Chapter

# Evolutionary Strategies of Highly Functional Catalases for Adaptation to High $H_2O_2$ Environments

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## Abstract

Enzymatic evolutionary strategies for adaptation to a high $H_2O_2$ environment have been evaluated using catalases with high catalytic efficiency isolated from two $H_2O_2$-tolerant bacteria, *Exiguobacterium oxidotolerans* and *Psychrobacter piscatori*. The entrance size of the narrow main channel in catalase has been estimated by determining the formation rate of the intermediate state of peracetic acid (b), which is a larger substrate than $H_2O_2$ versus that of catalase activity with $H_2O_2$ (a) (calculated as b/a). The ratio of b/a in *E. oxidotolerans* catalase (EKTA) is much higher than that of *P. piscatori* catalase (PKTA). To elucidate the structural differences between the catalases, the amino acids present in the main channel have been compared between the two catalases and other catalases in the database. The combination of amino acid residues, which contribute high catalytic efficiency in the narrow main channel of EKTA were different from those in PKTA. In this review, we discuss strategic differences in the elimination of high concentration of $H_2O_2$ owing to differences in the phylogenetic positions of catalases. In addition, we describe the relationships between the environmental distributions of genera involved in $H_2O_2$-resistant bacteria and their catalase functions based on the main channel structure of catalase.

**Keywords:** $H_2O_2$-tolerant bacteria, *Exiguobacterium*, *Psychrobacter*, *Vibrio*, catalase, narrow main channel, bottleneck size

## 1. Introduction

Oxygen is important for metabolism, acting as a terminal electron acceptor in aerobic bacteria, and these bacteria produce intracellular reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), superoxide ($O^{2\bullet-}$), and hydroxyl radical ($OH^{\bullet}$) as by-products of oxygen metabolism [1–4]. $H_2O_2$ is not a strongly harmful substance; however, the presence of $H_2O_2$ in bacterial cells may lead to the generation of harmful ROS, such as $OH^{\bullet}$, via the Fenton reaction. Therefore, the presence of catalase is critical for the protection of cellular components, such as DNA, RNA, proteins, and lipids, from strongly harmful $OH^{\bullet}$ [5–7]. Moreover, the production of intracellular catalases is important for the metabolism of aerobic microorganisms to conduct their metabolisms.

Bacteria possess catalases for the elimination of toxic by-products of oxygen metabolism produced inside the cells and for preserving their niches by eliminating the $H_2O_2$ produced by host organisms [8–10]. This function is important, particularly for pathogenic and symbiotic microorganisms or microorganisms needing to maintain their niches in the host. In such cases, bacterial catalases may have evolved during interactions with the host (to degrade active oxygen species generated by the host for parasites elimination) or parasitic/symbiotic microorganisms (to eliminate active oxygen species generated by the parasites/symbionts). For example, *Aliivibrio fischeri* (formerly *Vibrio fischeri*) exhibits a symbiotic relationship with the host squid by colonising the light-emitting organs of the squid. The host squid possesses a protective mechanism associated with the production of $H_2O_2$ to prevent the colonization of unfavourable pathogenic bacteria. In contrast, *A. fischeri* produces highly efficient catalase in the periplasmic space to eliminate $H_2O_2$ produced by the host squid. Thus, production of catalase in the vicinity of the cell surface is important for helping microorganisms to establish their niche.

The oral biofilm community consists of various microorganisms, including foe and companion bacteria and functions to maintain the ecological balance among constituents [11]. Among these community members, *Streptococcus gordonii* is known to produce $H_2O_2$ to expel its competitors. Additionally, *Veillonella atypica* is able to support the growth of the obligate anaerobe, *Fusobacterium nucleatum* under microaerophilic conditions and can also protect the microorganism from *S. gordonii* via production of catalase. Thus, extracellular catalase production is important for protection not only of the niche of the producer but also of other companion microorganisms to facilitate the formation of microbial communities within biofilm.

Catalase is commonly observed in various aerobic bacteria. Bacteria that do not possess catalase cannot grow on the agar plates owing to the presence of $H_2O_2$ on agar plates [12]. However, many bacterial strains have been isolated from agar plates, suggesting that these bacteria likely express catalase and these bacteria are likely to encounter $H_2O_2$. Moreover, these data suggest that $H_2O_2$ may be ubiquitously present in various environments in which many microorganisms live. Accordingly, investigation of the molecular strategies through which catalase eliminates $H_2O_2$ in various physiological, ecological, and taxonomic background is essential.

In this review, we evaluate the relationships between catalase evolution and structural changes in the main channel structure of catalases, based on various catalases including those isolated from $H_2O_2$- tolerant bacteria. In addition, considering the taxonomic backgrounds of $H_2O_2$-tolerant bacteria, we compared the main channel structures of catalases derived from the same genera of $H_2O_2$-tolerant bacteria and discussed the reasons for the distribution of these $H_2O_2$-tolerant bacteria. This systematic approach will bring deeper understanding in strategic evolutionary changes in bacterial catalases and strategic bacterial distributions in the environment.

## 2. Phylogeny of catalases

The dismutation of $H_2O_2$ in microorganisms occurs mainly via three phylogenetically unrelated catalases: monofunctional catalase, catalase-peroxidase, and Mn-catalase [2, 13]. Here, we focus on monofunctional catalases.

Bacterial monofunctional catalases are classified into clades 1–3 according to phylogenetic analysis based on their amino acid sequences [14, 15]. Clade 1

catalases contain approximately 500 amino acid residues per subunit and are mainly of plant origin, except a subgroup that is of bacterial origin, including Firmicutes group A and Proteobacterial minor group (*Sinorhizobium* clade). Clade 2 catalases, which exhibit larger molecular masses than catalases from other clades, consist of approximately 750 amino acid residues. The catalases in this clade originated from fungi, bacteria including Actionbacteria, Bacteroides, and Proteobacteria (*Polaromonas, Burkholderia* and *Akkermansia*) and archaea. Clade 3 catalases, with nearly 500 residues per subunit, occur in fungi, bacteria including Chloroflexi, Firmicutes group B and Proteobacteria, fungi, and some eukaryotes. Reports have shown that pathogenic or symbiotic bacteria possess only one clade 3 catalase (e.g., *Haemophilus influenzae*, *Neiseria gonorrhoeae*, and *A. fischeri* [described above]). These catalases evolved through interactions between the host and parasite. Moreover, many prokaryotic clade 3 catalases exhibit distinct NADP(H) binding compared with clade 1 catalases, discrimination of catalases between the two clades based on apparent molecular features and enzymatic characteristics is difficult.

## 3. Reaction mechanisms of catalases

Catalase consists of four identical subunits and each subunit, each of which possesses heme *b* or *d* at the reaction centre. The catalytic reaction cycle consists of the following two steps. The first step involves the formation of compound I, which is produced by oxidation of $Fe^{3+}$ ($Fe^{3+}$ Pro) in the heme moiety to an oxoiron (IV) porphyrin π-cation radical species, $Fe^{4+}$ = O $Pro^{+\bullet}$, by the first reacted $H_2O_2$ molecule [16]. During this reaction, the oxygen–oxygen bond in the peroxide (R–O–O–H) bound to the heme, that is the first $H_2O_2$ molecule, is cleaved heterolytically. As a result, one oxygen binds to the ion with the by-product of a water molecule. This reaction intermediate, compound I, is subsequently reduced by second reaction of $H_2O_2$ to the resting state ($Fe^{3+}$ Pro). This reaction leads to the production of molecular oxygen ($O_2$) and water molecules ($H_2O$) [17, 18]. Compound I can also be observed if organic peroxides are used as substrates instead of $H_2O_2$. The compound I formation rate decreases as the molecular size of the substrates increases (i.e., $H_2O_2$ > $CH_3COO_2H$). Therefore, estimation of the compound I formation rate may be an indicator of the size of the bottleneck structure of the narrow main channel, which is directly accessible to the reaction centre, heme.

## 4. Characteristics of $H_2O_2$-resistant bacteria

Catalase is important for cellular protection intra- and extracellular elimination of $H_2O_2$. Because $H_2O_2$-tolerant microorganisms may evolve in artificially high $H_2O_2$ environments, we have studied $H_2O_2$-tolerant microorganisms and their catalases. First, strain S-$1^T$ can survive downstream of drain pools (sedimentation tank [8°C, 1.5–6 mM]) from herring egg processing factory, which uses $H_2O_2$ as a breaching agent [19, 20]. This strain was identified as a new species, *Vibrio rumoiensis* S-$1^T$. The growth temperature range of strain S-$1^T$ is 2–34°C. The catalase activity of cell extracts of strain S-$1^T$ was found to be 4000–8000 U/mg protein, which was one or two orders of magnitude higher than those of *Alcaligenes faecalis*, *Corynebacterium glutamicum*, and *Pseudomonas fluorescens*. Strain S-$1^T$ possesses only one type of clade 3 catalase, which accounts for 1.8% of the protein in cell extracts. The isolate produces catalase not only inside the cell but also in

the periplasmic space and on the cell surface [21–24]. Therefore, *V. rumoiensis* S-1$^T$ cells exhibit catalase activity, and expression of catalase on the surface of *V. rumoiensis* cells may help to protect the cell in high $H_2O_2$ environments. According to several reports on symbiotic or pathogenic strains involving the genus *Vibrio* and its related genus *Aliivibrio*, strain S-1$^T$ was predicted to be derived from marine environments or organisms.

Strain T-2-2$^T$, an $H_2O_2$-tolerant microorganism, was isolated from the upstream region of a water treatment system (pretreatment tank to decrease $H_2O_2$ concentration [8°C, 6–38 mM]) of a herring egg processing factory [25]. The isolate was identified as a new species, *E. oxidotolerans* T-2-2$^T$. The growth temperature range of this strain was 4–40°C (optimum 34°C). The cell extract of strain T-2-2$^T$ exhibited catalase activity of 28,000 U/mg protein and catalase accounted for 6.5% of protein in the cell extract. The bacterium produced catalase (*E. oxidotolerans* [EKTA]) both intercellularly and extracellularly [26–29]. The immunolocalization of catalase suggests that the enzyme is present on the inner surface of the cells [28]. Catalase that bind to the cell surface and localise to the inner surface are also important for defence against extracellular $H_2O_2$ in *E. oxidotolerans* T-2-2$^T$. The localisation of catalase changes from inside of the cells to the cell surface as the culture period is extended. The catalase is induced by $H_2O_2$ stimulation prior to initiation of growth and low aeration growth condition [27, 29]. Thus, catalase activity is required inside the cells and is essential for extracellular defence as the cell age increases. *Exiguobacterium* spp. are distributed in various environments, including marine environments [30, 31]. Therefore, strain T-2-2$^T$ may have originated from marine environments or organisms. Additionally, although strain T-2-2$^T$ possesses a catalase gene sequence belonging to clade 2, only clade 1 catalase can be purified [32].

Strain T-3-2$^T$, an $H_2O_2$-tolerant microorganism, was isolated from the upstream of the water treatment system (pretreatment tank to decrease $H_2O_2$ concentration [8°C, 6–38 mM]) of a herring egg processing factory [33]. The growth temperature range of strain T-3-2$^T$ is 0–30°C, and the localisation of catalase has not yet been clarified. However, strain T-3-2$^T$ exhibits high resistance against $H_2O_2$. The isolate was identified as a new species, *P. piscatorii* T-3-2$^T$ and cell extracts of strain T-3-2$^T$ exhibit much higher catalase activity (12,000 U/mg protein) than those of other stains belonging to the same genus, including *Psychrobacter nivimaris* (15 U/ mg protein), *Psychrobacter proteolyticus* (29 U/mg protein) and *Psychrobacter aquamaris* (1800 U/mg protein). Strain T-3 belongs to *P. piscatorii* as well [34, 35] and exhibits higher catalase activity (19,700 U/mg), with catalase accounting for 10% of all proteins in the cell extract. Several reports have described *Psychrobacter* spp. were isolated from marine origins [36]; therefore, it is possible that strains T-3-2$^T$ and T-3 originated from marine environments or organisms. Although the strain T-3 possesses catalase gene sequences belonging to clade 2, only clade 3 catalase can be purified [32].

## 5. Characteristics of catalases from $H_2O_2$-resistant bacteria

Catalases derived from $H_2O_2$-tolerant microorganisms in clade 3 and clade 1 have been purified from *V. rumoiensis* S-1$^T$, *P. piscatorii* T-3, and from *E. oxidotolerans* T-2-2$^T$. The kinetic parameters ($k_{cat}/K_m$) of these catalases were higher or equivalent to the highest values comparing with those of catalases reported by Switala and Loewen (2002) [37]. In addition, these catalase activities exhibited distinctive temperature dependencies comparing with ordinary catalase such as *Micrococcus*

*luteus* catalase (MLC) and bovine liver catalase (BLC) [32]. These characteristics reflect the environmental conditions in which these bacteria were isolated (8°C, 1.5–38 mM $H_2O_2$). Thus, multiple environmental factors (including low temperature and high $H_2O_2$) have affected the characteristics of enzymes via evolutionary and/or environmental selection processes.

The catalase from *V. rumoiensis* S-1$^T$ (VKTA) can be purified by two steps of anion-chromatography and one step of gel filtration chromatography [25]. The purified VKTA exhibits 395,000 U/mg protein under standard reaction conditions (30 mM $H_2O_2$, pH 7), with a $V_{max}$ and $K_m$ of 8.0 × $10^5$ μmol $H_2O_2$/μmol heme/s and 35 mM for $H_2O_2$, respectively, as determined spectrophotometrically. The catalytic efficiency $k_{cat}/K_m$ of VKTA is 2.3 × $10^7$/s/M, which is the highest among reported clade 3 catalases owing to the low $K_m$ value [31]. Additionally, because of the fragility of *V. rumoiensis* S-1$^T$ cells, high affinity to $H_2O_2$ and high catalytic efficiency are required for protection of the cells. It is known that catalase activity is not as dependent on temperature as the activity of ordinary enzymes. Moreover, VKTA exhibits an obvious temperature dependence between 10°C and 70°C with an optimum temperature at 40°C. The amino acid sequence of VKTA contains active sites ($H^{61}$, $T^{100}$ and $N^{134}$), proximal sites of heme ($Y^{344}$ and $R^{351}$), and binding sites for the distal region of heme ($V^{102}$, $T^{124}$ and $F^{139}$). VKTA possesses NADPH- binding sites ($H^{180}$, $R^{189}$, $V^{288}$ and $K^{291}$). The active site containing "$T^{100}$" is unique compared with that of the other catalases listed in **Figure 1**. Indeed, other catalases contain an "S residue at this position", making the site less hydrophobic. However, the effect of this amino acid substitution on the function is unknown.

EKTA can be purified by two steps of anion-chromatography and one step of gel filtration chromatography. The purified EKTA exhibits an activity of 430,000 U/mg protein under standard reaction condition [26] with a $V_{max}$ and $K_m$ of 1.5 × $10^6$ μmol $H_2O_2$/μmol heme/s and 40 mM for $H_2O_2$, respectively, as determined by spectrophotometry [28]. The catalytic efficiency $k_{cat}/K_m$ of EKTA is 3.8 × $10^7$/s/M, which is the highest among reported clade 1 catalases owing to the high $k_{cat}$ and low $K_m$ values. EKTA exhibits a temperature dependency between 10°C and 70°C with an optimum temperature of 45°C. Catalase activity decreases from 100–60% as the temperature increases from 45–50°C and then is further decreased to approximately 10% at 70°C. Moreover, this catalase exhibits the highest temperature sensitivity among the three catalases purified from the three $H_2O_2$- tolerant bacteria. The amino acid sequence of EKTA contains active sites ($H^{56}$, $S^{104}$ and $N^{138}$), proximal sites of heme ($Y^{339}$ and $R^{346}$) and binding sites for the distal region of heme ($V^{97}$, $T^{119}$ and $F^{142}$), as shown in **Figure 1**. There is no NADPH-binding site in the amino acid sequence of this catalase. These important residues for catalase activity are well conserved in EKTA.

The catalase from *P. piscatorii* T-3 (PKTA) can be purified by one step of anion-chromatography and one step of hydrophobic chromatography [35]. The purified PKTA exhibits an activity of 222,000 U/mg protein under standard reaction conditions, with $V_{max}$ and $K_m$ of 2.4 × $10^5$ μmol $H_2O_2$/μmol heme/s and 75 mM for $H_2O_2$, respectively. The catalytic efficiency $k_{cat}/K_m$ of PKTA is 3.2 × $10^6$/s/M as determined with $O_2$ electrode [34]. PKTA exhibits a temperature dependency between 10°C and 80°C with an optimum temperature of 45°C. The activity decreases at temperature over 50°C, showing approximately 10% at 70°C and complete deactivation at 85°C. The amino acid sequence of PKTA contains active sites ($H^{65}$, $S^{104}$ and $N^{138}$), proximal sites of heme ($Y^{348}$ and $R^{355}$), and binding sites for the distal region of heme ($V^{106}$, $T^{128}$ and $F^{143}$). This PKTA also contains NADPH-binding sites ($H^{184}$, $R^{193}$, $V^{292}$ and $K^{295}$), as shown in **Figure 1**. These important residues for catalase activity are well conserved in PKTA.

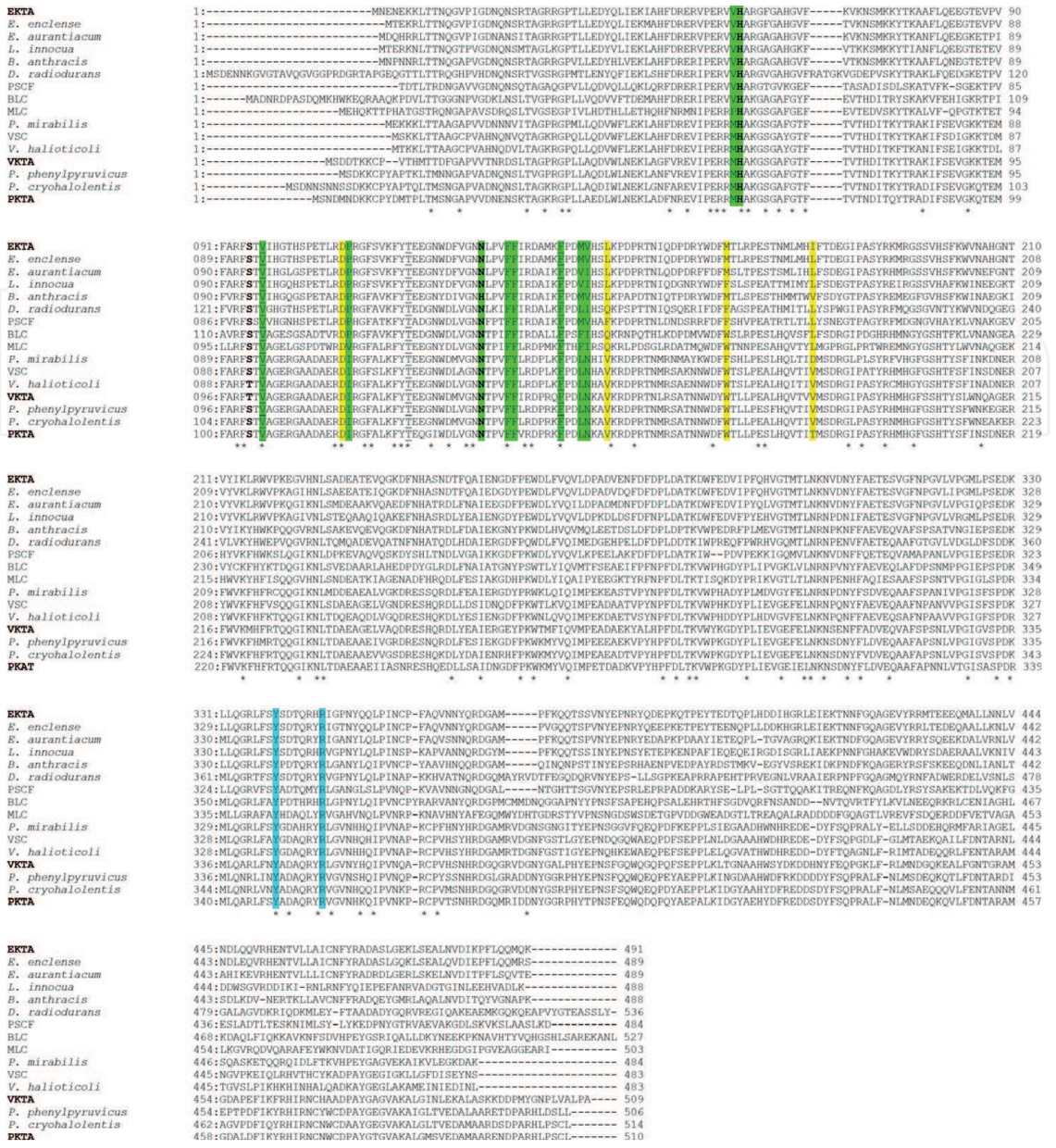

**Figure 1.**
*Amino acid sequence alignment of EKTA,* Exiguobacterium enclense *catalase,* Exiguobacterium aurantiacum *catalase,* Listeria innocua *catalase,* Deinococcus radiodurans *KatA, PSCF, MLC,* Proteus mirabilis *catalase,* Aliivibrio salmonicida *(VSC) catalase,* Vibrio halioticoli *catalase, VKTA,* Psychrobacter phenylpyruvicus *catalase and* Psychrobacter cryohalolentis *catalase. The amino acid residues involved in the narrow main channel are highlighted by green or yellow (bottleneck residues). The active sites are indicated in bold font, the proximal sites of heme are marked in blue and the binding sites of distal region of heme are marked by underlined text.*

## 6. Relationship between the compound formation rate with peracetic acid and the bottle neck amino acid residue in the narrow main channel

Catalase is known to have high activity owing to its superior substrate selectivity for $H_2O_2$. The interactions of substrate molecules larger than $H_2O_2$ are strongly inhibited due to selection of the substrate by the narrow main channel, which reaches the active site. The formation rate of the reactive intermediate (compound I) in the reaction of EKTA with peracetic acid is 77 times higher than that of BLC and 1200 times higher than that of MLC [26]. A comparison of the structural and functional data on EKTA (a clade 1 catalase) with the data for two clade 3 catalases (BLC

and MLC) revealed that the size of the bottleneck defines the compound I formation rate, which corresponds to the size of the substrate molecule. The atom-to-atom distance for combinations of amino acid residues showed that, the $L^{149}$ (BN [bottleneck] 2) to $I^{180}$ (BN4) and $D^{109}$ (BN1) to $M^{167}$ (BN3) combinations at the bottleneck of EKTA resulted in larger bottleneck sizes than the combinations in BLC and MLC [26]. The sizes of the amino acids and the probability of occurrence of the corresponding amino acids (based on a comparison of catalase sequences in the database) indicated that $M^{167}$ may play a key role in determining the size of the bottleneck of EKTA. Clade 3 catalases, i.e., BLC and MLC contain W (Phe) in the corresponding position of $M^{167}$ in EKTA. The volume of W (Phe) is 231.7 $Å^3$, whereas that of M (Met) is 167.7 $Å^3$ [38, 39]. Therefore, the size of the key residue $M^{167}$ in EKTA is the major reason for the high the compound I formation rate with peracetic acid.

## 7. Comparison of amino acid residues in the narrow main channel of catalase

The main channel of catalase consists upper and lower narrow parts. The narrow part, which is nearer to the reaction centre, heme consists of 14 amino acid residues [26] (**Figures 1** and **2**). The seven residues forming the channel ($H^{56}$, $V^{97}$, $D^{109}$, $N^{129}$, $F^{134}$, $F^{135}$ and $F^{142}$ in EKTA) are well conserved (≥95% homology). $V^{55}$ is relatively highly conserved (≥ 80%) followed by $P^{110}$ (54%). The other amino acid residues, including $M^{145}$ (approximately 20%), $V^{146}$ (approximately 30%) and $L^{149}$ (approximately 20%) are relatively rarely conserved. Both $M^{167}$ and $I^{180}$ are very rarely conserved (≤ 3%) among catalases. Among the 14 amino acid residues described above, $D^{109}$ (BN1), $L^{149}$ (BN2), $M^{167}$ (BN3) and $I^{180}$ (BN4) are located in the bottleneck structure in between the upper and lower parts of the main channel of catalase. Among these four amino acid residues only $D^{109}$ is well conserved. Therefore,

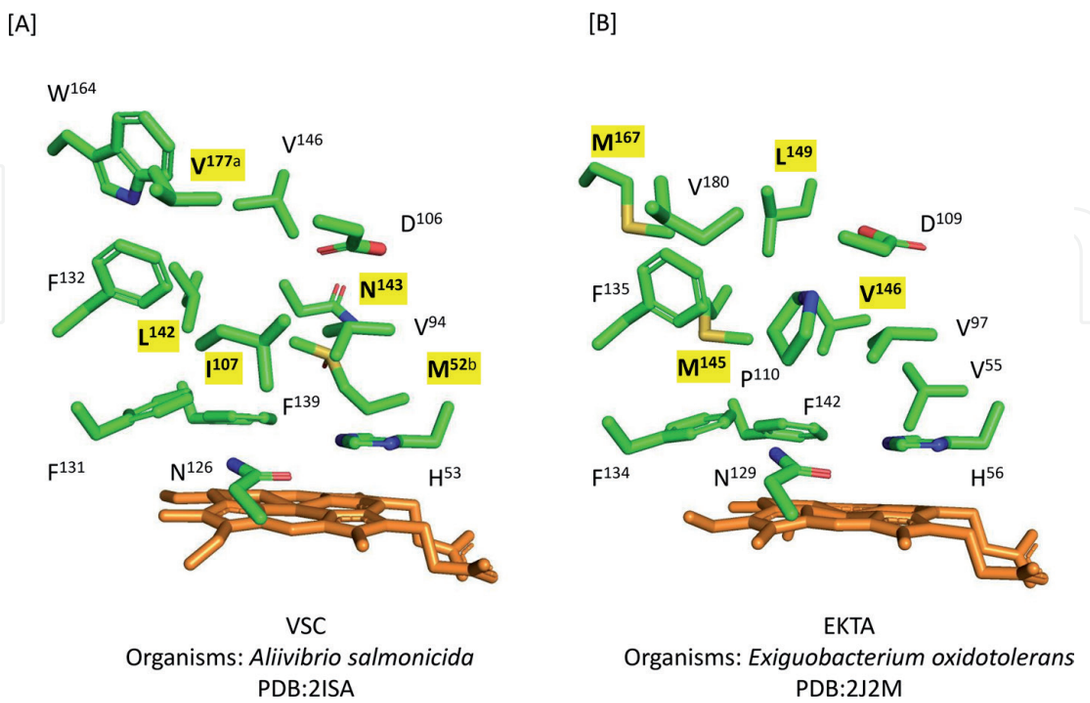


**Figure 2.**
*Structural model of narrow main channels of catalases of VSC [A] and EKTA [B]. Each characteristic amino acid residues are indicated by yellow marker. Number for amino acid residues was accordance with* ***Figure 1****. The amino acid residues of narrow main channels in VSC are the same as VKTA. [a]This amino acid residue is substituted to "T" in PKTA. [b]This amino acid residue is modified as S-dioxymethionine.*

variations in amino acid residues, except $D^{109}$, define the size of the bottleneck structure and the reaction rate with substrates larger than $H_2O_2$. Based on the alignment of multiple catalases including other catalases derived from other species belonging to the genus *Exiguobacterium*, there are several common amino acid residues between EKTA and *Exiguobacterium enclense* catalase ($M^{145}$, $V^{146}$, $L^{149}$ and $M^{167}$). Owing to the lower volumes of these residues compared with the corresponding residues in other catalases, these residues are thought to be related to the genus-specific efficiency catalytic reactions in the presence of high concentrations of $H_2O_2$.

In contrast, $M^{64}$, $I^{119}$, $L^{154}$, $N^{155}$ and $T^{158}$ are specific amino acid residues in the narrow main channel of PKTA. These amino acid residues are corresponding to $M^{60}$, $I^{115}$, $L^{150}$, $N^{151}$ and $V^{154}$ in VKTA and catalases from *Proteus mirabilis*, *Aliivibrio salmonicida*, *Psychrobacter phenylpyruvicus* and *Psychrobacter cryohalolentis* catalases. Although the activities of the latter two catalases are not known, the other three catalases exhibit high catalytic efficiency for $H_2O_2$ [40]. Therefore, these residues are specific to the catalase of Proteobacteria and affect the efficiency of these catalases.

## 8. Relationship between catalase phylogeny and the main channel structure of catalases

The clade 1 catalase EKTA exhibits a higher ratio (b/a = 1.4) of the compound I formation rate using peracetic acid (a) to catalase activity using $H_2O_2$ (b) than the clade 3 catalase PKTA (b/a = 0.0056) [29]. Although the size of the bottleneck of PKTA is unknown, the difference in the catalytic characteristics can be attributed to the seize of the bottleneck, which this can be ascertained from the amino acid residues in the bottleneck. In addition to EKTA and PKTA, the b/a ratio was estimated using the clade I catalases, *Pseudomonas syringae* catalase (PSCF) and *Deinococcus radiodurans* catalase and the clade 3 catalases BLC and MLC. Differences in the b/a ratio are related to the intensity of the degree of the extended branch in the phylogenetic tree of catalase (**Table 1** and **Figure 3**). This indicates that catalases from $H_2O_2$-tolerant bacteria evolved in different directions depending on the bacterial taxonomic phylogenetic position. Thus, the phylogenetic position can be

| | **EKTA** | **PKTA** | **VKTA** |
|---|---|---|---|
| **Bottle neck structure** | | | |
| BN2–BN4 | $L^{149}$, **M167**, $I^{180}$ | $V^{158}$, **W176**, $T^{189}$ | $V^{154}$, **W172**, $V^{185}$ |
| The size of BN2–BN4 | 164.6, **167.7**, 164.9 | 150.6, **231.7**, 120.0 | 150.6, **231.7**, 139.1 |
| **Enzymatic feature** | | | |
| b/a ratio[a] | **1.4** | **0.0056** | ND |
| Kinetic parameters for $H_2O_2$ | | | |
| $V_{max}$ (/s) | $1.5 \times 10^{6}$ [b] | $2.4 \times 10^{5}$ [c] | $8.0 \times 10^{5}$ [b] |
| $K_m$ (mM) | 40[b] | 75[c] | 35[b] |
| $k_{cat}/K_m$ (/M/s) | $3.8 \times 10^{7}$ [b] | $3.2 \times 10^{6}$ [c] | $2.3 \times 10^{7}$ [b] |
| **Cellular features** | | | |
| Percentage of catalase in cell extract | **6.5%** | **10%** | 1.8% |
| **Location of isolation** | Upstream of the drain (**6–38 mM $H_2O_2$**) | Upstream of the drain (**6–38 mM $H_2O_2$**) | Downstream of the drain (1.5–6 mM $H_2O_2$) |
| **Involved bacteria** | Gram positive | Gram negative | Gram negative |

| | EKTA | PKTA | VKTA |
|---|---|---|---|
| **Phylogeny** | | | |
| Clade | 1 | 3 | 3 |
| Extended of phylogenetic position | **Yes** | **Yes** | **Yes** |
| Purified catalase activity (U/mg)[c] | | | |
| Catalase activity of cell extract (U/mg)[d] | **28,000** | **20,000** | 7,300 |

[a]*The ratio of compound I formation rate using peracetic acid (a) to catalase activity using $H_2O_2$ (b).*
[b]*Determined by spectrophotometry.*
[c]*Determined by oxygen electrode analysis.*
[d]*Standard reaction conditions of 30 mM $H_2O_2$ at pH 7.*

**Table 1.**
*Summary of the characteristics of catalases from $H_2O_2$-tolerant bacteria.*

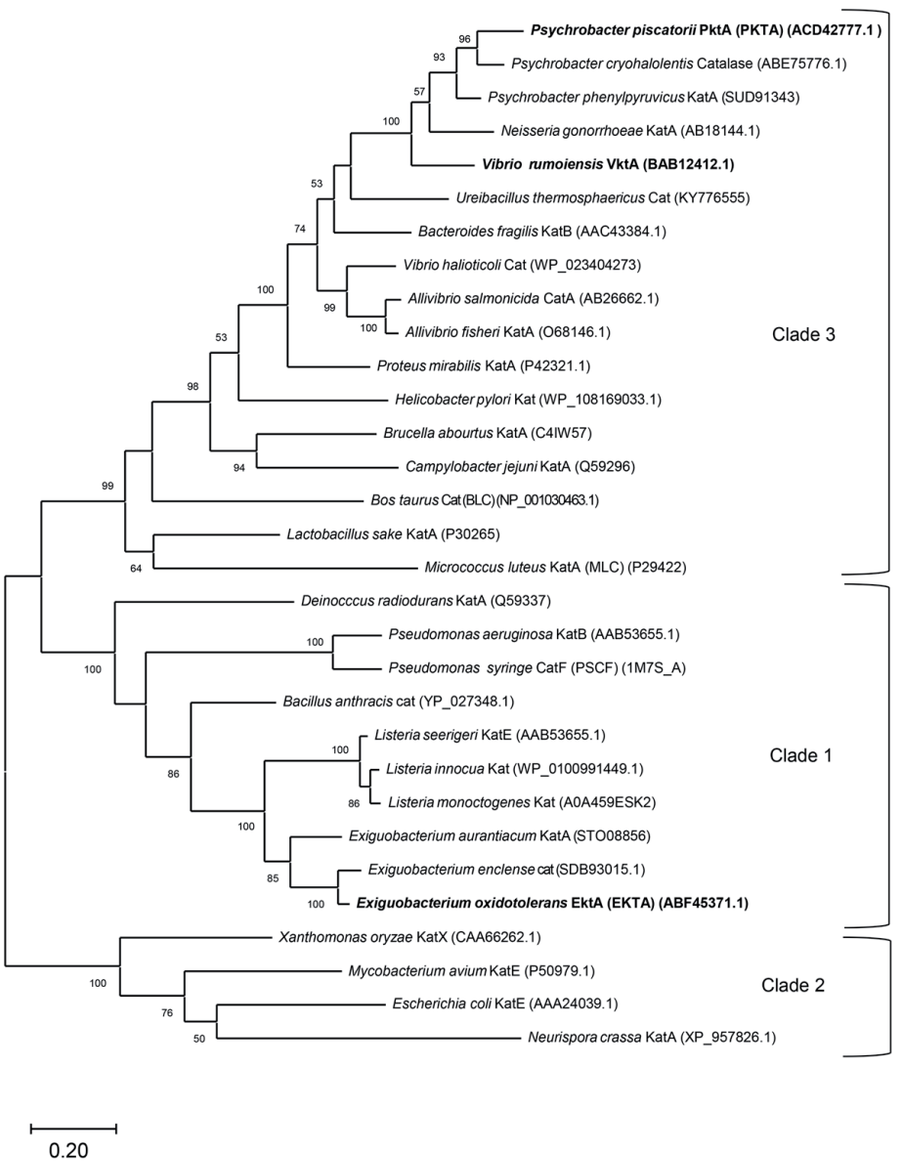


**Figure 3.**
*Phylogenetic position of catalases clades 1–3. The phylogenetic tree was constructed using the Maximum Likelihood method and JTT matrix-based model [41]. Multiple alignments of the sequences were performed using the MUSCLE program [42]. The numbers in the branches indicate bootstrap percentages based on 500 replicates. Bar, 0.20 changes per amino acid position. Evolutionary analyses were conducted in MEGA X [13].*

ascertained based on the amino acid sequences of catalase from *Exiguobacterium* spp. and *Psychrobacter* spp. However, it has been difficult to discriminate clade 1 and clade 3 catalase except phylogenetic position based on amino acid sequences. Indeed, these catalases can be discriminated based on differences in the catalytic efficiency for $H_2O_2$ according to the structure of the narrow main channel.

## 9. Environmental distribution and catalase function of $H_2O_2$-resistant bacteria

Results of a screening of bacterial strains adapted to high $H_2O_2$ environments (8°C, 6–38 mM $H_2O_2$), *E. oxidotolerans* T-2-2$^T$ and *P. piscatorii* T-3$^T$ and T-3-2 were isolated. Some microorganisms have been shown to thrive under extreme environments such as high and low temperatures and high and low pH. However, *Exiguobacterium* spp. and *Psychrobacter* spp. are known widely distributed in polar regions, permafrost, deep sea regions, temperate and tropical soils, and ordinary marine environments [43, 44]. Therefore, several strains belonging to the genera *Exiguobacterium* and *Psychrobacter* have been identified as psychrophilic or psychrotolerant bacteria. In addition to the cold-adapted variations of these genera, our studies revealed that there were variations in the $H_2O_2$ tolerance of these genera.

Although these genera exhibit common physiological characteristics and environmental distributions, phylogenetic positions are completely different from a taxonomical point of view [43]. Gram-positive *Exiguobacterium* belongs to the phylum Firmicutes, class Bacilli, and order Bacillales, whereas *Psychrobacter* belongs to the phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Moraxellaceae. Dias et al. analysed and compared four genomes of *Exiguobacterium* and *Psychrobacter* [44] and showed that *Psychrobacter* exhibited higher genomic plasticity, whereas *E. antarcticum* exhibited a large decrease in genomic content without changing its adaptability to cold environments. These results suggest that the $H_2O_2$ tolerance and molecular features of catalases and their productivities in $H_2O_2$-tolerant bacteria belonging to *Exiguobacterium* and *Psychrobacter* were related to the intrinsic genomic architectural dynamics of these taxa.

*V. rumoiensis* was isolated from an environment containing lower $H_2O_2$ concentration (1.5–6 mM) than the other two strains. The genus *Vibrio* and the closely related genus *Aliivibrio* are known for involving species of their pathogenicity and symbiosis with marine organisms. Thus, these organisms may have high capacity for adaptability to high $H_2O_2$ environment. Moreover, bacterial genome analysis of six bacterial species belonging to the rumoiensis clade revealed that there are ecogenomic signatures inferring the ongoing habit expansion in two strains (*V. rumoiensis* included) [45]. Thus, this microorganism may have adapted to environments containing high $H_2O_2$ by genomic altering specific characteristics.

## 10. Conclusion and future studies

It has been shown that completely different taxa of bacteria evolve catalases in different directions improving productivity of catalases in the same or similar environment (i.e., low temperature and high $H_2O_2$ concentration). Adaptations to environments with high concentration of $H_2O_2$ has been achieved by certain groups of bacteria, including psychrotolerant bacteria originating from marine environments, which are widely distributed and can survive under various environmental conditions (e.g., low temperature and high $H_2O_2$ concentration). This adaptability is observed in terms of enzymatic features, productivity and localisation of catalase.

Future studies are necessary to analyze the evolutionary process in more detail and determine the relationship of this evolutionary process with the functions of specific enzymes. Furthermore, detailed studies of the microbiota present in environments containing high $H_2O_2$ concentrations may provide insight into the mechanisms through which bacteria adapt to artificial extreme environments.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Appendices and nomenclature

Website resources:
Catalase website (http://www.catalase.com/index.htm)
EMBEL-EBI Catalase
(http://pfam.xfam.org/family/PF00199#tabview=tab3)
Catalase (enzyme nomenclature designation [EC] 1.11.1.6)

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