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Review Article

Environmental factors, oxidative stress and the effects of mutation on *Vibrio cholerae*

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ABSTRACT

Vibrio cholera is a gram negative, motile, non spore forming, non capsulated, curved or comma shaped rod with rounded or slightly pointed ends, about 1.5-2.4×0.2-0.4µm in size. *Vibrio cholera* has been isolated from a variety of clinical and environmental samples. The majority of *Vibrio cholera* strains isolated from the environment are non-01serovars, although 01 serovars have also been observed in areas, where limited outbreaks of cholera have occurred. During last fifty years, cholera has disappeared from most developed countries, but is remerging in many parts of the world in epidemic form, especially in tropical areas. It is possible because *Vibrio cholera* strains are still mutating. There are about 45 strains in the world (according to WHO, July 2012) but these strains were not mutated within a year, now a day environmental factors are so fluctuating that *Vibrio cholera* strains are mutating. Different biotic and abiotic factors influence on mutation of *Vibrio cholera*. Poisonous atmosphere arising from swamps and putrid matters as a source of disease influence the mutation of *Vibrio cholera*. It is due to wet, poorly drained and raw waste material. Antibiotic resistance among pathogens influences bacterial mutation. In this study when an environmental as well as genetic factor affects spontaneous mutation of *Vibrio cholera* that either sensitive or resistance to antibiotics then coding property of DNA double helix in replication is changed. Antibiotic resistance arises among bacterial population by endogenous or exogenous mechanism induces spontaneous mutation. Organisms that grow aerobically are exposed to oxidative stress in the form of reactive oxygen species (ROSs) (e.g peroxide, superoxide) that are the unavoidable by products of aerobic respiration. ROSs damages a variety of cellular macromolecules and thus elicits adaptive oxidative stress responses in bacteria intended to permit survival in the presence of this stressor. The present review briefly discusses about environmental factors, oxidative stress and the effects of mutation of *Vibrio cholera*e.

1. Introduction

Vibrio cholera, the etiologic agent of the disease cholera, is a rod shaped motile bacterium with a single sheathed polar flagellum. Vibrio cholera elaborateoles a toxin, cholera toxin, responsible for most of the diarrhea associated with this disease. However *v.cholerae* strains unable to produce the toxin still cause a mild diarrhea in humans, indicating the cholera toxin is not the only pathogenic factor[1]. Historically, seven pandemics of cholera have been recorded, the worst being the recent and continuing seventh pandemic that began in 1961, after a hiatus of 33 years[2]. The most important pathogens of man are *Vibrio cholera*. Cholerae is caused by this bacterium. This highly contagious disease is caused when the bacterium *Vibrio cholera* is ingested via contaminated water or food. Cholerae is a serious and ancient disease that has killed millions of people. During a cholera epidemic in 1854 in Florance, Pacini first described the comma shaped gram negative vibrio the "comma bacillus" responsible for cholera which was subsequently named *Vibrio cholera* by Robert Koch visited in India and examined faces of cholera patient in Medical college, Kolkatta.

Vibrio cholera is divided into two biotypes, classical and E1 Tor, distinguished on the basis of hemolytic activity, bacteriophage susceptibility, and the level of resistance to the antibiotic polymyxin B[3]. Although strains of the classical biotype were associated with previous cholera pandemics, in the present pandemic (1961 to present) classical strains have been replaced by E1 Tor strains as the causative agent[3]. However, occasional outbreaks caused by classical *V.cholerae* still occur[4]. *Vibrio cholera*, causative agent of epidemic cholera, has been isolated from a variety of clinical and environmental samples[5-8]. Cholerae is not properly disappeared from most developed countries, because different environmental biotic factors influence the mutation of *Vibrio cholera* strain.

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As a result *Vibrio cholera* strains are still mutating and cholera is remerging in many parts of the world in epidemic form, especially in tropical areas. The type II secretion (T2S) system plays an important role in the pathogenesis of *V.cholerae* by secreting cholera toxin**[9]**, which is largely responsible for the symptoms of the disease**[10]**. The T2S system is widespread and well conserved in gram negative bacteria inhabiting a variety of ecological niches and likely contributes to environmental survival as well as to virulenc**[11-12]**. In *v.cholerae*, secretion via the T2S machinery is supported by a trans envelope complex of 12 Eps proteins (EpsC to EpsN) and the type 4 prepilin peptidase PilD (VcpD)**[13,14,15]**. Transport of exoproteins by the T2S system occurs via a two step process. The first step, which is either Sec or Tat dependent, requires recognition of the N-terminal signal peptide of the exoproteins and translocation through the inner membrane to the extracellular milieu**[16,17]**.

1.1 *Influence of environmental factors on mutation*

Environmental factors are divided into two types, Intrinsic factors and extrinsic factors. Extrinsic factors influence the mutation of *Vibrio cholera*. Extrinsic factors are those that refer to the environmental surrounding food. Environmental factor or ecofactor is any factor that influences the virulence gene of *Vibrio cholera*, as a result the mutation of *Vibrio cholera* is changed for newly adaptation. These factors include temperature and pH.

1.2 *Influence of temperature on mutation of Vibrio cholerae*

Spontaneous mutation of *Vibrio cholerae* is greatly influenced by temperature. *Vibrio cholerae* grows well on ordinary media within a wide range of temperature 16^0 to 40^0C (optimum temperature is 37^0C). Temperature influences the rate of chemical reactions and protein structure integrity thus affecting rates of enzyme activity. At low temperature enzymes are not denaturated, therefore every 10^0C rise in temperature results in rise of metabolic activity and growth of microorganisms. However enzymes have a range of thermal stability and beyond it there denaturation take place and mutation of bacteria is influenced. Thus the high temperature kills bacteria by denaturating enzymes, by inhabiting transport carrier molecules or by change in membrane integrity.

1.3 *Influence of pH on mutation of Vibrio cholerae*

Vibrio cholerae grows well in strongly alkaline media (pH 8.2 to 9.5), which suppresses the growth of other intestinal bacteria. The range of pH for growth is 6.4 to 9.6 (optimum pH 8.2). Too much acid or base disrupts cellular activities and influence the mutation of *Vibrio cholerae*.

1.4 Oxidative stress/Importance of oxidative stress

Challenge of bacteria with reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals and hypochlorous acid (HOCl) causes a stress condition generally termed oxidative stress**[18]**. Efficient killing of Vibrios by host macrophages depends on a number of mechanisms including production of reactive oxygen species (ROS) by the phagosomal NADPH oxidase. ROS like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO) are toxic compounds produced by the incomplete reduction of oxygen during oxidative metabolism**[19]**. Cells have acquired the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. The protective mechanisms include both non-enzymatic (ascorbic acid, β-carotene glutathione, and α-tocopherol) and enzymatic superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) antioxidant systems. Bacteria employ mainly enzyme mechanisms to eliminate the damaging effects of oxidative stress, such as superoxide dismutase**[20,21,22]**, NADH oxidase**[23]**, CAT**[24]**, GPx**[25]**, glutathione reductase**[26]** thiol peroxidase**[27]** and alkyl hydroperoxidase**[28]**.The question of the mechanisms by which certain vibrio species survive oxidative stress has been under intense investigation**[29]**.Oxidative stress in animals have been found under different environmental conditions when they are fed on different drinks**[30-32]**.

1.5 *Influence of oxidative stress on Vibrio cholera*

Aerobic and facultative anaerobic microorganisms face constant risk from reactive oxygen species (ROS), including superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) that may be formed through the univalent reduction of molecular oxygen (O_2). These radicals may cause oxidative damage by oxidizing biomolecules and results in cell death**[33]**. Therefore elimination of ROS is definitively necessary for survival of cells. Superoxide dismutase (SOD) as part of the defense systems against oxidative damage in aerobic organisms, catalyzes superoxide anion(O_2^-) to O_2 and (H_2O_2), which then is reduced to H_2O by H_2O_2 scavenging enzyme catalase (CAT**)[34]**. Under stress conditions, the balance between oxidative impact and the antioxidative defense system could be disturbed leading to oxidative stress.

Vibrio cholerae non 01 serotypes are autochthonous bacteria of aquatic environments that are presently recognized as potential pathogens**[35]**. These species have been associated with cholera-like diseases and other extra intestinal infections, not only in humans but also in higher aquatic organisms**[36]**.

Various Vibrio species isolated of natural aquatic habitats possess a number of protecting mechanisms against oxidative stress or to different inducers of oxidative stress from the environment. There are contradictory data about the role of both antioxidant enzymes SOD and CAT in the cell response of Vibrio strains against stress**[37]** demonstrate that SOD expression in *V.shiloi* is temperature regulated (i.e the enzyme is produce at 30^0C, but not at 16^0C).

1.6 *Effect of temperature on the growth of Vibrio strains*

At 10^0C *Vibrio cholerae* 26/06 was the most sensitive to low temperature. A similar behavior was described for *V.vulnificus* which exhibited morphological changes at low temperature[38]. In contrast it was found that *V.cholerae* could adapt and grow at temperature down to 15^0C below which the growth was completely arrested[39].

1.7 *Effect of temperature on the enzyme of antioxidant activities*

All strains of *Vibrio cholerae* are exposed both antioxidant enzymes under normophysiological and stress conditions, but the cell response is more strain dependent than dependent on temperature.SOD activity in *V.cholerae* 29 is increased by 7.5 fold under stress conditions in comparison to that at optimal temperature. The strain *V.cholerae* 29T did not show any significant difference in the cell response depending on the growth temperature.

CAT activity in the cells of *V.cholerae* non 26/06 and *V.cholerae* 29T exhibited no temperature dependence. The strain of *V.cholerae* 29 is in accordance with the hypothesis that the cold stress induces production of ROS[40] and increases antioxidant enzyme synthesis[41]. SOD and CAT activities in *V.cholerae* 26/06 and *V.cholerae* 29T did not increase at low temperature. Moreover, the cold stress induces significant reduction of SOD activity in *V.cholerae* 26/06, paralleling the delayed growth.

1.8 *Vibrio cholerae* gene expression in response to inactivation of the T2S system

The *Vibrio cholera* type II secretion system (T2S) is amultiprotein complex that plays an important role in the pathogenesis of Vibrio cholera by secreting cholera toxin[42]. This system is wide spread and well conserved in gram negative bacteria likely contributes to environmental survival as well as to virulence[12]. Inactivation of the T2S system, by removal of either single *eps* genes or the entire *eps* operon, results not only in the loss of extracellular secretion but also in cell envelope perturbation, upregulation of the σ^E mediated stress response, and a reduced growth rate[42-43]. To further investigate the cellular response, to inactivation of the T2S system, we performed a global transcriptome analysis of NΔeps, in which the entire *eps* operon has been removed[43] and the parental wild-type strain, *V.cholerae* N16961, grown with aeration in LB medium. The gene expression data were analyzed by applying SAM software[44]. Inactivation of the T2S machinery resulted in changes in the expression of genes coding for many members of the σ^E regulon, some of which are outer membrane constituents, and genes encoding proteins participating in chemotaxis/motility, biofilm formation, transport of amino acids and carbohydrates, central metabolic process, and stress responses that involve heat shock proteins, chaperones, and proteases.

1.9 *Induction of oxidative stress in T2S mutants*

Iron is crucial for the structure and function of many proteins. Excess iron can be deleterious to the cells, because it rapidly reacts with ROS, which are a natural consequence of aerobic metabolism and generates highly toxic hydroxyl radicals via the Fenton reaction[45-46]. Therefore tight control of iron metabolism, is an integral part of the antioxidant defense response[47]. Fur provides protection against oxidative damage by inducing the expression of several genes encoding proteins that confer resistance to oxidative stress[48-49]. Microarray results revealed that the NΔeps mutant displayed significant upregulations of genes coding for antioxidant proteins, such as catalase (KatB), alkylhydroperoxidase (AhPC), and Superoxide dismutase (SodB)[50]. The level of ROS is higher in T2S mutants provides additional evidence that under conditions where the T2S machinery is inactivated, cells are suffering from internal oxidative stress.

2.0 *Paraquat (PQ) and hydrogen peroxide (H_2O_2 affect ROS production*

ROS used as a marker of oxidative stress, changes in the level of O_2^- and H_2O_2 in *V.cholerae* non 01 26/06 treated by different concentrations of PQ and H_2O_2. Exposure to PQ resulted in enhanced ROS levels in bacterial cells in a dose dependent manner. Superoxide anion redical level (µM per mg d.w. per 1h) increased steadily in PQ concentration range of 0.1-3mM. Even in low concentration (0.1-0.5mM), induced 1.2-1.8 fold higher generation of O_2^- compared with the control that has been reported. It has been shown that in the presence of 3mM PQ, bacterial cells accumulated 3.5 fold more O_2^- than the control cells and at the same time PQ induced about 2.4 fold increases in H_2O_2 content (mM per mg d.w per 1h) compared with the control.

Exposure to H_2O_2 indicate an opposite trend of O_2^- changes with PQ that has been reported .After 60 min in the presence of H_2O_2 in the concentration range of 0.1-3.0mM, a 20 to 50% decrease in O_2^- production. On the other hand, it has been shown that 2-fold increased H_2O_2 content in treated Vibrio cells compared with the control.

Exposure of *V.cholerae* cells to PQ and H_2O_2 promoted oxidative stress. The unstressed bacterial cells seemed to produce O_2^- and H_2O_2 presumably due to single electron reduction of 2% of the oxygen[51]. ROS can be generated via a variety of physiological and pathological conditions, including PQ and H_2O_2 exposure[52-53]. Direct assay of ROS showed that all variants of PQ treatment bacterial cells for 1h do clearly cause oxidative stress, which induced both O_2^- and H_2O_2 generation. Presence of second stress agent significantly inhibited O_2^- production and enhanced intracellular H_2O_2 content. Excessive ROS level has been linked to lipid peroxidation of the cell membrane, resulting in a loss of membrane fluidity, structure and function[51]. On the other hand the increased ROS level could be a direct consequence of the exogenous H_2O_2, but could also result from the generation of ROS by damaged cells and mitochondria[52].

2.1 *PQ and H_2O_2 induce protein oxidation*

Reaction of proteins with oxygen radicals leads to the appearance of carbonyl groups in polypeptide chains[54]. Measuring the content of these groups in intracellular proteins is one of the accepted assays for oxidative damage in microbial cells. When Vibrio strain was exposed to enhanced concentration of PQ and H_2O_2, the amount of carbonyl groups in cell proteins was changed in a dose-dependent manner.

The enhanced carbonylation damage to intracellular proteins is a marker for accumulation of oxidatively modified proteins. Environmental agents such as ionizing, near- UV radiation, or numerous compounds that generate intracellular O2- (redox-cyclining agents such as menadione and paraquat) can cause oxidative stress, which accelerates oxidation of proteins in pro- and eukaryotic cells[52].

2.2 *V.cholerae rescue aerobic growth defect of the OxyR mutant*

Many bacteria control oxidative stress through *OxyR*, a *LysR* type transcriptional regulator. OxyR is involved in the oxidative stress response of *V.cholerae*. It has been reported that *OxyR* homolog mutants in several gram negative bacteria including *E.coli*, *Xanthomonas campestris*, *Haemophilus influenza*, and *Pseudomonas aeruginosa* display aerobic growth defects in rich media[52-53].

This is not surprising since H_2O_2 is produced as an auto oxidation product of aerobic rich broth[52] and *OxyR* is critically involved in oxidative stress resistance. It has been shown that addition of spent culture restore *OxyR* mutant growth in *P.aeruginosa*[55]. It has been found that in the presence of cell free culture of wild type *V.cholerae OxyR* mutants grew normally on both solid and liquid LB medium. This suggests that *OxyR* is critical for aerobic survival and that the *OxyR* growth defect can be rescued by addition of wild type *V.cholerae* culture.

2.3 *Involvement of two catalases in rescuing OxyR aerobic growth defect*

Catalases affect *V.cholerae* growth. Two catalase genes are annotated in the *V.cholerae* genome, KatG (VC1560), and KatB (VC1585). Detecting either KatG or KatB, as well as both KatG and KatB together did not affect growth, a deletion was examined in prxA (VC2637), a gene that is divergently transcribed from oxyR and whose product has been shown to be regulated by H_2O_2[58] KatG and KatB gene products must play some role in ROS resistance[55]. KatG-KatB double mutant was weaker than that of the KatG single mutant. Both KatG and KatB in *V.cholerae* play a role in detoxifying H_2O_2 and promote OxyR mutant growth and KatG is more potent than KatB[55]. Catalase genes are regulated by oxidative stress signals and OxyR[56]. Both KatG and KatB were induced by H_2O_2, but interestingly OxyR was not required for the induction of these genes, at least under the growth condition tested[56].

It has been shown that expression of KatG and KatB was only significantly lower in the OxyR mutant than in wild type and PrxAluxCDABE was strongly induced by H_2O_2 and deletion of OxyR abolished PrxA expression[56].

2.4 *Stress responsive proteins*

Temperature stress in bacteria induces the generation of a heat shock response which involves the expression of a set of very conserved proteins called heat shock proteins(H_SPs)[59]. HSPs are commonly grouped into families based on their molecular weight: HSP10 (10KDa) or GroES-homologue proteins, HSP60(~60KDa) or GroEL-homologue proteins, HSP40(~40KDa) or DnaJ-homologue proteins, HSP70 (70KDa) or DnaK-homologue proteins, HSP90 or HPtG-homologue proteins (~90KDa) and clp ATP- dependent proteases (HSP100).

3. Conclusion

The role of environmental factors in the occurrence of cholera is also being studied further to gain greater precision in the predictive model. Recently published analyses showed that temperatures of≥25 ℃ and P^H of ≥7.0 enhance *Vibrio cholera* counts in the Chesapeake Bay. Earlier studies in Bangladesh and Peru identified temperature as a key factor associated with increased counts of *Vibrio cholerae* and cases of cholera[60-61]. Factors affecting mutability of chromosomal genes conferring resistance to antibiotics have a potential role among different *Vibrio cholerae* species. The nature of spontaneous mutation rates which are affected by different environmental and genetic factors.

The spontaneous mutation frequencies to antibiotic resistance among *Vibrio cholera* strains were shown. *In-vitro* spontaneous mutation frequencies of *Vibrio cholera* strains to antibiotics can be enhanced according to the composition of growth medium and selective antibiotic concentration, suggests that under specific in vivo condition, where lower antibiotic concentrations and high osmotic conditions might prevail, selection of chromosomal mutants coferring multiple resistance among bacterial species. Sensitivity and spontaneous mutability to antibiotic resistance were altered according to the composition of growth medium under environmental factors. Mutator genotypes increase mutation rates among bacterial populations of different bacterial species both in nature and in laboratory. Spontaneous mutations conferring multiple resistances to antibiotics can arise at high frequencies under environmental conditions at low selective antibiotic concentrations. Environmental factors as well as genetic factors induced mispair formation in the DNA double helix. As a result coding property of DNA base pair would be changed and effected spontaneous mutation among bacterial population and therefore spontaneous mutation frequency in DNA replication would be either increased or decreased. Organisms that grow aerobically are routinely exposed to oxidative stress in the form of reactive oxygen species (ROSs).

[e.g peroxide, superoxide] that are the unavoidable by products of aerobic respiration. Oxidative stress responses have the potential to contribute to antimicrobial resistance in a variety of ways. *Vibrio cholerae* is an opportunistic human pathogen that has two elistinct life styles: in aquatic environments, often associated with plankton and other marine organisms, and propagating in human small intestines**[62]**. In both environments, however, oxidative stress induced by reactive oxygen species (ROSs) must be a common stress condition to which *Vibrio cholerae* encounters. In many microorganisms, sub lethal exposure to a stress (e.g starvation of marine vibrios) can confer resistance to a lethal exposure to the same stress (adaptive response) or to other stresses (cross-protection response)**[63]**.

As reviewed by Oliver**[64]** high salt concentrations represent a significant stress to enteric. Osmotic stress has some physiological features in common with starvation including the induction of some starvation proteins by osmotic shock in *E.coli***[65]**.

In conclusion mutational effects are magnified under environmental stress. Some mutations with deleterious effects across most environments appear to have beneficial effects in other environment**[66]**. Thus oxidative stress responses as well as environmental factors effect mutation in *Vibrio cholera*.

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