (Fig. S6 and (221)) probably because the lack of m5C prevents deamination of cytosines and formation of T:G mismatches (218, 221). Thus, the high frequency of Δ*vchM* suppressors cannot

be a consequence of a high mutation rate, but rather the result of a very strong selection of these mutations.

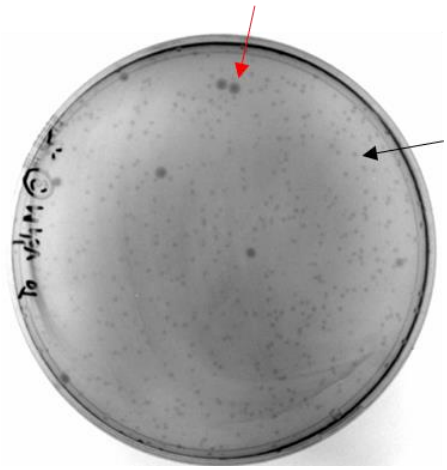

FIG. 18 Plating of a 10^{-6} dilution of a Δ*vchM* overnight culture (i.e. ≈ 16 hours) grown in 10mL of MH, at 37°C, 180rpm. Small colonies (black arrow) and larger, WT-like colonies (red arrow) are visible.

3.2. Δ*vchM* suppressors lose the tolerance towards aminoglycosides

Next, we picked five large colonies originated from five independent Δ*vchM* cultures and put them to grow overnight, as well as a colony picked from a WT streak and a small colony picked from a Δ*vchM* streak. Then we tested their growth in liquid cultures in absence or presence of subMIC doses of tobramycin (Fig. 19) As we can observe in the absence of antibiotic, Δ*vchM* exhibits the typical growth defect, while all of the Δ*vchM* suppressors (Δ*vchM* sup) grow similar to the WT, with the exception of Δ*vchM* suppressor #3 (Fig. 19A). Moreover, while the Δ*vchM* culture presented the typical tolerance to increasing concentrations of subMIC tobramycin, Δ*vchM* suppressors became even more susceptible than the WT (Fig. 19B). Thus, suppression of the growth defect of Δ*vchM* also suppresses the increased tolerance to low doses of aminoglycosides.

To confirm these results, we randomly chose one of Δ*vchM* suppressors, and assessed its ability to compete against a WT strain in subMIC TOB (Fig. 20A), as well as its tolerance to lethal doses of this aminoglycoside (Fig. 20B). As we can observe, and accordingly with the results obtained in monocultures, Δ*vchM* sup #1 displays no longer a growth defect in the absence of stress. Moreover, it even loses the competition in subMIC TOB (Fig. 20A). In addition, it displays no longer a higher tolerance to lethal doses of tobramycin, as its survival after 4 hours of treatment with 20X MIC TOB is similar to that of the WT (Fig. 20B).

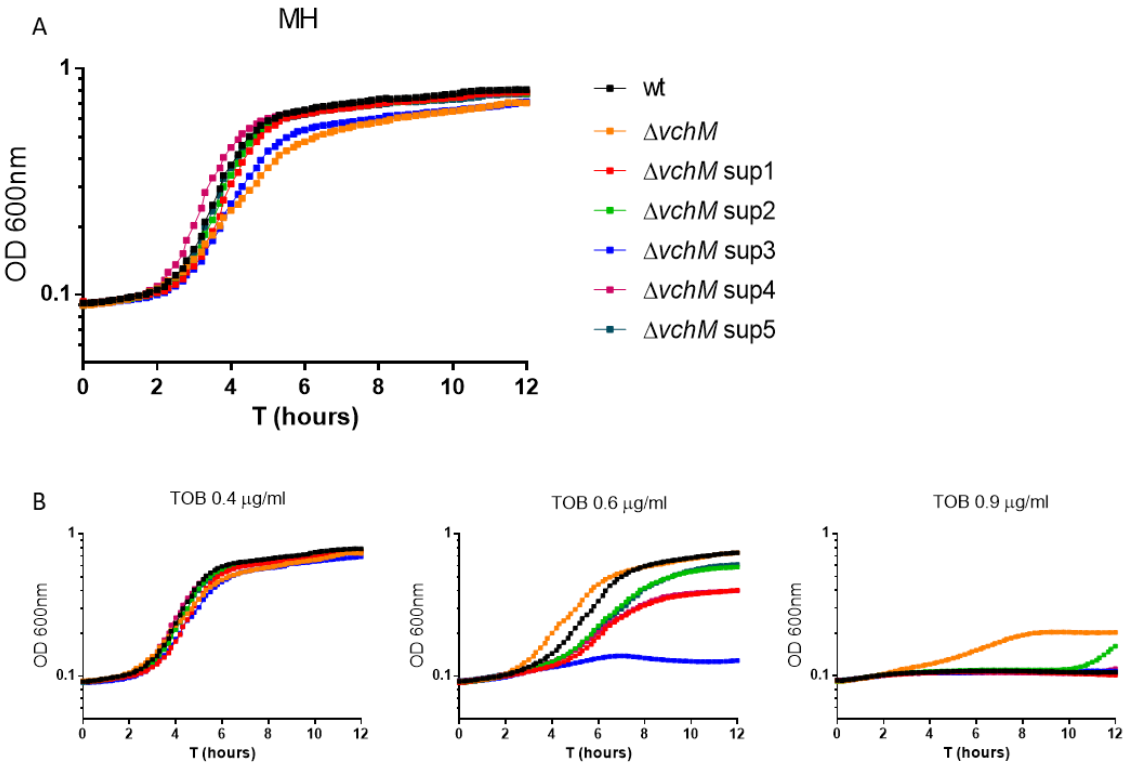

FIG 19. Growth of *V. cholerae* WT, Δ*vchM* and Δ*vchM* suppressors in absence **(A)** or presence of subMIC concentrations of tobramycin (TOB) **(B)**; Growth was measured with the Tecan Infinite plate reader. This experiment was repeated at least 3 times.

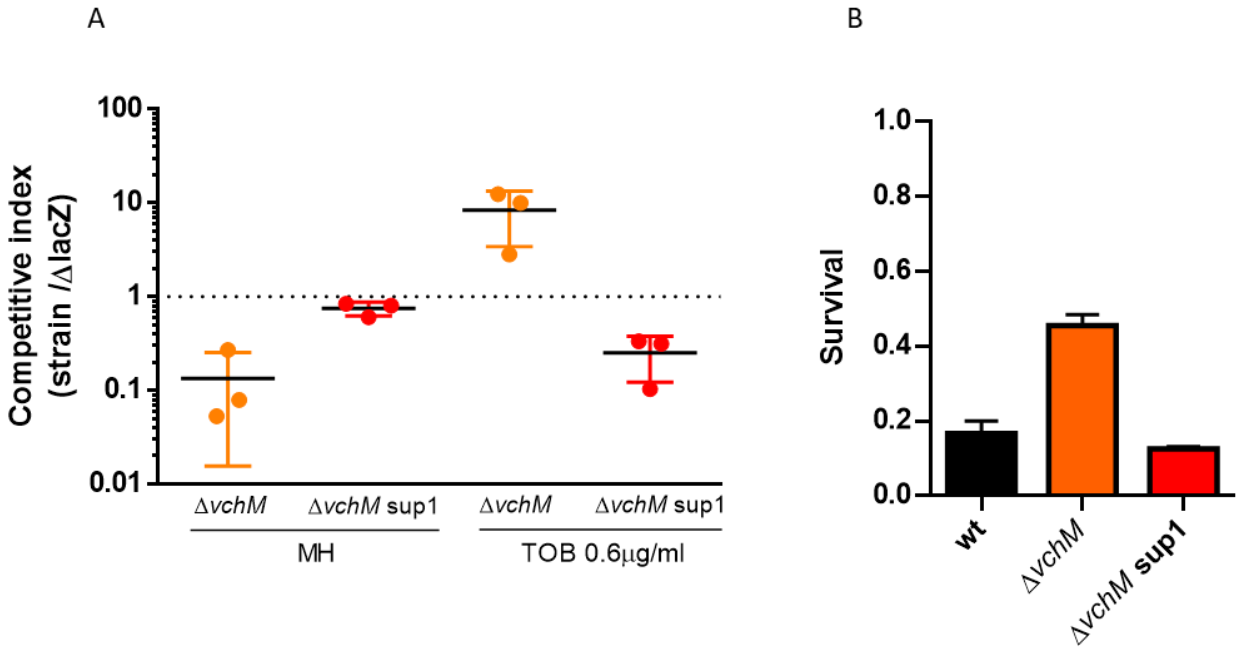

FIG 20. **A**. Competitions of *V. cholerae* Δ*vchM* and Δ*vchM* suppressor #1 against a *lacZ*- mutant in the presence or absence of TOB 0.6 µg/ml. After 20 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain, n=3. **B**. Survival of stationary-phase *V. cholerae* WT, Δ*vchM* and Δ*vchM* suppressor #1 cells exposed for 4 hours to lethal doses of TOB 20X MIC (20µg/ml). Survival is determined by dividing the cfu/ml at 4 hours post-treatment by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. This is a representative experiment of at least two independent experiments.

3.3. Suppressor mutations occur in an operon located downstream of *vchM*

In order to identify the mutations that suppress the growth defect and aminoglycoside tolerance in Δ*vchM*, we extracted genomic DNA of WT, ΔvchM and ΔvchM suppressors #1-5 and sent it for Whole Genome Sequencing (WGS).

Table 3 shows the mutations detected in Δ*vchM* suppressors #1-5 and absent in Δ*vchM* genome. Suppressor #1 contains a rearrangement between gene *vca0201* and *vca0371* leading to disruption of *vca0201;* suppressor # 2, 4 and 5 contain all mutations that generate truncated forms of a protein encoded by *vca0200* and suppressor # 3 contained a 1 bp deletion in *vc0747*, a locus encoding the iron-sulfur cluster regulator IscR. The *del* mutation in *iscR* led to the most dramatic effect in subMIC TOB (Fig. 19B) showing that regulation of iron-sulfur proteins may play an important role in cell response to aminoglycosides. However, the majority of suppressor mutations (4 out of 5) occurred in neighbor genes (*vca0200* and *vca0201*) suggesting a higher biological significance of these genes in suppressing Δ*vchM* phenotypes. For this reason, we focused on these.

According to Kyoto Encyclopedia of Genes and Genomes (KEGG) database (299), *vca0200* and *vca0201* encode hypothetical proteins of unknown function. Vca0200 contains several predicted AAA ATPase domains. Interestingly, these two genes are member of an operon (*vca0199-vca0200-vca0201*), which lies immediately downstream of *vca0198*, the *locus* encoding *vchM*. Moreover, the region containing *vchM* and *vca0199-vca0201* constitutes a genomic island with a GC content of 36%, which is lower than the remainder of the genome (GC content of 47%). This suggests this segment may have been acquired horizontally and that the function of all of its constituents are intertwined.

Thus, mutations that suppress the growth phenotype of Δ*vchM* tend to occur mostly in the neighboring downstream genes. As these are mostly nonsense mutations, this tells us that inactivation of either Vca0200 or Vca0201 is strongly selected in the absence of *vchM*. In agreement with this, in our TI-seq experiment the number of transposon insertions in these genes is overwhelmingly increased in Δ*vchM* mutant (Table 2). Strikingly, this operon is also strongly upregulated in Δ*vchM* (Table S1).

Specifically, in Δ*vchM* stationary phase cells, *vca0199, vca0200* and *vca0201* are 5.32, 6.10 and 3.97 times more expressed than in WT. We thus considered the possibility that deletion of Δ*vchM* caused a polar effect on the downstream genes, leading to their upregulation and consequently affect bacterial growth. In order to exclude this hypothesis, we cloned *vchM* in a high copy plasmid and overexpressed *vchM in trans* in Δ*vchM* strain. Remarkably, Δ*vchM* transformants with the control empty plasmid yielded only small colonies while transformants with the complementation plasmid only originated larger, WT-colonies (data not shown). Complementation was confirmed by genomic DNA restriction analysis with a restriction enzyme sensitive to methylation in RCCGGY motifs (Fig .S7). Moreover, complemented Δ*vchM* strain showed WT-level tolerance to treatment with lethal doses of tobramycin (Fig. 21), suggesting that the lack of VchM, and not a polar effect due to deletion at its endogenous locus, determine expression of *vca0199-vca0201* genes. No RCCGGY motifs can be found within the sequence of this operon, which suggests its regulation by VchM occurs in an indirect way.

Table 3. Mutations that suppress growth defect and tolerance to aminoglycosides in Δ*vchM* background

Suppressor	Locus	Gene	Mutation	Predicted effect
#1	vca0201	Unknown	Rearrangement vca0201-vca0371	Inactivation of VCA0201
#2	vca0200	Putative ATPase	Q219* (CAG→TAG)	Early stop codon - Truncated form of VCA0200
#3	vc0747	iscR	Δ1 bp	Early stop codon – Truncated form of IscR
#4	vca0200	Putative ATPase	(A)7→6	Early stop codon - Truncated form of VCA0200
#5	vca0200	Putative ATPase	(A)7→6	Early stop codon - Truncated form of VCA0200

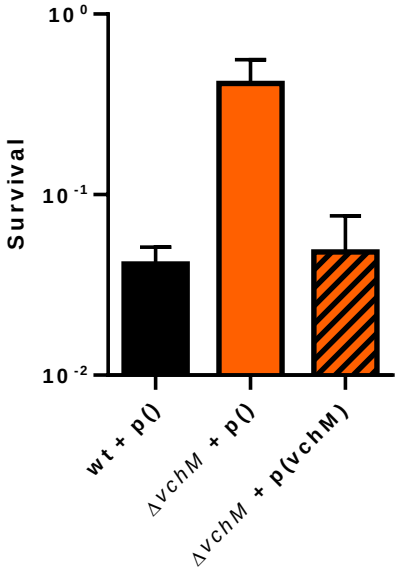

Fig. 21 Survival of stationary-phase *V. cholerae* WT, Δ*vchM* and Δ*vchM* complemented strains exposed for 4 hours to lethal doses of TOB 20X MIC (20µg/ml). Survival is determined by dividing the cfu/ml at 4 hours post-treatment by the cfu/ml before treatment. Mean and standard deviations are represented, n=3.

3.4. Overexpression of *vca0201* affects growth of *V. cholerae*

The data obtained implies that the high expression of *vca0200* and *vca0201* genes determines the intrinsic growth defect and the tolerance to aminoglycosides observed in *V. cholerae* cells lacking VchM. We thus asked whether the overexpression of either gene in a WT strain would render *V. cholerae* cells a similar phenotype. To answer this question, we cloned *vca0200* or *vca0201* in a pSC101, a low copy plasmid, under the control of a constitutive promoter, and transformed *V. cholerae* WT cells. Unfortunately, for unknown reasons, until this moment we could not get a plasmid expressing *vca0200-vca0201*. We then assessed growth of the obtained strains in MH or MH supplemented with low doses of antibiotics. In Fig. 22 we can observe that overexpression of *vca0201,* but not overexpression of *vca0200*, causes a growth defect in *V. cholerae* cells, similar to what we observe in a Δ*vchM* mutant. However, the overexpression of each of these genes did not increase tolerance to subMIC doses of tobramycin (as all strains were affected by subMIC TOB at the same extent). A concerted overexpression of *vca0200-vca0201* may be needed for the tolerance to subMIC TOB. This matter is currently under study.

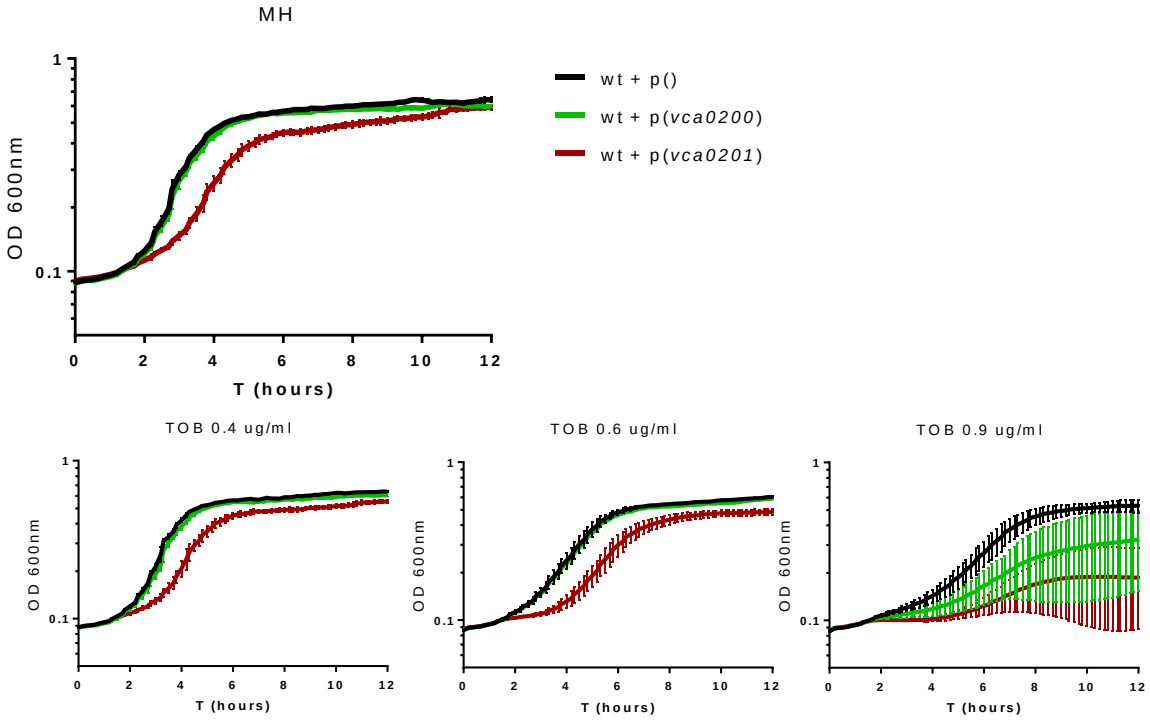

FIG 22. Growth of *V. cholerae* cells overexpressing *vca0200* or *vca0201* in absence **(A)** or presence of subMIC concentrations of tobramycin (TOB) **(B)**; All cultures contained Carbenicilin (100 µg/ml) for plasmid maintenance. Growth was measured with the Tecan Infinite plate reader. This experiment was repeated at least 3 times.

3.5. Discussion – Part III

Together, these results suggest that the growth defect observed in *V. cholerae* cells lacking VchM, is likely caused by the high expression of the downstream operon *vca0199-vca0200-vca0201*. It seems that the high expression of these genes in Δ*vchM* mutant constitutes a high burden to the cell, since mutations that inactivate these genes suppress this growth defect and are rapidly selected in the population. In addition, suppressors of Δ*vchM* no longer exhibit higher tolerance to subMIC or lethal doses of tobramycin, which shows that in these growth defect suppressors, the mechanisms that participate in the high tolerance phenotype are also affected. In fact, it would be interesting to measure *groESL-2* expression in these suppressors to unveil a possible regulation of the chaperonins by *vca0200* or *vca0201*. Another hypothesis is that Vca0200 and Vca0201 regulate the expression of factors involved in lethal aminoglycoside tolerance which are themselves substrates of GroESL-2,

needing its chaperonin activity to properly exert their function. Assessing aminoglycoside tolerance of a Δ*vchM* suppressor overexpressing *groESL-2* should give an answer to this.

The genomic island comprising *vchM-vca0199-vca0200-vca0201* is only present at the species level, and gene organization is highly conserved, which indicates their functions may be somehow intertwined. The functional relationship between these genes and their regulation is a mystery. Why is *vca0199-vca0201* upregulated in absence of VchM? Can VchM directly regulate these genes independently of DNA methylation? Or, on the other hand, does Vca0200 or Vca0201 influence methyltransferase activity of VchM? What are the consequences for the cell upon deletion of the entire genomic island? The functions of the proteins encoded in *vca0199-vca0201* are unknown. Interestingly, single point mutations in *vca0199* have been identified as suppressors of *rpoE* essentiality in *V. cholerae* (109). However, transcription activity and protein levels of RpoE are augmented in strains containing *vca0199* mutations. In addition, an insertional mutation in *vca0199* abolishes transcription of *vca0200* and in this context, *rpoE* deletion mutants could not be obtained (109). Thus, it is tempting to hypothesize that depletion of *vca0200* is the determining factor that allows for *rpoE* deletion while at the same time it causes physiological changes that leads to activation of RpoE stress response. A recent study by the same lab showed through TI-Seq (Transposon Insertion Sequencing) that disruption of *vca0200* and vca0201 is highly detrimental in a *vchM rpoE* double deletion mutant, but not in *vchM* single mutant (110). This led the authors to classify *vca0200* and *vca0201* as genes necessary for envelope stability in *V. cholerae*. Further examination showed that transposon insertion mutants of *vca0200* and *vca0201* displayed high levels of RpoE in the cell, while a Δ*vchM* has barely detectable levels of this sigma factor (110). The data obtained in our work adds to the plot, showing that these genes are highly expressed in Δ*vchM* mutant and probably help maintain envelope stability in these cells. However, this comes at a cost, since high expression of *vca0201* results in a growth defect. Notwithstanding, it is tempting to propose a link between higher envelope stability and tobramycin tolerance, in which high envelope stability could counteract the insertion of aminoglycoside-induced misfolded proteins in the membrane and delay aminoglycoside diffusion across the outer membrane (76, 237). However, such possibility needs to be empirically tested.

Part IV – "Vca0447 – A putative DNA methyltransferase induced by aminoglycosides"

Low doses of antibiotics are important modulators of gene expression in bacteria, as previously stated. In fact, subMIC doses of aminoglycosides, an important class of antibiotics widely used in the clinical context, were shown to affect important stress responses in *V. cholerae*, differently from what was observed for *E. coli* (139, 300). In order to characterize the response of *V. cholerae* to low doses of aminoglycosides, our lab conducted a RNA-seq experiment in *V. cholerae* WT cells in absence or presence of very low doses of tobramycin, i.e. 2% of the MIC for this antibiotic (unpublished data). Such a low concentrations of tobramycin was shown to trigger a wide range of transcriptomic changes, which highlights the role of antibiotics as important signaling molecules (148, 149).

Interestingly, among the most significantly affected transcripts, we found *vca0446* and *vca0447* to be upregulated in *V. cholerae* WT cells exposed to 2% MIC of TOB (Fig. 23). These two genes are part of an operon, localized within the Super Integron (SI) in Chromosome 2 of *V. cholerae*. *vca0446* encodes a small peptide of 63 amino acids of unknown function while *vca0447* encodes a putative site-specific DNA-methyltransferase. Since no sign of a restriction endonuclease is found in the vicinity of *vca0447*, it is probable that Vca0447 is an Orphan DNA methyltransferase. Although the methylation targets of this enzyme were unknown, preliminary results of a bisulfite sequencing experiment in our lab pointed for a lower frequency of methylated cytosines in *vca0446-0447* deletion mutants. This result suggested that low doses of the aminoglycoside tobramycin were able to induce a putative DNA methyltransferase and hypothetically lead to differential DNA methylation in *V. cholerae* under stress of these antibiotics. The fascinating idea that subMIC aminoglycosides could induce an epigenetic response in bacteria, led us to better characterize this new Orphan putative DNA methyltransferase.

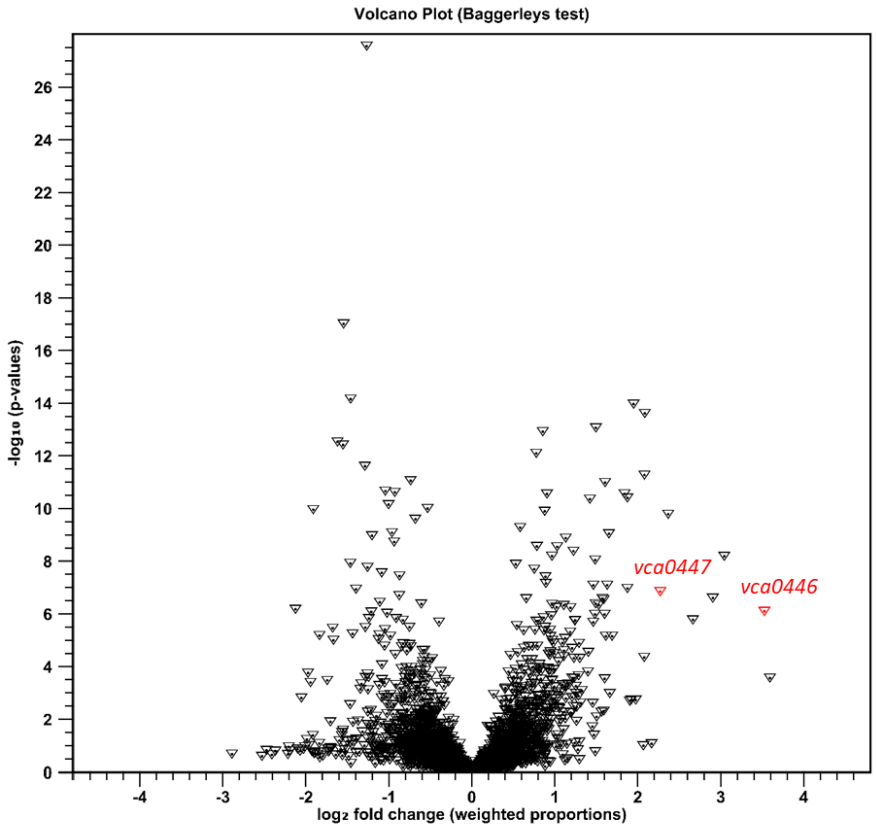

FIG 23. Volcano plot depicting gene expression changes in *V. cholerae* WT treated with 2% MIC TOB. *vca0446 and vca0447* genes are among the most affected genes. Figure gently ceded by Sebastian Aguilar Pierlé.

Vca0447 was previously shown to be dispensable for optimal growth of *V. cholerae* (110), but its role under stress caused by aminoglycosides was never investigated. Thus, we constructed a *V. cholerae vca0446-0447* deletion mutant and we assessed its relative fitness in competitions with a WT *lacZ*- strain, both in presence or absence of subMIC aminoglycosides (Fig.24A). The results show that a Δ*vca0446-0447* equally competes with the WT strain independently of the presence of tobramycin. We also tested this mutant's tolerance to lethal doses of TOB (Fig. 24B) and no biologically relevant differences was observed for the survival frequency of WT or the mutant. On the other hand, overexpression *in trans* of *vca0446-0447* in a plasmid resulted in a higher number of colony-forming units (cfus) after the plating of early-stationary phase cultures (Fig. 24C). Intriguingly, this effect was independent of the presence of antibiotic.

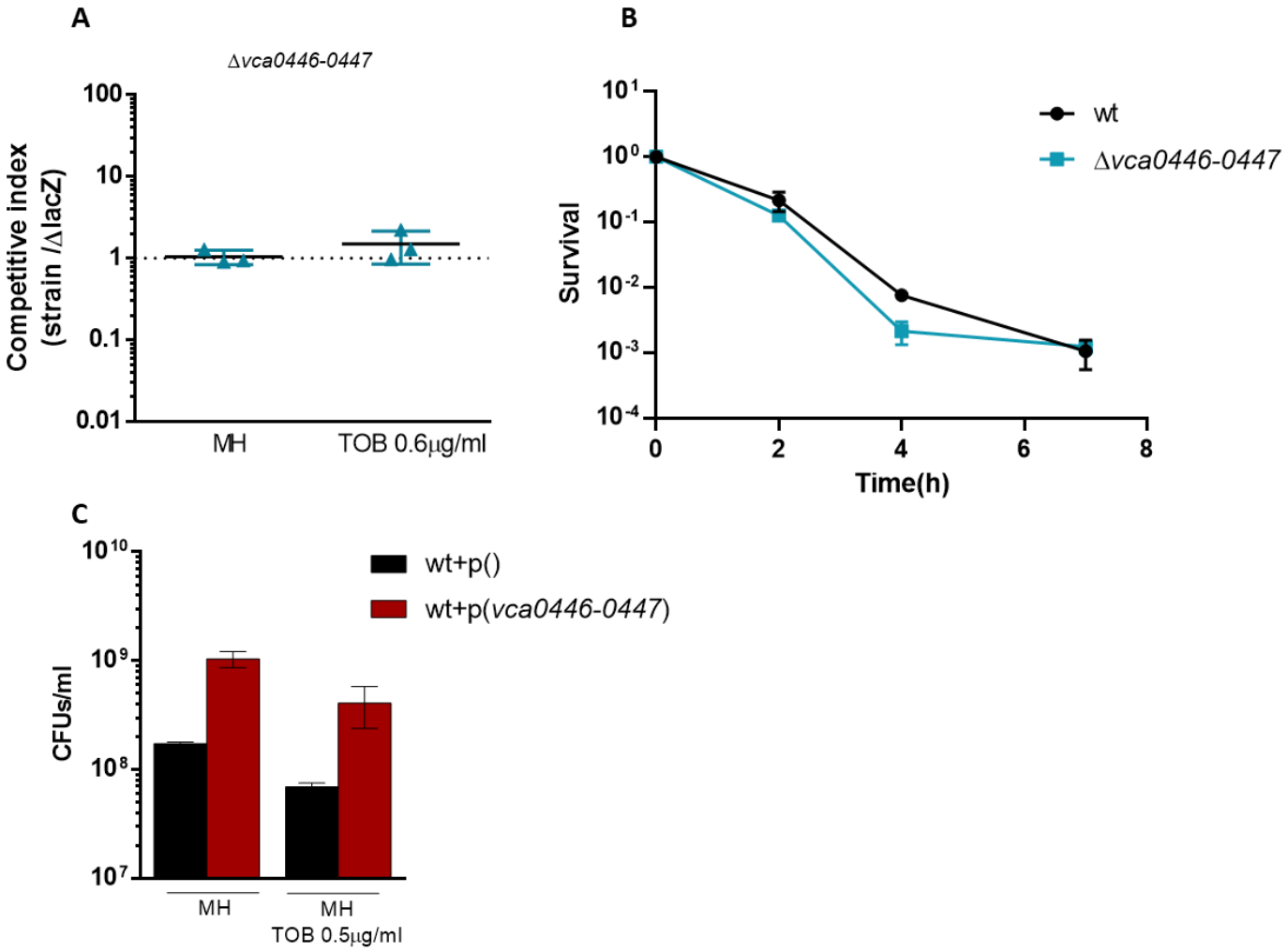

FIG 24. **A**. Competitions of *V. cholerae* Δ*vca0446-0447* against a *lacZ*- mutant in the presence or absence of TOB 0.6 μg/ml. After 20 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains (WT or Δ*vchM*) to lacZ- strain, n=3. **B**. Survival of stationary-phase *V. cholerae* WT and Δ*vca0446-0447* cells exposed to lethal doses of TOB 20X MIC (20μg/ml). Survival is determined by dividing the cfu/ml at each time point by the cfu/ml before treatment. Mean and standard deviations are represented, n=3. **C.** cfu/ml of early-stationary phase cultures of WT cells transformed with an empty plasmid or overexpressing *vca0446-0447* genes, both in MH or MH with 0.5ug/ml tobramycin. Chloramphenicol was added to the cultures for plasmid retention.

Together, these results show that, although induced by very low doses of tobramycin, deletion of *vca0446-0447* does not seem to be affect growth or survival following aminoglycoside treatment. On the other hand, overexpression of these genes seems to favor growth of *V. cholerae* cells, independently of the presence of antibiotic.

vca0446-vca0447 is part of the heat-shock regulon of *V. cholerae*

As said above, *vca0446-0447* is a cassette of the Super Integron of *V. cholerae.* Usually, genes in cassettes of the SI don't have a promoter of its own (except the ones that are part of a toxin-Antitoxin system) (145) but our lab identified a TSS (transcription start site) upstream of the coding region of *vca0446* (and none upstream of *vca0447*) which suggests that transcription of these coding regions occurs from a promoter upstream of *vca0446* (301). Moreover, Slamti et al. 2007 showed *vca0446* and *vca0447* were part of RpoH regulon of *V. cholerae* by observing a dramatic reduction of mRNA levels of *vca0446-0447* in a *rpoH* mutant (241). These authors further showed a putative binding site for RpoH on the promoter of *vca0446*. Thus, it is possible that *vca0446-0447* induction by low doses of aminoglycosides occurs as a consequence of the heat-shock response activation by these antibiotics. In agreement with this hypothesis, most of the genes showed by Slamti et al. 2007 to be regulated by RpoH, were also upregulated by subMIC TOB in our RNA-seq data. Examples of these genes include *ibpA*, *dnaK*, *dnaJ, htpG* and the two operons of *groESL*. Thus, very low doses of aminoglycosides are able to induce the heat-shock regulon of *V. cholerae*, including *vca0446-0447* genes.

vca0446-0447 mutant has a lower spontaneous mutation frequency

It has been previously shown that the presence of methylated cytosines in the DNA constitute a hotspot for C -> T mutations, thus contributing to a higher mutation rate. This happens because the rate of deamination of m5C is higher than its not methylated form (219). We checked whether the deletion of Δ*vca0446-0447* would affect this phenotype by assessing frequency of spontaneous mutations (Fig. 25A). In fact, Δ*vca0446-0447* strain seems to yield a lower spontaneous mutation frequency. Expression *in trans* of *vca0446-0447* in the mutant restored the frequency of spontaneous mutants to the level of the WT Fig. 25B). Furthermore, it also seems that the expression of *vca0447* alone is enough to restore this phenotype, which indicates that the activity of Vca0447 alone contributes for the mutation rate in *V. cholerae*.

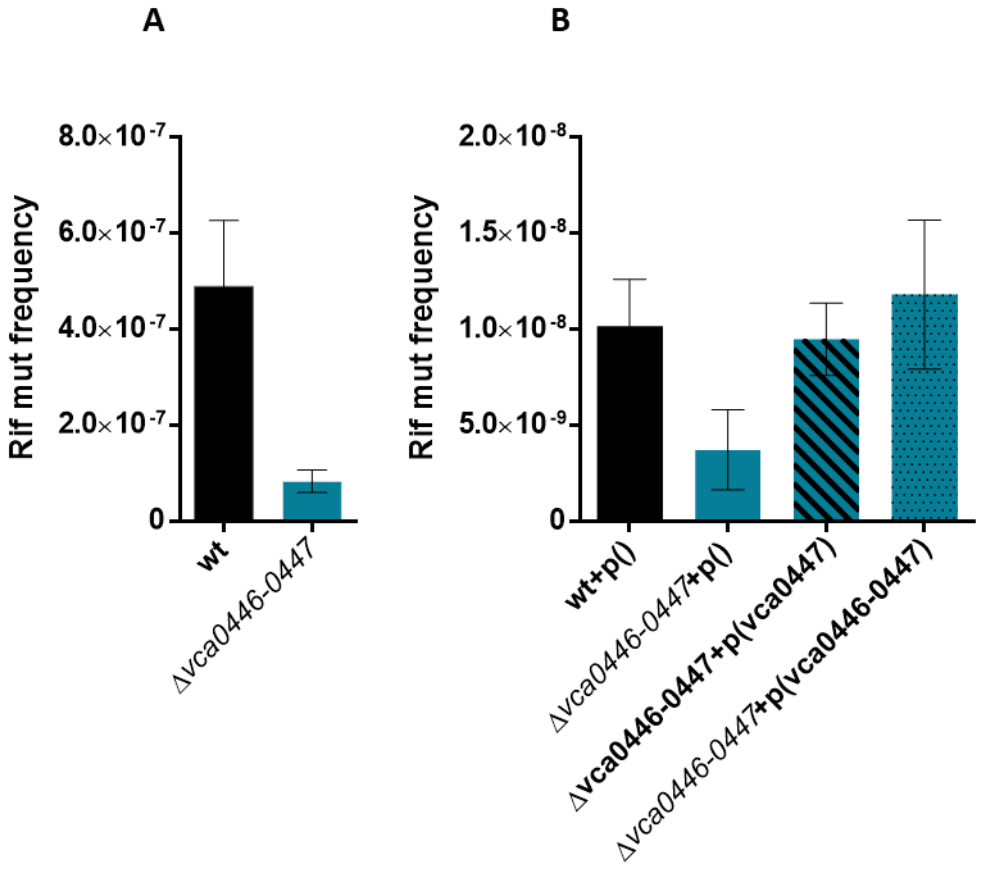

FIG 25. A and B. Frequency of Rifampicin resistant (RifR) mutants of indicated strains after grown in MH for 24 hours. Rif mutation frequency is calculated by dividing the number of clones in rifampicin by the total number of clones in MH plates, at T=24. n=5. Standard deviations are represented. These are representative experiments of at least three independent experiments.

Vca0446-0447 does not affect DNA m5C methylation levels

The preliminary results in our lab and the lower mutation frequency observed in Δ*vca0446-0447* cells were good indications that Vca0447 could be a DNA cytosine methyltransferase. Thus, we decided to test whether Vca0447 was really able to modulate the frequency of m5C in DNA of *V. cholerae*, together with VchM. We thus conducted a Bisulfite Sequencing experiment to assess frequency of m5C of WT, Δ*vca0446-0447* and Δ*vchM* strains with an empty plasmid or with a plasmid overexpressing *vca0446-0447.* Bisulfite Sequencing consists on the whole genome sequencing of previously bisulfite-treated DNA. Treatment of DNA with bisulfite converts unmethylated cytosine in

uracil, but leaves m5C untouched. Thus, after sequencing, detected cytosines correspond to only methylated cytosines. Figure 26 shows the distribution of methylated cytosines in the different strains. Unexpectedly, deletion of Δ*vca0446-0447* does not alter the distribution methylated cytosines, which were all located within RCCGGY motifs, targeted by Δ*vchM.* Moreover, overexpression of *vca0446-0447* did not change this trend (not shown). Thus, we conclude that Vca0447 is not a m5C DNA methyltransferase, and that VchM seems to be in fact the only cytosine methyltransferase in *V. cholerae*. Importantly, since our bisulfite sequencing experiment was done in stationary phase cells, we cannot exclude a possible transient methylation in exponential phase.

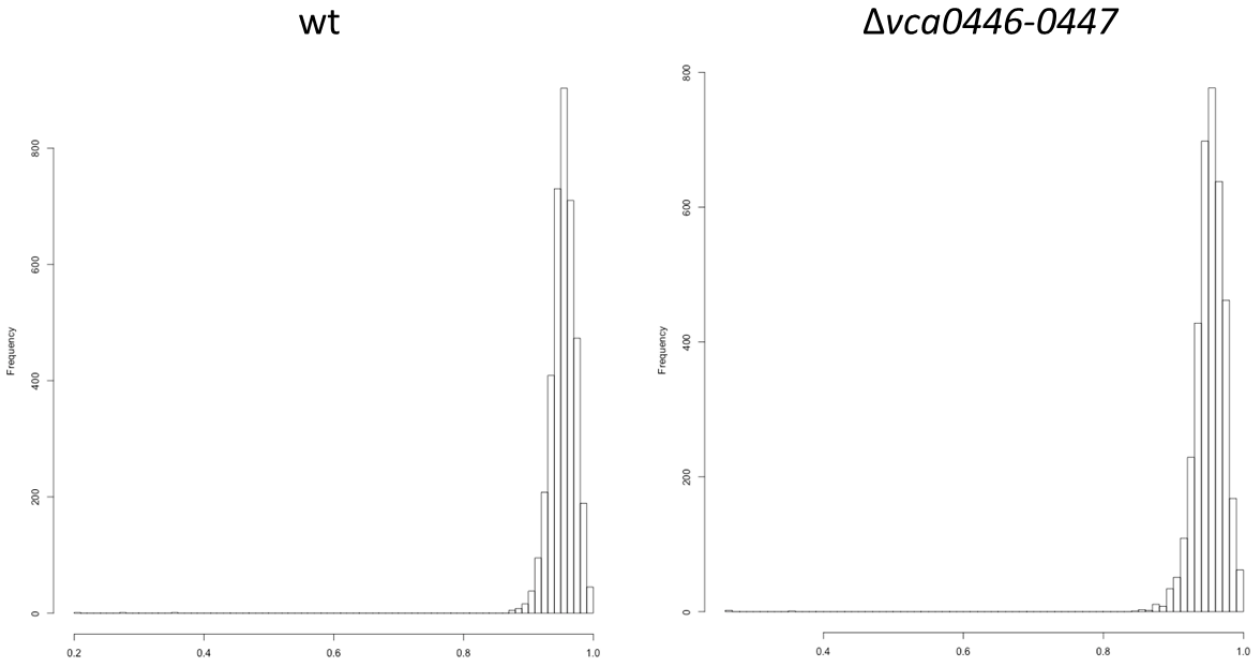

FIG 26. Histograms with distribution of methylated cytosines in WT and Δvca0446-0447 genomes. Y-axis represent the number of sites with methylated cytosines and the X-axis represent the frequency with which those sites were methylated. In WT and Δvca0446-0447 strains all methylated cytosines corresponded to the first C within RCCGGY motifs.

Vca0447 is not involved in DNA adenine methylation

Knowing that Vca0447 is not involved in DNA m5C methylation, we decided to test whether it methylates adenines in the DNA. The existence of colorimetric tests that use N6-methyladenosine specific antibodies to quantify m6A in the DNA allows for a first, fast assessment of differential adenine methylation. Typically, in these tests, m6A is detected using capture and detection antibodies. After the adding of an enhancer solution, the signal is quantified by absorbance readings. Thus, the amount of m6A is proportional to the OD intensity measured.

We used this test to quantify m6A in genomic DNA extracted from *V. cholerae* WT and Δ*vca0446-0447* strains grown until stationary phase in rich media. As a control, we included the gDNA from a *dam* mutant obtained in a different background, as well as its isogenic strain (Fig. 27) The results showed no differences in absorbance between the Δ*vca0446-0447* mutant and the WT, while a mutant for the DNA adenine methyltransferase Dam showed a clear reduction in absorbance (thus m6A levels) comparatively to the WT isogenic strain. Hence, we conclude Vca0447 does not methylate adenines in the DNA of *V. cholerae.*

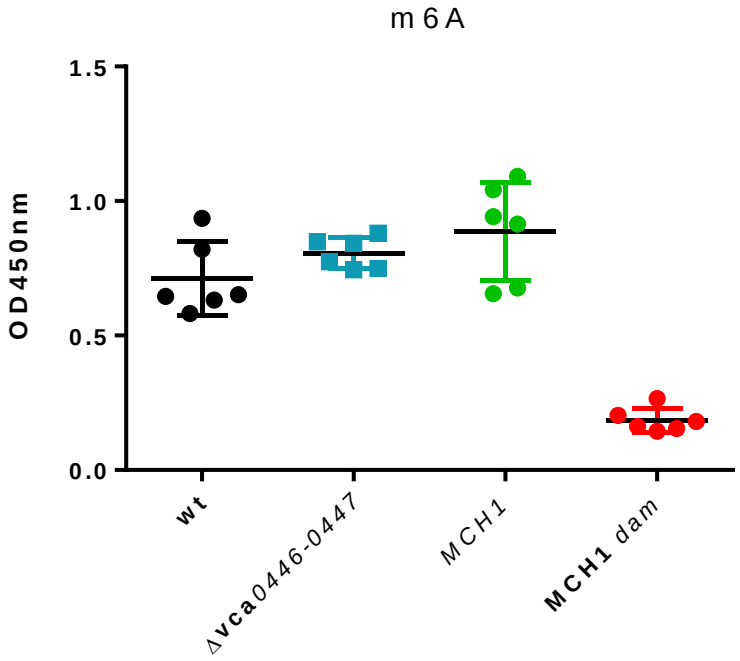

FIG 27. Relative quantification of m6A in the genomes of the indicated strains. The amount of m6A is proportional to the OD intensity measured. Absorbance was read in a microplate reader at 450 nm.

Discussion – Part IV

In this study we looked to characterize a putative *V. cholerae's* orphan DNA methyltransferase, encoded by *vca0447*. This gene is downstream of *vca0446*, a small peptide of unknown function and, together, they form an operon, which is also a cassette of the SI of *V. cholerae*. The role and the real function of Vca0447 is unknown. Interestingly, previous results from our lab observed this operon was highly upregulated in response to subMIC doses of tobramycin. Although a deletion mutant of *vca0447* was shown to not affect fitness of *V. cholerae in vitro* and *in vivo* in a mouse model (110), the fact that this gene was upregulated by tobramycin, suggested it might have an important role in *V. cholerae*'s adaptation to aminoglycosides. However, our results showed deletion of *vca0446-0447* did not affect neither growth in subMIC doses nor survival to lethal doses of aminoglycosides. On the other hand, we observed an increase of cfus in early stationary phase cultures of cells overexpressing *vca0446-0447*. However, this effect seems to be independent of the presence of aminoglycosides.

The reason why overexpression of *vca0446-0447* favors growth (or prevents death) are currently not known, but it may be related with the fact that these genes are part of the heat-shock regulon of *V. cholerae*. In fact, the promoter of *vca0446* contains a predicted RpoH binding site and this operon was concomitantly upregulated with other genes of heat-shock response of *V. cholerae*. Heat-shock genes are not only important to deal with external stress but are equally important in housekeeping functions, like cell division, ribosome biogenesis, envelope stability, etc. Thus, *vca0446-0447* overexpression may favor some of these processes in the same way.

Vca0446 encodes a small peptide of unknown function. On the other hand, Vca0447 is annotated as a site-specific DNA methyltransferase and the protein structure predicted by Phyre2 web-server (302) is modeled with a 100% confidence as a S-adenosyl-L-methionine-dependent methyltransferase from the Type 2 DNA methylase family (which include orphan methyltransferases). As a previous, preliminary bisulfite-sequencing experiment, pointed for differential cytosine methylation in *vca0446-0447* mutant, we decided to properly study cytosine methylation in the mutant and overexpression strains, in order to characterize possible motifs and processes affected. However, the results clearly show neither deletion nor overexpression of *vca0446-0447* or *vca0447* alone affect the frequency of m5C in the genome, which is solely dictated

by VchM activity. Moreover, Vca0447 does not seem to affect the levels of adenine methylation in the DNA of *V. cholerae*, assessed with a m6A detection commercial kit. Is thus not obvious which is the target of Vca0447. One possibility is that Vca0447 methylates cytosines as N4-methyl-cytosine (4mC). In fact, this epigenetic mark is harder to detect by bisulfite sequencing, given that m4C is partially resistant to bisulfite conversion (303). However, new cutting-edge methods, such as SMRT-seq, allow for the systematic detection of all the three existent forms of methylated DNA (225), and has been shown useful in the identification of m4C in *H. pylori* (170). Thus, assessing the whole methylome of *V. cholerae vca0446-0447* mutant, should give us insights about the possible role of Vca0447 as a DNA methyltransferase. Additionally, we cannot discard the possibility of Vca0447 being a RNA methyltransferase. In fact, a previously thought-to-be DNA methyltransferase was shown to additionally methylate tRNA (304). Interestingly, we have promising preliminary data indicating increased levels of translational -1 frameshifting in the strain deleted for *vca0446-vca0447*, which can be affected by tRNA modifications (305). We will address this possibility in the future.

Although we showed no link with DNA methylation, our results suggest Vca0447 controls the spontaneous mutation frequency of *V. cholerae*. Dam, Dcm, VchM, and other bacterial DNA methyltransferases are known to affect DNA repair pathways and mutation frequencies. Such effects are mostly inherent to the chemical modification of the DNA molecule caused by these enzymes. It is thus likely that Vca0447 contributes to the spontaneous mutation frequency of *V. cholerae* in a similar way, although the exact mechanism remains a mystery. The study of the mutational landscapes of WT and *vca0446-0447* mutant may thus be an interesting approach to gain a better insight on such mechanism.

DISCUSSION

During this PhD I have explored the link between bacterial stress caused by sub-inhibitory doses of aminoglycosides and the role of orphan DNA methyltransferases in response to such stress. I studied this link using *Vibrio cholerae* as a model. *V. cholerae* is a human pathogen and the causative agent of cholera disease. In our lab, several studies on the response of *V. cholerae* to low doses of antibiotics have shown this pathogen responds to sub-lethal doses of aminoglycosides in a way different from that of the common model *Escherichia coli* (300) and, interestingly, several of these results have pointed for a role of specific *V. cholerae* DNA methyltransferases in the response to these antibiotics. I thus tried to unveil how DNA methylation could affect the response to aminoglycosides and how this class of antibiotics could, in turn, modulate *V. cholerae*'s DNA methylation.

We discovered that deletion of *V. cholerae*'s *vchM* gene, encoding a DNA cytosine methyltransferase, provided cells a growth advantage against the WT, but only in presence of subMIC doses of aminoglycosides. In absence of these antibiotics, this mutant displays a clear growth defect. Moreover, in addition to the displayed competitive advantage in low doses of aminoglycosides, cells lacking VchM are also able to better withstand lethal doses of these antibiotics. We have studied the transcriptome of a Δ*vchM* mutant and came up with a model explaining the tolerant phenotype of this mutant. Indeed, we suggest that lack of *vchM* affects tolerance to aminoglycosides through two distinct mechanisms: in response to lethal doses of aminoglycosides, where the rate of protein misfolding is very high, the upregulation of the chaperonins *groESL-2* seems essential for a better survival. On the other hand, in presence of subMIC doses of aminoglycosides, the competitive advantage observed in the Δ*vchM* mutant is likely explained by the higher expression levels of the ribosome promoting hibernation factor, HPF. However, this model is far from being complete, as essential questions remain to be answered.

Upregulation of *groESL-2* and *hpf*

One of the questions that remained to be answered is why are these specific genes upregulated in *V. cholerae* cells lacking VchM? Knowing that VchM is a DNA cytosine methyltransferase and that m5C is strongly associated to gene repression both in eukaryotes and

prokaryotes (110, 168, 216), it is tempting to assume that in absence of VchM, lack of m5C in the DNA would lead to de-repression of these genes. Indeed, the four RCCGGY sites present in *groESL-2* operon, were fully methylated in WT strain and unmethylated in the *vchM* mutant in our bisulfite sequencing data (not shown). However, disruption of three of these sites in the WT strain, didn't seem to affect *groESL-2* transcription levels. Similarly, disruption of the site present at the 5' UTR region of this operon equally failed to modulate *groESL-2* expression. We recognize several caveats to these particularly results. In fact, we haven't managed to disrupt all the four sites at once, which could impact the DNA mechanical properties and conformation and, subsequently, inhibit the binding of certain proteins essential to transcription of this locus. Secondly, we could not achieve disruption of these three sites at the endogenous locus and rather compared the expression of methylated or unmethylated sequences of *groESL-2* cloned in plasmids and under the control of a constitutive promoter. It can happen that methylation of these sites cause an effect at the regulatory region of this operon, which is absent in our constructs. Thus, in general, we cannot conclude that the upregulation of *groESL-2* is completely independent of the methylation states of these RCCGGY sites. A more precise analysis and study of these methylation sites is needed for a proper conclusion.

The reason why *hpf* is upregulated in Δ*vchM* mutant may not be so obvious, considering the absence of RCCGGY motifs within the coding region and in the vicinity of this gene. Thus, this gene is likely upregulated due to a pleotropic effect of *vchM* deletion. Our TI-seq experiment revealed certain translation-related processes were affected in Δ*vchM*, which makes us wonder about a possible link between VchM and translation.

Translation-related processes affected in Δ*vchM*

The TI-seq performed in ΔvchM revealed that disruption of *rnr* and *rhlE* could not be tolerated in this mutant. On the other hand, a higher number of transposon insertions in several tRNA and rRNA modification genes and important chaperones was observed in *ΔvchM* cells, suggesting disruption of this processes is favored, or inconsequential, in these cells. One of the main functions of RNase R in bacteria is degradation of faulty RNAs, including rRNA and tRNA. Thus RNase R is considered to be critical for ribosome integrity and translation accuracy (281, 285). Even if it is not considered an essential enzyme (due to compensation by other RNases) (280), its deletion was shown

to be co-lethal with other exoribonucleases (306) and mutants of RNase R were found to accumulate rRNA fragments. Moreover, tRNA or rRNA modification is an important step of translational quality control (69, 307). Regarding this, we observed an enrichment of RCCGGY motifs on DNA sequences encoding certain tRNAs (preliminary observation). Whether VchM-mediated methylation of these sites affect these tRNAs expression or is even able to methylate these tRNAs is a question we will try to answer in the future.

The overall of these observations indicates that translation (likely at the levels of translation accuracy) is somehow affected in *V. cholerae* cells in absence of VchM. It is possible that these cells become pre-adapted to conditions where translation accuracy is affected, as for example, in presence of aminoglycosides. Thus, the link between VchM and translational efficiency/accuracy clearly needs to be explored. For that, we will assess rRNA maturation, translational fidelity and rate of protein synthesis in the Δ*vchM* mutant in presence and absence of aminoglycosides. We will also conduct a proteomics experiment in these conditions to look for the occurrence of specific patterns of translation errors in Δ*vchM*.

ΔvchM phenotypes and *vca0199-vca0201*

The growth defect associated to loss of VchM still needs to be understood. Here we have shown this phenotype is tightly associated with *vca0199-vca0201*, the three genes located immediately downstream of the gene encoding *vchM*. In fact, we observed that mutations preventing translation of these proteins are rapidly selected and suppress not only the growth defect, but also the higher tolerance to aminoglycosides. Given *vchM* and *vca0199-0201* genes belong to a genomic island marked by a lower GC content (relative to the rest of the genome), it is thought that all of these genes were acquired by horizontal gene transfer. Thus, it is tempting to assume that they may control related processes. In future work we will explore the role of these proteins in translational processes and in response to several stresses, including envelope stress, as it was shown RpoE levels are affected in this mutants (110).

Vca0447 – a methyltransferase that is a member of heat-shock regulon

In parallel to the study of VchM, I also tried to characterize Vca0447, a new putative DNA methyltransferase that we observed to be highly upregulated in presence of low doses of aminoglycosides. Despite the preliminary results indicating a role in cytosine methylation, we showed here that Vca0447 does not seem to contribute to DNA methylation, at least in the conditions tested, i.e. rich media, stationary phase and in absence of stress. Vca0447 is part of the heat-shock regulon of *V. cholerae* and thus it would be interesting to evaluate differential DNA or RNA methylation in presence of stresses inducing this response. Moreover, we observed this enzyme contribute to the spontaneous mutation rate in *V. cholerae* and we will investigate the mechanisms behind it.

CONCLUSION AND PERSPECTIVES

Bacteria are constantly reacting to changes in their surroundings. In response to several sources of stress, bacteria deploy a plethora of different mechanisms to help them thrive in the challenging environments. It is known that low doses of antibiotics are one important source of stress in bacteria and strong modulators of gene expression. Knowing that this may lead to the evolution of antibiotic resistance, the scientific community should focus on two important questions in this field: first, which are the specific molecular responses induced by bacteria in response to antibiotic treatment? and second, which are the mechanisms these bacteria evolved to fight such antibiotics?

In this work we show that modification of DNA and/or RNA molecules may be an answer to both questions. For the past decades it has becoming clear how ribonucleic methyltransferases can play crucial roles in main processes like DNA replication, transcription and translation. Here we contributed to this field, showing that low doses of aminoglycosides were able to induce the expression of a putative DNA/RNA methyltransferase (Vca0447*)*, which is part of the heat-shock regulon of *V. cholerae*, contributing to a higher spontaneous mutation frequency. The most recent, preliminary results showed that deletion of Vca0447 results in increased translation frameshifting, thus suggesting an effect of Vca0447 in translation quality control. Additional work is needed to finally uncover the target molecule of this methyltransferase.

Additionaly, we showed the lack of VchM - mediated m5C DNA methylation of RCCGGY motifs confers *V. cholerae* cells a higher level of tolerance to stresses known to affect proteostasis, including proteotoxic stress caused by aminoglycosides. VchM's modulation of these phenotypes seems to be integrated with the translational and protein quality control processes. However, important questions still need clarification. For example, how biologically relevant is this observation? Is there any situation during *V. cholerae*'s life cycle where methylation of RCCGGY sites is reduced? For that, we will need to know more about the expression of *vchM*. Is it controlled by any known stress regulator? Interestingly, a QS regulator was recently found to bind *vchM* sequence, suggesting the possibility of QS-dependent regulation. A subsequent question arises: is a downregulation of this gene enough to prevent methylation of cytosines at RCCGGY sites? How is the stoichiometry of this enzyme? What is the processivity of this enzyme? Is it able to methylate several motifs without release the DNA? And lastly, is it able to additionally methylate RNA? All of these aspects should be the focus of future work.

MATERIALS AND METHODS

Strains, media and culture conditions

Strains and plasmids used in this study and their constructions are listed in Table 4 and Table 5, respectively. Oligonucleotides used in this study are listed in Table 6.

For routinely cloning we used chemically competent *E. coli* One Shot® TOP10 (Invitrogen).

All *V. cholerae* strains are derived from *Vibrio cholerae* serotype O1 biotype El Tor strain N16961 hapR+. *V. cholerae* mutants were constructed by homologous recombination after natural transformation or with a conjugative suicide plasmid as follows:

Homologous recombination: fragments of around 500 bp upstream and downstream of the gene of interested were amplified by PCR from genomic DNA of *V. cholerae* N16961 El tor hapR+. In addition, the *aadA1* gene, conferring resistance to spectinomycin, or the *aph* gene, conferring resistance to kanamycin, have been amplified from the plasmids pAM34 and pKD4, respectively, with sequences homologous to the regions flanking the gene of interest. The three products generated were then assembled by PCR with specific primers. The assembled DNA fragment was introduced into the *V. cholerae* N16961 El tor hapR+ by natural transformation as previously described (308) and the deletion mutants were selected in LB Lennox containing the respective antibiotics.

Suicide conjugation: a DNA fragment was obtained as described above, sub-cloned in a pTOPO vector and cloned using EcoRI sites in the suicide conjugative plasmid pMP7, which was then introduced by electroporation in competent *E. coli* β3914 cells ((F-) RP4-2-Tc::Mu ΔdapA::(erm-pir116)). Conjugation between this strain and *V. cholerae* N16961 El tor hapR+ was performed for 16 hours at 37°C in LB agar plates supplemented with 0.3 mM Diaminopimelic acid (DAP) and glucose 1% w/v. The content of these plates was then collected in 5ml of LB, vortexed, and non-diluted and diluted aliquots were plated in LB agar plates supplemented with glucose 1% w/v. These plates were incubated overnight at 37°C. Next day several clones were picked in LB and cultures were incubated at 37°C with agitation until fully grown. These cultures were then plated in LB agar supplemented with 0.2% arabinose and antibiotic according to the selection marker, if applicable. Plates were incubated at 37°C overnight or until visible clones appeared.

All strains and plasmids were confirmed by sanger sequencing.

Growth curves

Overnight cultures from single colonies were diluted 1:100 in Mueller-Hinton (MH) rich media or MH + subMIC antibiotics at different concentrations, in 96-well microplates. OD_{600} was measured in a Tecan Infinite plate reader at 37°C, with agitation for 12 hours. Measurements were taken every 10 minutes.

Competitions experiments

Overnight cultures from single colonies of lacZ- and lacZ+ strains were washed in PBS (Phosphate Buffer Saline) and mixed 1:1 (500µl + 500µl). At this point 100µl of the mix were serial diluted and plated in MH agar supplemented with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 µg/ml to confirm T0 initial 1:1 ratio. At the same time, 10 µl from the mix were added to 2 ml of MH or MH supplemented with subMIC tobramycin at 0.6µg/ml and incubated with agitation at 37°C or 42°C, for 20 or 6 hours, respectively. Cultures were then diluted and plated in MH agar plates supplemented with X-gal. Plates were incubated overnight at 37ºC and the number of blue and white cfus was assessed. Competitive index was calculated by dividing the number of blue cfus (lacZ+ strain) by the number of white cfus (lacZ- strain).

Survival assays

Bacterial cultures from single colonies were cultured at 37°C for 16 h with agitation in 10 ml of MH medium. Aliquots from these cultures were removed, serial diluted and plated in MH agar plates to assess cfus formation prior antibiotic treatment (T0). In addition, 5 ml of these aliquots were subjected to antibiotic treatment and incubated with agitation at 37°C. At the indicated time points, 500uL of these cultures were collected, washed in PBS, serial diluted and plated in MH agar plates. The plates were then incubated overnight at 37°C. Survival at each time point was determined by dividing the number of cfus/ml at that time point by the number of cfus/ml prior treatment. Antibiotics were used at the following final concentrations: 20 µg/ml Tobramycin (TOB) and 10 µg/ml Gentamicin (GEN).

Digital qRT-PCR

For RNA extraction, overnight cultures of three biological replicates of strains of interest were diluted 1:100 in MH media and grown with agitation at 37°C until an OD_{600} of 0.3 (exponential phase) or an OD_{600} of 2.0 (stationary phase). 0.5 mL of these cultures were centrifuged and supernatant removed. Pellets which were homogenized by resuspension with 1.5 ml of cold TRIzol™ Reagent. Next, 300 µl chloroform were added to the samples following mix by vortexing. Samples were then centrifuged at 4°C for 10 minutes. Upper (aqueous) phase was transferred to a new 2mL tube and mixed with 1 volume of 70% ethanol. From this point, the homogenate was loaded into a RNeasy Mini kit (Quiagen) column and RNA purification proceeded according to the manufacturer's instructions. Samples were then subjected to DNase treatment using TURBO DNA-free Kit (Ambion) according to the manufacturer's instructions. RNA concentration of the samples was measured with NanoDrop™ spectrophotometer and diluted to a final concentration of 1ng/µl.

qRT-PCR reactions were done with 1 ng of RNA using the qScript^{TM} XLT 1-Step RT-qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA) within a Sapphire chips. Digital PCR was conducted on a Naica Geode (programmed to perform the sample partitioning step into droplets, followed by the thermal cycling program suggested in the user's. Primer and probe sequences used in digital qRT-PCR reaction are listed in Table 7. Image acquisition was performed using the Naica Prism3 reader. Images were then analyzed using the Crystal Reader (total droplet enumeration and droplet quality control) and the Crystal Miner software (extracted fluorescence values for each droplet).

RNA-seq

For RNA extraction, overnight cultures of three biological replicate of WT and Δ*vchM* strains were diluted 1:100 in MH media and grown with agitation at 37ºC until an OD_{600} of 2.0. Total RNA extraction, library preparation, sequencing and analysis were performed as previously described (301).

Transposon insertion sequencing (TI-seq)

A saturated mariner mutant library was generated by conjugation of plasmid pSC1819 from *E .coli* to *V. cholerae* WT and *ΔvchM*. Briefly, pSC189 (309) was delivered from *E. coli* strain 7257 (β2163 pSC189::spec, laboratory collection) into the *V. cholerae* F606 strain and the *ΔvchM* H505 strain. Conjugation was performed for 4 h on 0.45 µM filters. The filter was resuspended in 2 ml of MH broth. Petri dishes containing 100 µg/ml spectinomycin were then spread. The colonies were scraped and resuspended in 2 ml of MH. When sufficient single mutants were obtained (>600 000 for 6X coverage of non-essential regions), a portion of the library was used for gDNA extraction using Qiagen DNeasy® Blood & Tissue Kit as per manufacturer's instructions. The libraries were passaged in MH media for 16 generations with or without subMIC tobramycin at 0.6 µg/ml, in triplicate. gDNA from time point 0 and both conditions after 16 generation passage in triplicate was extracted. Sequencing libraries were prepared using Agilent's sureselect XT2 Kit with custom RNA baits designed to hybridize the edges of the Mariner transposon. The 100 ng protocol was followed as per manufacturer's instructions. A total of 12 cycles were used for library amplification. Agilent's 2100 bioanalzyer was used to verify the size of the pooled libraries and their concentration. After confirmation of correct size of pooled libraries, these samples were sent to high throughput sequencing (Illumina PE150).

Reads were filtered through transposon mapping to ensure the presence of an informative transposon/genome junction using a previously describe mapping algorithm (236). Detection of at least 10 nucleotides of the transposon sequence were considered sufficient o to retain a read. Informative reads were extracted, mapped and counted. Fitness scores were then calculated according to (310). Expansion or decrease of fitness of mutants was calculated in fold changes with normalized insertion numbers. Baggerly's test on proportions was used to determine statistical significance as well as a Bonferroni correction.

Suppressors whole genome sequencing

A WT colony and five independent colonies of Δ*vchM* strain were grown with agitation at 37°C overnight in 20 mL of MH broth. These cultures were then serial diluted and a 10^{-6} dilution was plated in MH agar plates, following incubation overnight at 37ºC. Next day one colony from the WT culture, one small colony from one of the Δ*vchM* cultures and five normal size colonies (suppressors) from

the 5 independent Δ*vchM* cultures, were picked in 2ml MH and grown overnight. 1 mL of each culture was then used for gDNA extraction using Qiagen DNeasy® Blood & Tissue Kit as per manufacturer's instructions. Samples were sent for whole genome sequencing at Institut Pasteur. DNA concentration and quality were measured using Qubit® Fluorometer. Analysis of the results was conducted by the Bioinformatics and Biostatistics Hub at Institut Pasteur.

Bisulfite sequencing

For bisulfite sequencing, overnight cultures from three independent colonies of strains of interest were diluted 1:1000 in MH and incubated for 16 hours in absence of presence of chloramphenicol (5 µg/ml) when appropriate, for plasmid retention. 1 mL of each culture was then used for gDNA extraction using Qiagen DNeasy® Blood & Tissue Kit as per manufacturer's instructions. DNA concentration and quality were measured using Qubit® Fluorometer. Samples were sent for Bisulfite Sequencing at an external company. Analysis of the results was conducted by the Bioinformatics and Biostatistics Hub at Institut Pasteur.

Spontaneous mutation frequency

Bacterial cultures from single colonies were cultured overnight at 37°C with agitation. Appropriate dilutions were plated on MH agar plates, and 1 ml of culture was centrifuged and plated on MH agar supplemented with 100 µg/ml rifampicin plates. The mutation frequency corresponds to the rifampicin-resistant CFU count over the total number of CFU.

MIC assessment with E-tests

Stationary phase cultures were diluted 20 times in PBS, and 300 µL were plated on MH plates and dried for 10 minutes. E-tests (Biomérieux) were placed on the plates and incubated overnight at 37°C.

Adenine methylation Kit

For relative quantification of m6A, single colonies of indicated strains were grown in triplicate at 37°C with agitation, overnight. 1 mL of culture was used for DNA extraction using Qiagen DNeasy® Blood

& Tissue Kit DNA. 200ng of DNA was used as input. Relative quantification of m6A was assessed with *MethylFlash™ m6A DNA Methylation ELISA Kit (Colorimetric)* following manufacturer's instructions. Briefly, in this assay DNA is bound to strip wells using DNA high binding solution. m6A is detected using capture and detection antibodies. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of m6A is proportional to the OD intensity measured.

Table 4. *V. cholerae* strains used in this study

	Strain	Relevant genotype	Construction	Source
WT	F606	*N16961 hapR+*		Gift from Melanie Blokesch
Δ*vchM*	H505	*N16961 hapR+ vchM::kan*	PCR on pH237 with primers zip136/139 followed by natural transformation in F606	This study
Δ*vchM*	H507	*N16961 hapR+ vchM::spec*	PCR on pE352 with primers zip136/139 followed by natural transformation in F606	This study
lacZ-	K329	*N16961 hapR+ ΔlacZ*		Lab collection
Δ*groESL-2*	M958	*N16961 hapR+ vca0819-0820::kan*	Suicide conjugation with pM591 in F606	This study
Δ*groESL-2* Δ*vchM*	N330	*N16961 hapR+ vca0819-0820::kan vchM::spec*	PCR on pE352 with primers zip136/139 followed by natural transformation in M958	This study
WT + pSC101()	N308	*N16961 hapR+* pSC101 empty	Electroporation of pSC101 in F606	This study
Δ*vchM* + pSC101()	N754	*N16961 hapR+ vchM::spec* pSC101 empty	Electroporation of pSC101 in H507	This study
Δ*groESL-2* + pSC101()	N314	*N16961 hapR+ vca0819-0820::kan* pSC101 empty	Electroporation of pSC101 in M958	This study
Δ*groESL-2* Δ*vchM* + pSC101()	N756	*N16961 hapR+ vca0819-0820::kan vchM::spec* pSC101 empty	Electroporation of pSC101 in N330	This study
Δ*groESL-2* Δ*vchM* + pSC101(*groESL-2*)	N757	*N16961 hapR+ vca0819-0820::kan vchM::spec* pSC101-*vca0819-0820*	Electroporation of pN752 in N330	This study
WT + pTOPO()	M512	*N16961 hapR+* pTOPO empty	Electroporation of pTOPO in F606	This study
WT + pTOPO(*groESL-2*)	L839	*N16961 hapR+* pTOPO- *vca0819-0820*	Electroporation of L837 in F606	This study
Δ*groESL-2* + pSC101-groESL-2	N766	*N16961 hapR+ vca0819-0820::kan* pSC101-*vca0819-0820*	Electroporation of pN752 in M958	This study
Δ*groESL-2* + pSC101-groESL-2 (VchM sites #2-4 mut)	N767	*N16961 hapR+ vca0819-0820::kan* pSC101-*vca0819-0820* (VchM sites #2-4 mutated)	Electroporation of pN754 in M958	This study

WT – VchM site #1 mutated	L900	*N16961 hapR+* C->T point mutation of VchM site #1 in *vca0819-0820* region	Suicide conjugation with pL442 in F606	This study
Δ*hpf*	M566	*N16961 hapR+ hpf*::kan		Lab collection
Δ*hpf* Δ*vchM*	N332	*N16961 hapR+ hpf*::kan *vchM::spec*	PCR on pE352 with primers zip136/139 followed by natural transformation in M566	This study
Δ*vchM* sup1	L980	*N16961 hapR+ vchM::kan* junction *vca0201-vca0371*	Natural suppressor of H505	This study
Δ*vchM* sup2	L981	*N16961 hapR+ vchM::kan* vca0200 Q219* (CAG→TAG)	Natural suppressor of H505	This study
Δ*vchM* sup3	L982	*N16961 hapR+ vchM::kan iscR (del 1bp)*	Natural suppressor of H505	This study
Δ*vchM* sup4	L983	*N16961 hapR+ vchM::kan (A)7→6* in *vca0200*	Natural suppressor of H505	This study
Δ*vchM* sup5	L984	*N16961 hapR+ vchM::kan (A)7→6* in *vca0200*	Natural suppressor of H505	This study
Δ*vchM +* pTOPOblunt()	M514	*N16961 hapR+ vchM::kan* pTOPOblunt	Electroporation of pTOPOblunt	This study
Δ*vchM +* pTOPOblunt (*vchM*)	M515	*N16961 hapR+ vchM::kan* pTOPOblunt-*vchM*	Electroporation of pM365	This study
WT + pSC101(*vca0200)*	N309	*N16961 hapR+* pSC101-*vca0200*	Electroporation of pN306 in F606	This study
WT + pSC101(*vca0201)*	N310	*N16961 hapR+* pSC101-*vca0201*	Electroporation of pN307 in F606	This study
Δ*vca0446-0447*	F946	*N16961 hapR+ vca0446-0447*::spec		Lab collection
WT + pSU18()	I752	*N16961 hapR+* pSU18empty	Electroporation of pSU18 in F606	This study
WT + pSU18(*vca0446-0447*)	I754	*N16961 hapR+* pSU18-*vca0446-0447*	Electroporation of pI748 in F606	This study
Δ*vca0446-0447* + pSU18()	I751	*N16961 hapR+ vca0446-0447*::spec pSU18empty	Electroporation of pSU18 in F946	This study
Δ*vca0446-0447* + pSU18(*vca0447*)	I756	*N16961 hapR+ vca0446-0447*::spec pSU18-*vca0447*	Electroporation of pI746 in F946	This study
Δ*vca0446-0447* + pSU18(*vca0446-*vca0447)	I757	*N16961 hapR+ vca0446-0447*::spec pSU18-*vca0446-0447*	Electroporation of pI748 in F946	This study
MCH1	9821			Lab collection
MCH1 *dam*	9817			Lab collection

Table 5. Plasmids used in this study

Plasmids for allelic replacement		Source
pH237	pTOPO Δ*vchM::frt-kan-frt*	Lab collection
pE352	pTOPO Δ*vchM::spec*	Lab collection
pM591	pMP7 Δ*vca0819-0820*	Lab collection
pL442	pMP7- C->T point mutation of VchM site #1 in 5′ UTR region of *vca0819-0820*	Amplification of 500bp upstream of *vca0819* with primers 5923/5922; Amplification of 500bp downstream of *vca0819* with 5924/5921; PCR assembly of the two fragments with primers 5923/5924. Note: Primers 5922 and 5921 contain a different nucleotide to give origin to the C->T point mutation in VchM site #1
Plasmids for overexpression		
pSC101	pSC101 empty	Low copy plasmid – lab collection
pN752	pSC101-Ptrc-*vca0819-0820*	Amplification of *vca0819-0820* from *V. cholerae* gDNA with primers AFC029/AFC030. Cloning with EcoRI in pSC101
pTOPO	pTOPO empty	High copy plasmid – TOPO TA cloning kit
pL837	pTOPO-Plac-*vca0819-0820*	Amplification of *vca0819-0820* from *V. cholerae* gDNA with primers 5981/5982. Cloning in pTOPO through TOPO TA cloning.
pM365	pTOPO-PvchM-vchM	Amplification of *vca0198 (vchM)* with its own promoter from *V. cholerae* gDNA with primers 5983/5911. Cloning in pTOPOblunt through TOPO blunt cloning.
pN306	pSC101-Ptrc-*vca0200*	Amplification of *vca0200* from *V. cholerae* gDNA with primers AFC013/AFC014. Cloning with EcoRI in pSC101
pN307	pSC101-Ptrc-*vca0201*	Amplification of *vca0201* from *V. cholerae* gDNA with primers AFC015/AFC016. Cloning with EcoRI in pSC101
pSU18	pSU18 empty	mild copy plasmid – lab collection
pI746	pSU18-Plac-*vca0447*	Amplification of *vca0447* from *V. cholerae* gDNA with primers 5518/5519 and cloning in pSU18 using SmaI/SalI
pI748	pSU18-Plac-*vca0446-vca0447*	Amplification of *vca0447* from *V. cholerae* gDNA with primers 5518/5519 and cloning in pSU18 using SmaI/SalI

Table 6. Oligonucleotides used in this study

Oligonucleotide	**Sequence 5'-3'**
ZIP136	GCCGCCGAAGGAAAAACCGTACTATTGC
ZIP139	TTAATTTCTCGAGTTTCAGATGC
5518	GTACCCGGGATGTTAGGTTATGCGTTTCCC
5519	GTAGTCGACGGCATAAATAGTTTCAAGCTCTA
5921	AAACAATCCTA**T**CGGCCTTTTATC
5922	GATAAAAGGCCG**A**TAGGATTGTTT
5923	ACTTTGATGGTACGCGCGATG
5924	GATTTATTGAGCACAACATGGCG
5981	GTACCCGGGATGAATATTCGTCCTTTACATG
5982	GTAGTCGACATTACGCCGCAGACTCTTTGTC
AFC013	GTAAGTGAATTCTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCAC ACAGGAAACAGCGCCGCATGATTAACGAAGTAACTTTAAG
AFC014	GTAAGTGAATTCCTATAGTTTTCATTTATCCTGTCC
AFC015	GTAAGTGAATTCTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCAC ACAGGAAACAGCGCCGCATGAAAACTATAGTTTCCGTT
AFC016	GTAAGTGAATTCCTTCTAAGAAGGCGGATTT

Table 7. Primer and probe sequences used in digital qRT-PCR reactions.

Target	Primers (5'-3')	Probes (5'-3')
groES-1	CGTAGCTTTCTGCGAAGATC	[HEX] - AGCTCCAAAGGTTGAACTGAACCGTTCTCTA -[BHQ1]
	TGGTGGAATTGTTCTAACTG	
groES-2	GGCGACCAGATCATTTTCAAC	[FAM] - TGGACGGTAAAGAGTATCTGATCCTCTCC-- [BHQ1]
	TCTACAATCGCTAACACATCAG	
vchM	TGCTATCGTTCAGAATCAACC	[HEX] - AAGCTTACCAGCATAGAACTTGCCAGAGAT- [BHQ1]
	TTTACTTCTCGCTCCCAATCG	

SUPPLEMENTARY DATA

Table S1. RNA-seq of Δ*vchM* strain (upregulated genes)

Locus	Name	Annotation	Fold change Δ*vchM*/WT	p-value
vca1028	*lamB*	maltoporin	52,81	0,0E+00
vca0707	*uhpC*	MFS transporter	8,76	8,6E-06
vc1368		conserved hypothetical protein	8,18	0,0E+00
vca0819	***groES-2***	**chaperonin**	**6,78**	**0,0E+00**
vca0200		putative ATPase	6,10	0,0E+00
vca0957	*glcB*	putative Malate synthase	5,97	0,0E+00
vca0199		**conserved hypothetical protein**	**5,32**	**5,2E-09**
vca1027	*malM*	putative Maltose operon periplasmic protein MalM	4,14	0,0E+00
vca0201		conserved hypothetical protein	3,97	3,4E-11
vca0946	*malK*	Maltose/maltodextrin import ATP-binding protein malK	3,91	0,0E+00
vca0469	*higA2*	plasmid stabilization system HigA protein (antitoxin of TA system HigB-HigA)	3,91	0,0E+00
vca0468	*higB2*	plasmid stabilization system HigB protein (toxin of TA system HigB-HigA)	3,90	2,3E-10
vca0945	*malE*	maltose ABC transporter periplasmic binding protein	3,90	0,0E+00
vc1819	*aldA*	Aldehyde dehydrogenase	3,84	1,7E-12
vc1204	*hutG*	putative Formimidoylglutamase HutG (histidine utilization)	3,80	2,2E-16
vc1203	*hutU*	Urocanate hydratase (histidine utilization)	3,60	0,0E+00
vca0820	*groEL-2*	chaperonin	3,54	2,2E-16
vca0692	*secF*	fragment of putative SecD/SecF/SecDF export membrane protein (part 2)	3,48	3,2E-08
vc1809	*alpA*	putative Prophage CP4-57 regulatory protein (AlpA)	3,37	1,8E-09
vc1205	*hutI*	Imidazolonepropionase (histidine utilization)	3,24	1,1E-11
vc1740	*fadE*	acyl coenzyme A dehydrogenase	3,22	5,6E-15
vca0944	*malF*	maltose ABC transporter membrane subunit MalF	3,18	0,0E+00
vc1142	*cspD*	Cold shock-like protein cspD	2,88	0,0E+00
vc2530	*hpf*	putative ribosome hibernation promoting factor HPF/sigma 54 modulation protein	2,83	0,0E+00
vca0948	*yaeO*	putative Rho-specific inhibitor of transcription termination (YaeO)	2,78	0,0E+00
vca0998	*xenB*	Xenobiotic reductase B	2,73	4,4E-06
vc0486	*srlR*	putative Transcriptional regulator of sugar metabolism; DeoR family transcriptional regulator	2,65	9,9E-14
vc1874		putative SpoVR family protein	2,64	0,0E+00
vc1202	*hutH*	Histidine ammonia-lyase	2,61	0,0E+00
vc2265	*groES-1*	chaperonin	2,60	0,0E+00
vc2615		conserved hypothetical protein	2,55	4,6E-10

vc1678	*pspA*	Phage shock protein A	2,48	4,6E-10
vc1183		putative 2OG-Fe dioxygenase	2,43	0,0E+00
vca0470		putative Acetyltransferase	2,43	2,1E-08
vca0324	*relB1*	Plasmid stabilization system protein RelB (Anti Toxin of TA system RelB-RelE)	2,43	1,1E-11
vca0886	*kbl*	glycine C-acetyltransferase	2,40	2,3E-08
vca0185	*arfA*	putative Stalled ribosome alternative rescue factor ArfA	2,39	3,0E-13
vca0958		putative transcriptional regulator	2,38	3,9E-10
vca0013	*malP*	maltodextrin phosphorylase	2,37	3,9E-11
vca0881		conserved hypothetical protein	2,36	1,1E-13
vca0551		conserved hypothetical protein	2,35	0,0E+00
vca0391	*higB1*	Plasmid stabilization system HigB protein (Toxin of TA system HigB-HigA)	2,34	1,5E-10
vca0923	*mlp37*	chemoreceptor Mlp37	2,34	2,2E-12
vc2361	*grcA*	Autonomous glycyl radical cofactor	2,30	0,0E+00
vca0392	*higA1*	Plasmid stabilization system HigA protein (Antitoxin of TA system HigB-HigA)	2,28	0,0E+00
vc0665	***vpsR***	**Fis family transcriptional regulator (sigma-54 dependent transcriptional regulator)**	**2,28**	0,0E+00
vca0720	*hnoX*	Heme-Nitric Oxide/Oxygen Binding Protein (H-NOX)	2,27	5,1E-10
vc1433	*uspE*	universal stress protein UspE	2,26	0,0E+00
vc1248	*tar*	putative Methyl-accepting chemotaxis protein	2,25	0,0E+00
vc2264	*groEL-1*	chaperonin	2,24	0,0E+00
vc0734	*aceB*	Malate synthase A	2,24	1,4E-06
vc2507		PhoH family protein	2,23	0,0E+00
vc1344	*hppD*	4-hydroxyphenylpyruvate dioxygenase	2,22	6,9E-15
vca0219	*hlyA*	Hemolysin	2,20	5,7E-14
vca0159		putative Universal stress protein family 1	2,17	2,9E-15
vca0987	*ppsA*	phosphoenolpyruvate synthase	2,15	2,6E-09
vca0359	*parE2*	Plasmid stabilization system ParE protein (toxin of TA system ParD-ParE)	2,14	9,4E-10
vca0004		conserved hypothetical protein	2,14	0,0E+00
vc1222	*ihfA*	integration host factor subunit α	2,13	0,0E+00
vc2758	*fadB*	fatty acid oxidation complex subunit alpha FadB	2,10	6,1E-10
vc2704		conserved hypothetical protein	2,10	8,9E-09
vc1962	*nlpE*	copper resistance protein NlpE N-terminal domain-containing protein	2,08	3,9E-09
vca0278	*glyA*	serine hydroxymethyltransferase	2,04	0,0E+00
vca0280	*gcvT*	glycine cleavage system aminomethyltransferase	2,04	4,4E-16
vc0976	*qmcA*	putative Membrane protease subunits, stomatin/prohibitin homolog qmcA	2,03	6,5E-06
vc1539a		hypothetical protein	2,02	6,9E-10
vc0666		conserved hypothetical protein	2,02	1,8E-09
vc2144	*flaF*	Polar flagellin F	2,01	3,0E-13

Table S2. RNA-seq of Δ*vchM* strain (downregulated genes)

Locus	name	Function	fold change	p-value
vca0198	*vchM*	Cytosine-specific DNA methyltransferase	-12,22	1,4E-24
vca0933	*cspE*	transcription antiterminator and regulator of RNA stability	-8,41	1,0E-07
vca0017	*hcp*	Haemolysin co-regulated protein (putative Type VI secretion system effector, Hcp1)	-3,20	1,3E-10
vc2383	*ilvY*	putative Transcriptional regulator, LysR family	-3,14	5,9E-27
vca0804	*deaD*	Cold-shock DEAD box protein A	-3,12	6,5E-49
vca0874		conserved hypothetical protein	-3,10	1,1E-05
vc1704	*metE*	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	-3,02	7,6E-21
vc0768	*guaA*	GMP synthetase	-2,91	2,7E-72
vca0680	*napC*	Cytochrome c-type protein napC	-2,87	2,4E-07
vca0679	*napB*	Diheme cytochrome c napB	-2,82	2,1E-06
vca0935		hypothetical protein	-2,76	1,2E-10
vc1261		putative Sugar efflux transporter B	-2,63	3,9E-07
vc0069	*mdtL*	putative FLORFENICOL EXPORTER	-2,53	8,1E-14
vca1002		AzlC family ABC transporter permease	-2,49	2,0E-08
vc1415	*hcp*	type VI secretion system secreted protein Hcp	-2,48	1,0E-06
vc0433	*arcD*	Arginine/ornithine antiporter	-2,48	1,9E-06
vc1393	*sugE*	Quaternary ammonium compound-resistance protein sugE	-2,43	3,4E-22
vc1035		conserved hypothetical protein	-2,40	6,3E-06
vc1608		ABC transporter permease	-2,38	2,8E-11
vca0250		Alpha-amylase	-2,37	7,2E-08
vc0767	*guaB*	inositol-5-monophosphate dehydrogenase	-2,31	3,0E-22
vc1239	*cobU*	cobinamide-P guanylyltransferase / cobinamide kinase	-2,28	1,2E-09
vc0290	*fis*	DNA-binding protein fis	-2,27	1,4E-04
vc2227	*purN*	phosphoribosylglycinamide formyltransferase 1	-2,25	2,6E-10
vc2384		TSUP family transporter	-2,24	8,1E-16
vca1003		AzlD domain-containing protein	-2,23	1,5E-11
vca0678	*napA*	Periplasmic nitrate reductase	-2,22	2,6E-10
vca1031		Methyl-accepting chemotaxis protein	-2,21	7,0E-11
vc1843	*cydB*	Cytochrome d ubiquinol oxidase subunit 2	-2,21	5,9E-13
vc0191	*rhtC*	putative Lysine/Homoserine/Threonine exporter protein	-2,20	2,6E-10
vc1609		ABC transporter permease	-2,19	2,2E-10
vc0005	*yidD*	membrane protein insertion efficiency factor	-2,16	4,9E-10
vc0291	*dusB*	tRNA-dihydrouridine synthase B	-2,16	3,8E-16
vc1855	*dinG*	ATP-dependent DNA helicase DinG	-2,15	7,7E-07
vc2382		conserved hypothetical protein	-2,15	3,1E-07
vc1842	*cydX*	cytochrome bd-I oxidase subunit CydX	-2,14	1,8E-06

vc1629		ABC transporter permease	-2,13	3,0E-06
vc0706	*raiA*	Ribosome-associated inhibitor A	-2,13	1,1E-08
vc2047		SDR family oxidoreductase	-2,11	3,4E-23
vc1174	*trpE*	Anthranilate synthase component 1	-2,09	9,9E-18
vc1240		histidine phosphatase family protein	-2,09	1,5E-06
vc0940		conserved hypothetical protein	-2,07	1,3E-07
vc1487		glutaredoxin family protein	-2,06	8,0E-06
vca0214	*emrD*	multidrug efflux pump EmrD	-2,05	6,0E-12
vca0269		aspartate aminotransferase family protein	-2,04	2,2E-13
vc2647	*aphA*	Transcriptional regulator PadR family	-2,04	9,6E-56
vc2226	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase	-2,02	9,3E-10
vc0651		putative Peptidase U32	-2,02	2,4E-13
vc1630		putative SalX, ABC-type antimicrobial peptide transport system, ATPase component	-2,02	2,5E-19
vca0684		MFS transporter	-2,02	9,3E-14
vc0717	*yegQ*	tRNA 5-hydroxyuridine modification protein YegQ	-2,01	4,2E-09
vc1856		TSUP family transporter	-2,00	7,2E-10
vc0987	*hemH*	ferrochelatase	-2,00	1,9E-17

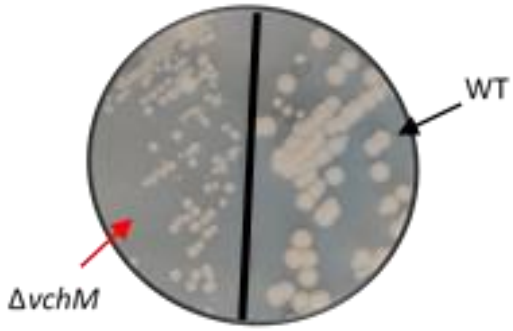

FIG S1. Colony size of WT and *ΔvchM* mutants.

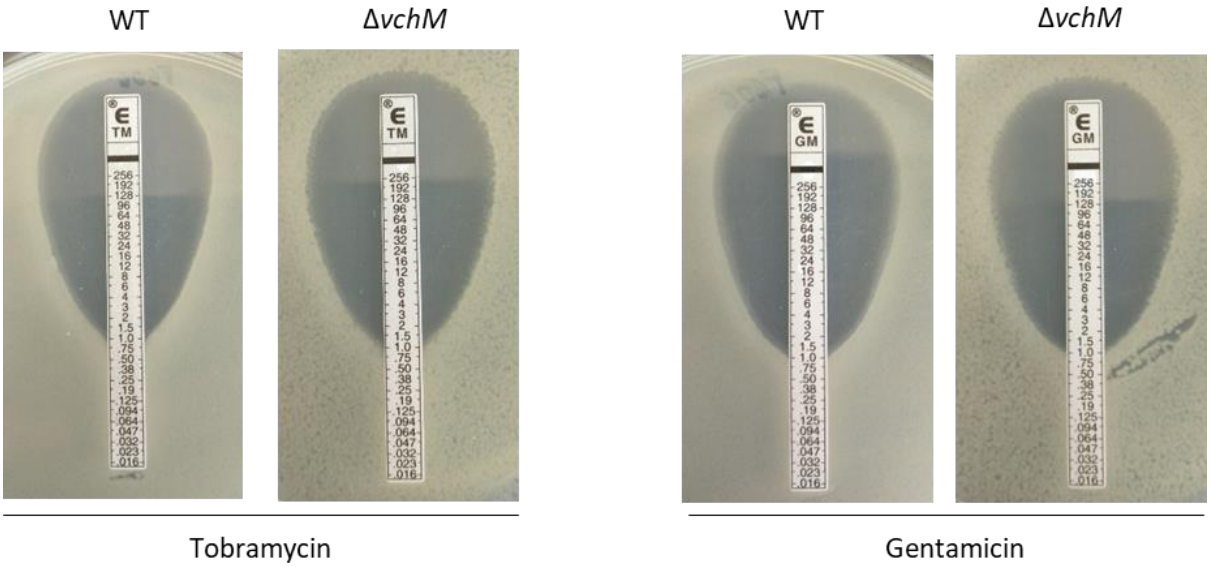

FIG S2.MIC of Tobramycin and Gentamicin of WT and *ΔvchM* mutants measured with E-tests (Biomérieux).

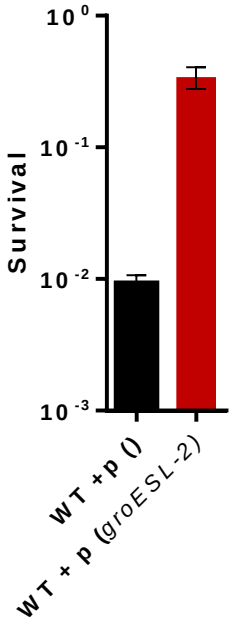

FIG S3. Survival of stationary-phase *V. cholerae* WT cells transformed with pTOPOempty plasmid (WT + p()) or with pTOPO-*groESL-2* (WT + p(*groESL*-2) exposed to lethal doses of TOB 20X MIC (20µg/ml). Survival is determined by dividing the cfu/ml at 4 hours post-treatment by the cfu/ml before treatment. Mean and standard deviations are represented, n=3.

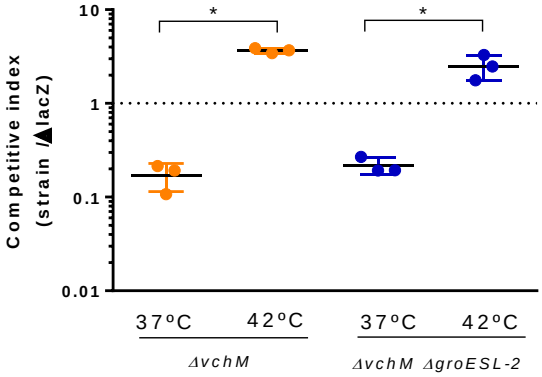

FIG S4. Competitions of *V. cholerae* Δ*vchM* and Δ*vchM ΔgroESl-2* cells (lacZ+) against a *lacZ*- mutant at 37°C or 42°C. After 6 hours in co-culture, cells were diluted and plated in MH agar supplemented with x-gal. The competitive index was calculated from the ratio of lacZ+ strains to lacZ- strain. * p-value < 0.05 Mann-Whitney test; n ≥ 3.

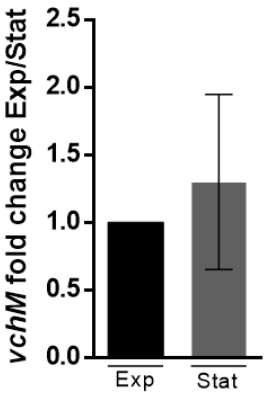

FIG S5. Relative expression levels of *vchM* in exponential (Exp) or stationary phase (Stat), measured by digital qRT-PCR. n=3.

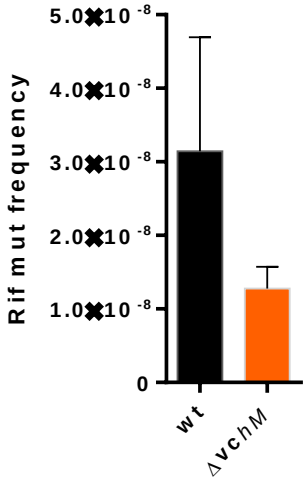

FIG S6. Frequency of Rifampicin resistant (RifR) mutants of WT and Δ*vchM* strains. Rif mutation frequency is calculated by dividing the number of rifampicin resistant clones by the total number of clones, at T=24. n=3. Standard deviations are represented.

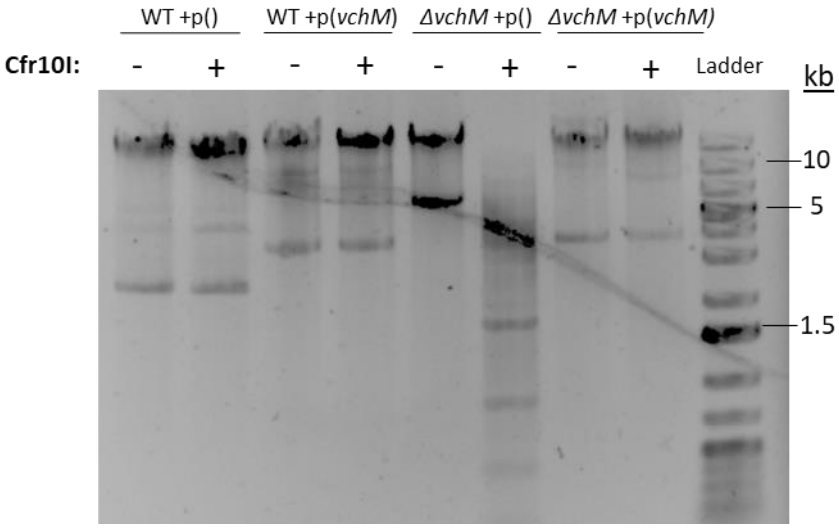

FIG S7. Genomic DNA from the indicated strains digested or not with the methylation-sensitive enzyme Cfr10I, which does not cleave methylated RCCGGY motifs.

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