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Exogenous Catalase Gene Expression as a Tool for Enhancing Metabolic Activity and Production of Biomaterials in Host Microorganisms

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1. Introduction

Heterologous gene expression is a widely used and vital biotechnology in basic and applied biology research fields. In particular, this technology (bioengineering) is emerging as a useful tool in fields of applied biology, such as medical, pharmaceutical, and agricultural sciences including food science. With this technology, animals, fishes, plants, and eukaryotic and prokaryotic microorganisms can be used as host organisms for transformation, and various types of vectors have been developed and have become commercially available. It is only about 40 years since a heterologous cloned DNA was expressed in *Escherichia coli* cells (Annie et al., 1974; Old & Primrose, 1989). Development of various types of gene transfer systems has assisted the widespread use of gene engineering technologies in all research fields.

The production of high-value compounds is a requisite purpose of gene engineering for all the fields of applied biology. Such production can occur in two ways: host cells are used to produce a product that is new to them by expressing a foreign gene(s); or host cells are enhanced to produce higher levels of the target product(s), which can be also inherently synthesized at normal levels by host organisms. The examples of the former are the production of insulin (Goeddel et al., 1979) or α-fetprotein (Nishi et al., 1988) by *E. coli* cells that were transformed with genes encoding these proteins. The literature on the latter means of production has accumulated rapidly. In such cases, two or more kinds of foreign gene carried in one or more vectors was used to transform the host organisms, by which a high-value compound(s) can be generated as the new product in the transformed host organisms or can enhance the metabolic activities of the host organisms.

Yumoto et al. (1998) isolated a bacterium with a remarkably high catalase activity from a waste pool at a fish-processing factory in Hokkaido, Japan. This bacterium was identified as *Vibrio rumoiensis* strain S-1^T (strain S-1 hereafter; Yumoto et al., 1999). Details of this

bacterium and its catalase protein (VktA**)** and gene (*vktA*) are described in the following section (**Section 2, VktA catalase and its gene)**. The VktA catalase had a significantly high specific activity after being purified (Yumoto et al., 2000). The *vktA* gene encoding VktA was cloned and expressed in various strains of *E. coli* (Ichise et al., 1999; Orikasa, 2002). Cell-free extracts prepared from *vktA*-transformed *E. coli* cells exhibited almost the same specific activity of catalase as those prepared from the parent strain S-1. Biochemical and molecular studies on VktA prepared from the parent and *vktA*-transformed *E. coli* transformants showed that strain S-1 could accumulate VktA protein at a level as high as a concentration of 2% of total soluble proteins and that the high catalytic activity of purified VktA enzyme is 4 times greater than that of bovine liver catalase (Yumoto et al., 2000).

In this chapter, we describe two biotechnological uses of the *vktA* gene as a foreign gene. First it was used to enhance the nitrogen-fixing activity in a root nodule bacterium (**Section 3, Enhancement of nitrogen fixation by *vktA* in root nodule bacteria**), which has its own catalase. Second, it was used to enhance eicosapentaenoic acid (EPA) biosynthesis in *E. coli* that had already been transformed with the EPA biosynthesis gene cluster (*pfa* genes) cloned from a marine bacterium (**Section 4, Enhancement of eicosapentaenoic acid production in *E. coli* through expression of *vktA***). The host *E. coli* cell has its own catalase but no ability to synthesize EPA. In both cases, the catalase activity of *vktA*-transformed host cells increased remarkably. Furthermore, the nitrogen-fixing ability in *R. leguminosarum* was definitely enhanced, and the EPA contents in *E. coli* transformed with *pfa* and *vktA* became greater than the EPA content in *E. coli* transformed with *pfa* genes only. The physiological and molecular roles of the increased catalase activity in catalase gene (*vktA*)-engineered bacterial cells are discussed in each section. **Section 5** contains concluding remarks and discusses possibilities for further use of VktA. Catalase comparable with VktA or those with much higher performance than VktA are described as a tool for producing biomaterials by biotechnologies in various research fields.

2. VktA catalase and its gene

The VktA catalase is the sole catalase protein detected in strain S-1 (Yumoto et al., 1999). This enzyme is characterized by its significantly high catalytic activity when compared with other known catalases. General information on catalases and detailed characteristics of VktA and its gene (*vktA)* are provided in this section.

2.1 Catalases and their genes

Aerobic organisms metabolize oxygen through respiration for production of energy to sustain their life. During aerobic respiration, organisms generate toxic reactive oxygen species (ROSs), such as superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•), as by-products. The presence of H_2O_2 in cells has a possibility to generate a more toxic ROS, OH•, by a Fenton-type reaction (Halliwell & Gutteridge, 1999). Excessive amounts of H_2O_2 and OH• are harmful to cell components. Therefore, aerobic organisms eliminate H_2O_2 with scavenging enzymes, such as catalase, peroxidase, and glutathione peroxidase. On the other hand, elimination of extracellular H_2O_2 by catalase is also important for either aerobic or anaerobic organisms to distribute their niche among several microorganisms. For example, parasitic and symbiotic microorganisms are attacked

by ROSs which are produced by the external defense system of their host organisms (Katsuwon & Anderson, 1992; Rocha et al., 1996; Visick & Ruby, 1998).

The dismutation reaction of H_2O_2 has evolved into three unrelated groups in the category of catalase (EC 1.11.1.6). The first group consists of so-called typical catalase. These catalases consist of four identical subunits each equipped with protoheme IX (heme *b*) or heme *d* in the active site as the prosthetic group, and their subunit molecular masses are 55-84 kDa. These catalases can be subdivided into small subunits (subunit molecular mass: 55-69 kDa) possessing heme *b* as the prosthetic group and large subunits (75-84 kDa) possessing mainly heme *d* as the prosthetic group. This group of catalases is the most widespread in nature and exhibits efficient catalytic reactions. These enzymes have a broad pH optimum range, are specifically inhibited by 3-amino-1,2,4-triazole, which reacts with a catalytic intermediate state, compound I (see below), and are resistant to reduction by dithionite (Kim et al., 1994; Nadler et al., 1986). The second group of catalases is catalase-peroxidase. Catalase-peroxidases exhibit a bifunctional character: catalase and peroxidase activities. The maximal catalatic activities of catalase-peroxidases are two or three orders of magnitude lower than those of typical catalases. These enzymes typically have a dimeric or tetrameric structure with a subunit with a molecular mass of approximately 80 kDa, containing only one or two hemes *b* per molecule. Catalase-peroxidases have been detected in Bacteria, Archaea and Eukarya domains (although only in fungi in Eukarya). In addition, these enzymes have a sharp pH optimum, are not inhibited by 3-amino-1,2,4-triazole, and are readily reduced by dithionite (Hochman & Shemesh, 1987; Kengen et al., 2001). The deduced primary structures of these enzymes are closely related to each other and their three-dimentional structures are similar to those of plant peroxidase. The third group of catalases consists of manganese catalase (Mn-catalase). Mn-catalases, in contrast with the other two catalase groups, are not equipped with heme as the prosthetic group; rather these enzymes use manganese ions. Therefore, activities of these enzymes are not inhibited by cyanide or azide, which are inhibitors of catalases in the other groups. Mn-catalases are mostly hexameric and the molecular size of their subunit ranges 28 kDa to 35 kDa (Kono & Fridovich, 1983; Allgood & Perry, 1986). These catalases, which are sometimes referred to as pseudocatalases, are distributed in lactic acid bacteria and thermophilic bacteria.

All catalases possessing heme as the prosthetic group commonly exhibit a two-step mechanism for the degradation of H_2O_2. In the first step, one H_2O_2 molecule oxidizes the enzyme in the resting state (Fe^{3+} Por) to ferryl porphyrin with a porphyrin π-cation radical (Fe^{4+}=O $Por^{+\bullet}$), so-called compound I. In the second step, compound I oxidizes a second H_2O_2 molecule to molecular oxygen and water (eqs. 1 and 2; Deisseroth & Dounce, 1970; Schonbaum & Chance, 1976).

$$\text{Enzyme } (Fe^{3+}\ \text{Por}) + H_2O_2 \rightarrow \text{Compound I } (Fe^{4+}\text{=O } Por^{+\bullet}) + H_2O \quad \text{(Reaction 1)}$$

$$\text{Compound I } (Fe^{4+}\text{=O } Por^{+\bullet}) + H_2O_2 \rightarrow \text{Enzyme } (Fe^{3+}\ \text{Por}) + H_2O + O_2 \quad \text{(Reaction 2)}$$

Phylogenic analysis based on the amino-acid sequence deduced from the gene sequence of typical catalases has revealed their subdivision into three distinct clades (Klotz et al., 1997). Clade 1 catalases are small-subunit catalases and are mainly of plant origin, but also includes one algal representative and a subgroup of bacterial origin. Clade 2 catalases are all large-subunit catalases of bacterial, archaeal, and fungal origins. The one archaeal enzyme

belonging to clade 2 catalase is postulated to have arisen in a horizontal transfer event from *Bacillus* species. This clade of catalases exhibits a strong resistance to denaturation by heat and proteolysis. Clade 3 catalases are small-subunit catalases and their origins are bacteria, archaea, fungi, and other eukaryotes. There are no pronounced functional difference between clade 3 and clade 1 catalase. Bacteria harboring clade 3 catalase as the single catalase isozyme are distributed to a restricted environment.

Most aerobic bacteria contain one or more catalases, which are produced in response to oxidative stress or depending on the growth phase. *Escherichia coli* possess two types of catalase gene, *katG* and *katE*. These encode periplasmic HPI (catalase-peroxidase) and cytoplasmic catalase HPII (typical catalase, clade 2), respectively. *KatG* is induced by H_2O_2, while *katE* is induced by the entry into the stationary phase of growth (Storz & Zheng, 2000). Both of the genes are regulated by the alternative sigma factor, σ^s, which is produced by the *rpoS* gene. The expression of *katG* is regulated by OxyR, a transcriptional regulator that senses H_2O_2 (Ivanova et al., 1994; Storz & Zheng, 2000). OxyR can switch rapidly between reduced and oxidized states, and only the oxidized form acts as a transcriptional activator for target genes (Aslund et al., 1999; Christman et al., 1985).

2.2 Characteristics of VktA catalase and its gene

Even though there have been many reports of bacterial oxidative stress responses, there had been few reports on the microorganisms that are able to survive in highly oxidative environments. Therefore, studies were conducted in order to understand how a bacterium adapts to an oxidative environment and what kind of H_2O_2 eliminating system it possesses. A facultatively psychrophilic bacterium exhibiting high catalase activity was isolated from a drain pool of fish egg processing factory that uses H_2O_2 as a bleaching agent (Yumoto et al., 1998, 1999). The isolate, strain S-1, was identified as a new species, *Vibrio rumoiensis*. The catalase activity in cell extract of strain S-1 was 2 orders higher than those of *E. coli* and *Bacillus subtilis*. Although S-1 cells exhibit high catalase activity, individual cells do not exhibit strong resistance to H_2O_2. It is probably due to the fragility of the cell structure (Ichise et al., 1999).

Catalase (VktA) from strain S-1 has been purified and characterized (Yumoto et al., 2000). Molecular mass of the subunit of the catalase is 57.3 kDa and the enzyme consists of four identical subunits. The enzyme was not apparently reduced by dithionite. The activity showed a broad optimum pH range (pH 6–10) and was inhibited by 3-amino-1,2,4-triazole.

Therefore, the enzyme belongs to the typical small subunit catalase. The catalase activity of VktA was 1.5 and 4.3 times faster than *Micrococcus luteus* and bovine catalases, respectively, under 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7) at 20°C. Therefore, VktA is considered to be a catalase with very high activity and one with the highest turnover numbers in all known catalases. The thermoinstability of VktA was significantly higher than that of *M. luteus* and bovine catalases. It is suggested that the unique properties of VktA may reflect protective strategies for the survival of strain S-1 under oxidative environmental conditions, where this bacterium was inhabited.

The gene of VktA, *vktA*, has been isolated and sequenced. The *vktA* consisted of an open reading frame of 1530 bp encoding a 508 amino-acid protein with a calculated molecular mass of 57657.79 Da (Ichise et al., 2000). A putative ribosome binding site (AGGAGA) was

found 5 bases upstream of the start codon (Fig. 1). In further upstream, putative promoter sequences, (TATAAT) and (TTGGCT), corresponding to -10 and -35, respectively were also found. The promoter sequences were probably recognized by RNA polymerase carrying the housekeeping sigma subunit σ^{70} (Hawley & McClure, 1983). Another putative promoter binding site for OxyR was in further upstream (Fig. 1).

Fig. 1. Nucleotide sequence of the *vktA* promoter region. Nucleotides in the putative ribosome binding site, Shine-Dalgarno ribosome-binding site (SD) is in the boxed. Arrow indicates the putative transcription initiation site. Potential promoter sites of -10 and -35 are indicated as underlined bold letters. An alignment with OxyR consensus sequences of OxyR consensus (Tartaglia et al., 1989; Toledano et al., 1994), *Haemophilus influenza* (Bishai et al., 1994); and *Salmonella typhimurium* (Tartaglia et al., 1989) is shown upstream of the putative -35 region.

The deduced amino acid sequence of VktA shows high similarity with that of typical small subunit catalases belonging to clade 3 (Fig. 2). Many calatases possessing as the sole isozyme in parasitic or symbiotic microorganisms belong to clade 3. Therefore, there is a possibility that strain S-1 inherently be a symbiont of fish, which is supported by the fact that the strain was originally obtained from the fish egg processing factory. It is also pointed that bacteria possessing clade 3 catalases as the sole isozyme like strain S-1 was naturally selected in the environment that is frequently exposed to H_2O_2.

Localization of the VktA catalase in the cytoplasmic and periplasmic spaces has been demonstrated by the enzymatic activity of fractionated cells and immunological detection methods (Ichise et al., 1999; Yumoto et al., 2000). In addition, it has been shown that cell density and release of VktA from disrupted cells play an important role in the survival of strain S-1 cells when they reacted with H_2O_2 (Ichise et al., 1999). However, almost no growth hindrance is observed when 100 mM H_2O_2 is introduced into the culture. Accounts of strain S-1's strong tolerance to H_2O_2 in high concentrations of H_2O_2 during cultivation, concomitant with very rapid elimination of H_2O_2 due to the strong catalase activity of the cells themselves, have remained unresolved. The contribution of catalase to H_2O_2 tolerance and the presence of cell surface catalase have been demonstrated by H_2O_2 tolerance of catalase-deficient *E. coli* strain UM2 carrying *vktA* and immunoelectron microscopic observation on strain S-1 and catalase-deficient mutant, strain S-4, derived from strain S-1 using an antibody for the intracellular catalase of strain S-1, respectively (Ichise et al., 2008). Cell surface catalase is considered to contribute to the elimination of extracellular H_2O_2.

To further characterize the VktA catalase protein, it was analyzed by polyacrylamide gel electrophoresis (PAGE) using partially purified bovine liver catalase (Sigma, St Louis, USA), two types of *Aspergillus niger* catalase (one from Sigma and the other from NAGASE Co., Ltd, Tokyo, Japan), and *Corynebacterium glutamicum* catalase (Sigma) as references. All samples except for strain S-4 exhibited one activity band detected by the method by Uriel (1958) in native gel (data not shown). Fig. 3 shows protein band profiles and indications of the activity

band position of all the samples in SDS-PAGE under different conditions. In the presence of SDS with neither 2-mercaptoethanol (2-ME) nor heating, all samples except for strain S-4 showed one activity band, whereas no activity band was observed in the presence of SDS, 2-ME and heating. The bands with activity are considered to be proteins with a dimeric form. It is interesting that two *A. niger* and C. *glutamicum* catalases were stained positive for activity in the presence of SDS, 2-ME, but no heating. The dimeric form of these catalases was resistant to reduction by 2-ME, at room temperature. By contrast, VktA was sensitive to 2-ME treatment at room temperature. A very faint oxygen bubble formation was observed for the position of the dimeric form of bovine liver catalase in the presence of SDS, suggesting that the tetrameric form of bovine liver catalase is apt to be easily dissociated into monomers in the presence of SDS only. These results suggest that VktA has an intermediate characteristic of bovine liver catalase and other microbial catalases against treatment with 2-ME at room temperature. The VktA catalase, which is tetrameric in its native form (Yumoto et al. 2000), can be dissociated into active dimers in the presence of SDS; however, the structure and activity of the dimers are sensitive to treatment with 2-ME at room temperature, suggesting that VktA could be a structurally and actively flexible enzyme.

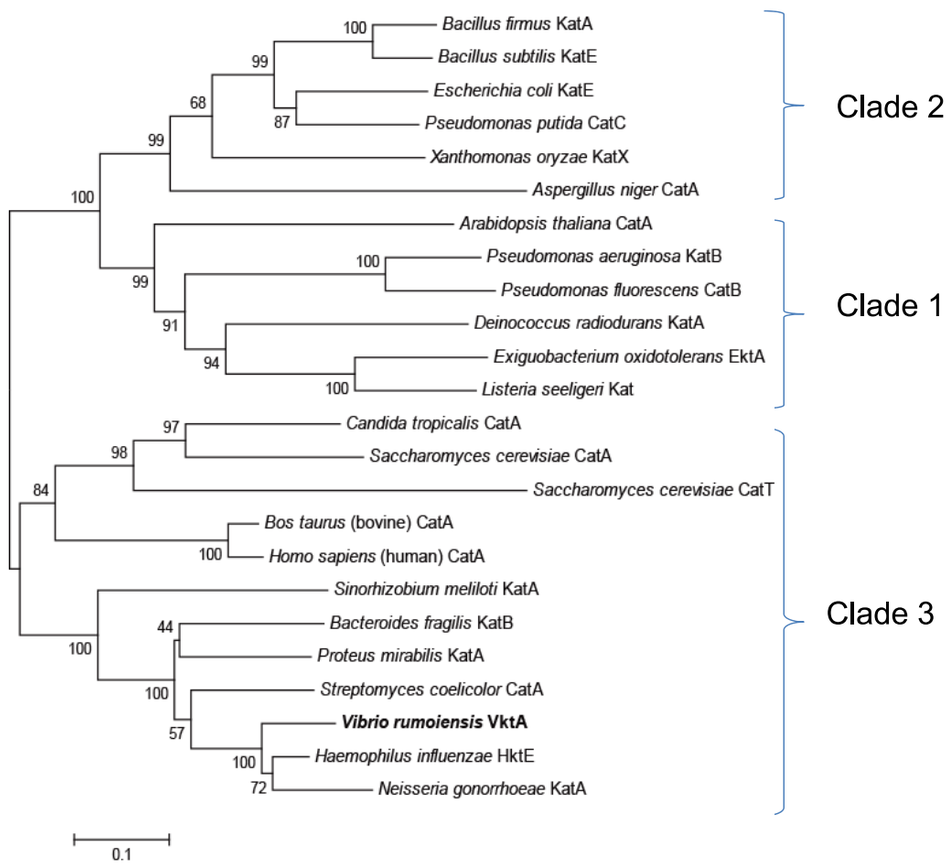

Fig. 2. Phylogenic tree of catalases. The tree was constructed by the CLUSTAL W program (Thompson et al., 1994) with multiple alignment using neighbor-joining method (Saitou & Nei, 1987). Numbers at the branches are bootstrap percentages based on 1000 replicates. Bar, 0.1 changes per amino acid position.

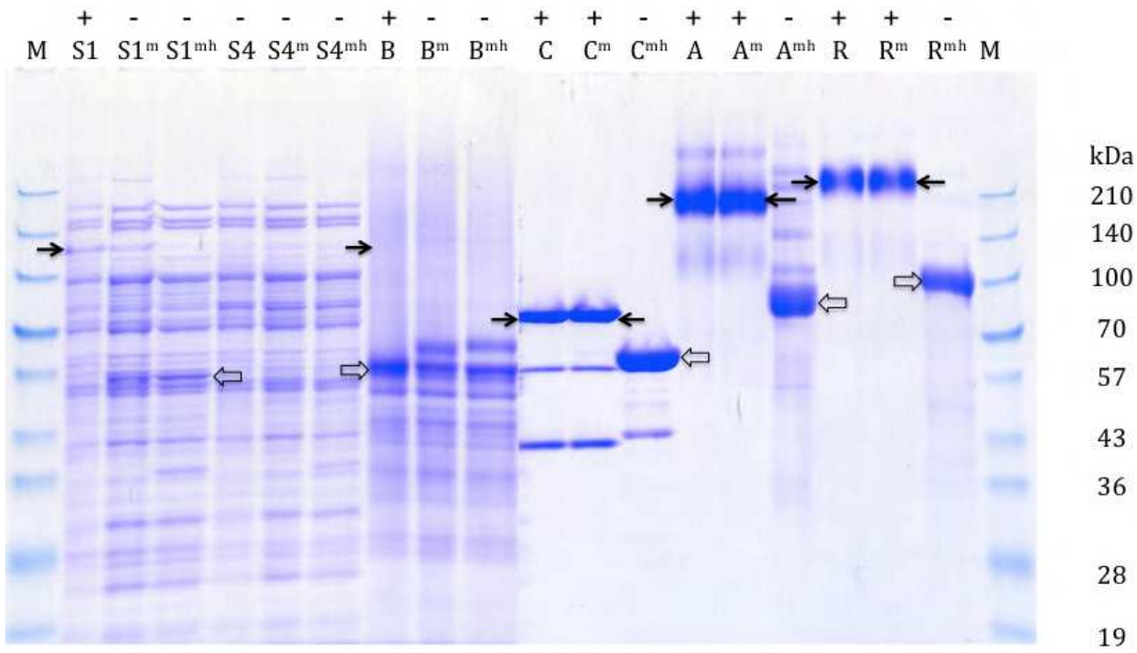

Fig. 3. SDS-PAGE profiles of cell-free extracts from *Vibrio rumoiensis* strain S-1 (S1) and its catalase-deficient mutant strain S-4 (S4), and commercially available catalases: B from bovine liver; C from *Corynebacterium glutamicum;* A from *Aspergillus niger* (Sigma); R from *A. niger* (Ryonet F Plus; Nagase Co., Ltd). +, catalase activity positive (oxygen bubble formation) at the band shown by solid arrows; –, no catalase activity band; superscripts, ^{m} and ^{mh}, SDS-PAGE conditions in the presence of 2-mercaptoethanol (2-ME) at room temperature for 5 min and in the presence of 2-ME with heating at 100°C for 5 min, respectively. No superscript means SDS-PAGE conditions with SDS but no 2-ME. The open arrows indicate the position of monomeric subunits of catalases. VktA, bovine liver (Sigma) and two *A. niger* catalases from Sigma and Nagase have a tetrameric native structure with a molecular mass of 230 kDa, 240 kDa, 250 kDa and approximately 350 kDa, respectively. Information on the native form of the *C. glutamicum* catalase is not available. Gels (e-Pagel, 10%; Atto, Tokyo) were stained with Coomassie Brilliant Blue. Lane M, molecular marker standard (kDa).

3. Enhancement of nitrogen fixation by *vktA* in root nodule bacteria

Root nodule bacteria are characterized by their ability to fix dinitrogen in root nodules, where microaerobic conditions are maintained. However, the nitrogen-fixing process requires a large amount of ATP, suggesting that molecular oxygen is requisite for oxidative phosphorylation. This section describes the use of *vktA* to modulate oxygen metabolism in root nodule bacteria. The *vktA* gene was introduced into *Rhizobium leguminosarum* cells and the strain with a remarkably high catalase activity was constructed. Results show that the increase of catalase activity in rhizobia could be a valuable way to improve the nodulation and nitrogen-fixing ability of nodules (Orikasa et al., 2010).

3.1 Nitrogen fixation and catalases in rhizobia: General information

Rhizobia infect the roots of leguminous plants and form nodules with elaborate control by exchanging molecular signals between two partners (Wei et al., 2008, 2010). The rhizobia

are present as bacteroids such as microsymbiotic organelles in the nodules and convert atmospheric dinitrogen into biologically available ammonia (nitrogen fixation). Nitrogen fixation is an energy-requiring process and needs large amounts of ATP, which is supplied by oxidative phosphorylation. This process is extremely oxygen-sensitive and the partial pressure of oxygen inside the nodules is maintained at very low levels, resulting in strongly reduced conditions with the production of ROSs such as H_2O_2 (Tjepkema & Yocum, 1974). Leghemoglobins present in nodules play a role in the effective diffusion of oxygen and their autoxidation results in the production of O_2•- and H_2O_2 (Appleby, 1984; Puppo et al., 1981). However, as the host plant can control the nodulation in such a way that the infection during symbiotic interaction is aborted due to a hypersensitive reaction, it has been suggested that the release of ROS such as H_2O_2, termed the oxidative burst, may occur in the early stage of the infection thread formation (Vasse et al., 1993). It was reported that a striking release of ROS occurred under the conditions of plant defenses against pathogens (Mehdy, 1994). Since it is believed that ROSs such as H_2O_2, O_2•- and OH• are also generated naturally during the metabolism of cells growing aerobically and that these ROS could damage the protein, lipids, and DNA components in organisms (Sangpen et al., 1995), these reports suggest that the response of rhizobia to ROSs such as H_2O_2 would exert a serious influence on both the nodulation process and nitrogen-fixing abilities.

Catalase in rhizobia cells was not previously considered to play a crucial role in the nitrogen-fixing abilities because *Sinorhizobium meliloti* has three kinds of catalase (KatA, KatB, KatC) and the *katA*-minus mutant did not impair both nodulation and nitrogen fixing abilities (Herouart et al., 1996). Rhizobia are symbiotic microorganisms. They were previously considered to be able to rely on their host plant for defense against toxic forms of oxygen such as H_2O_2; that is, plant-derived catalases, as well as defense systems against H_2O_2 toxicity such as the ascorbate-glutathione reaction system, were considered to be able to contribute to the removal of H_2O_2 toxicity. It was reported that the production of oxidative protection enzymes by plant cells in nodules was positively correlated with the increase in nitrogenase activity and leghemoglobin content (Dalton et al., 1986). In addition, parasitic bacteria were reported not to need their own catalase because they could depend upon their host catalase (Steiner et al., 1984). However, subsequent experiments showed that the double-catalase mutants (Δ*katA*Δ*katC* or Δ*katB*Δ*katC*) had considerably lower capability for both nodulation and nitrogen fixation (Jamet et al., 2003; Sigaud et al., 1999), and their activities decreased during nodule senescence (Hernandez-Jimenez et al., 2002). These results showed that the catalase in rhizobia could be important for the efficient function of both nodulation and nitrogen fixation. Another rhizobium strain, *Bradyrhizobium*, showed the important role of catalase for H_2O_2 decomposition, and the *katG*-minus mutant resulted in the loss of both catalase activity and exogenous H_2O_2 consumption (Loewen et al., 1985b; Panek & Obrian, 2004).

Unexpectedly, rhizobia had a tendency for less catalase activity than other genera of aerobic and facultative anaerobic bacteria, resulting in higher susceptibility to H_2O_2 (Ohwada et al., 1999). We compared catalase and peroxidase activities in rhizobia (11 strains) with those in other genera of bacteria (six strains). Catalase activities (units per mg protein) of the rhizobia strains tested were in the range of 0.9-5.8, although the addition of H_2O_2 increased activities in all strains to the range of 2.5-11.3. By contrast, the activities in the other genera strains tested ranged 12.3 to 893.3, and, in the presence of

H_2O_2, the levels of all strains were increased to the range of 16.1-1,460.2, indicating that the catalase activity of the tested rhizobia tended to be considerably lower than those of the other genera tested (Fig. 4). Results of Southern analyses imply that rhizobia have no DNA region similar to *katE* of *E. coli*, which is inducible tenfold during the stationary phase of growth (Loewen et al., 1985a), and the kinetics of catalase induction in *R. leguminosarum* bv. *phaseoli* was different from that in *E. coli* (Crockford et al., 1995; Hassan & Fridovich, 1978). Crockford et al. (1995) mentioned that, during growth, unidentified compounds accumulate in the cells and repress catalase activity, although the significance of growth-phase-dependent regulation of catalase activity remains obscure.

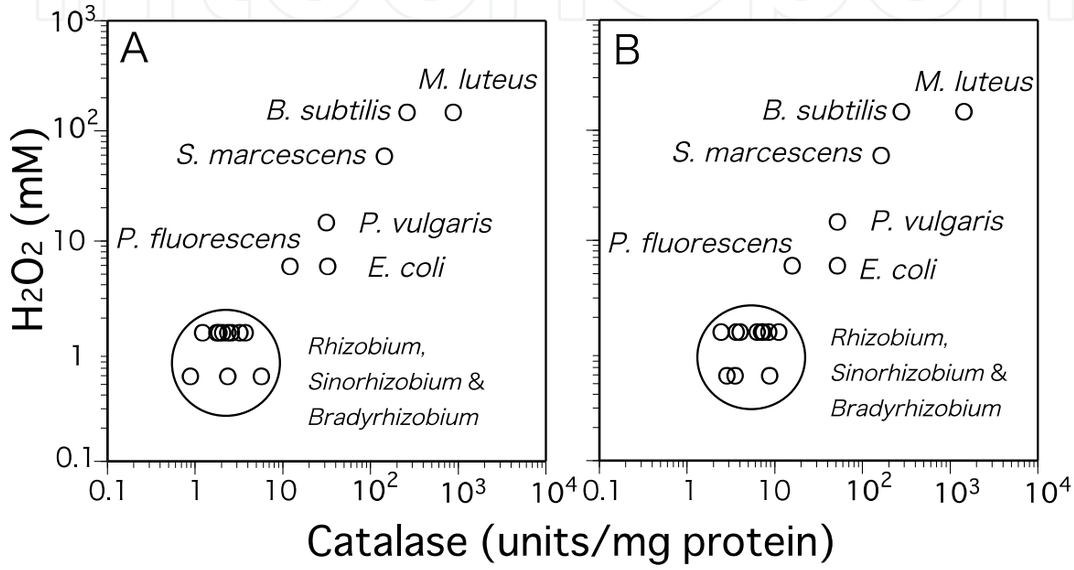

Fig. 4. Correlation between catalase activities and H_2O_2 tolerance. Cells were incubated with H_2O_2 (0.6, 1.5, 5.9, 14.7, 29.4, 58.8, and 147 mM) and the maximum concentration of H_2O_2 (mM) in which the cells could grow within 24 h (96 h for *Bradyrhizobium japonicum*) was plotted as ordinate. Catalase activities in the cells in the early stationary phase with (panel B) or without (panel A) H_2O_2 (0.6 mM) were plotted as the abscissa. *Rhizobium, Sinorhizobium* and *Bradyrhizobium* strains tested are enclosed within a circle. Data points are means of results from at least three trials. *Rhizobium, Sinorhizobium* & *Bradyrhizobium* strains used: *Rhizobium leguminosarum* bv. *phaseoli* USDA2667, 2676; *R. leguminosarum* bv. *trifolii* USDA2053, 2145; *R. leguminosarum* bv. *viciae* USDA2370, 2443; *Sinorhizobium fredii* USDA191, 206; *S. meliloti* USDA1021, 1025; *Bradyrhizobium japonicum* S32. Other strains used: *Bacillus subtilis* AHU1390; *Escherichia coli* JM109; *Micrococcus luteus* AHU1427; *Proteus vulgaris* AHU1144; *Pseudomonas fluorescens* AHU1719; *Serratia marcescens* AHU1488. Adapted with permission from Ohwada et al. (1999).

The relationships between catalase activities and H_2O_2 tolerance in all bacteria tested are shown in Figure 4. The results indicate that a tendency for a positive and mutual correlation between them for all strains tested. Particularly, both the catalase activities and the H_2O_2 tolerance of the rhizobia tested were lower than those of the others. On the other hand, peroxidase activities in cells with different electron donors (NADH, *o*-dianisidine, and *p*-phenylenediamine) were considerably lower than the catalase activities and there was no

significant difference between rhizobia and the others. In addition, the growth repression caused by the addition of catalase inhibitor in the presence of H_2O_2 was observed in rhizobia such as *R. leguminosarum* bv. *phaseoli*, bv. *trifolii* and *S. meliloti*. These results indicate that catalase could be mainly responsible for the defense mechanism against H_2O_2 toxicity. For *E. coli*, it was reported that catalase-deficient and catalase-overproductive mutants were more sensitive and resistant to H_2O_2, respectively (Greenberg & Demple, 1988; Loewen, 1984). The peroxisome-targeting signal (SKL sequence) of catalase (KatA) in *S. meliloti*, which is supposed to be connected with the export into the periplasmic region, suggests that the *S. meliloti* catalase is located in this region (Herouart et al, 1996). The location of protecting enzymes such as catalase against H_2O_2 seems to be important because periplasmic enzymes could be advantageous to the defense against exogenous H_2O_2. Therefore, the location of catalase in rhizobia would have an effect on the mutual correlation between catalase activities and H_2O_2 tolerance.

Since rhizobia are symbiotic microorganisms, they might be able to rely on their host plant in nodules to some extent for defense against toxic forms of oxygen such as H_2O_2. However, the catalase activity of *Bradyrhizobium* strain from effective nodules was reported to be higher than that in a strain from ineffective nodules (Francis & Alexander, 1972). These results prompted the construction of rhizobia with higher catalase activity to improve the nitrogen-fixing ability of nodules.

3.2 Enhancement of nitrogen fixation through expression of *vktA* in *Rhizobium leguminosarum*

The DNA fragment (4.9 kbp) including the catalase gene (*vktA*), which is controlled by its own promoter activity, was ligated into pBluescriptII SK+ to construct pBSsa1 (Ichise et al., 2000). The *Bam*HI-*Xho*I fragment including a coding region of *vktA* (4.9 kbp) was isolated from the pBSsa1 and ligated into a broad host range vector, pBBR1MCS-2 (Kovach et al., 1995) at the site of *Bam*HI-*Xho*I. Then, the recombinant plasmid was introduced into *R. leguminosarum* bv. *phaseoli* USDA2676 cells to construct the *vktA*-transformant by triparental mating (Simon, 1984). The result showed that the *vktA*-transformed *R. leguminosarum* exhibited a remarkably high catalase activity of up to around 10,000 units per mg protein. This activity was three orders of magnitude greater than that of the parent strain and comparable to that of strain S-1 (Yumoto et al., 1999). To confirm the production of VktA catalase, activity staining of the catalase and immunoblot analysis with anti-VktA antiserum were conducted. The activity staining was carried out according to Clare *et al*. (1984). Results showed an obvious band of VktA catalase in both logarithmic and stationary phases.

Although the parent strain of *R. leguminosarum* originally had two bands of different mobility from the VktA catalase, their intensities were considerably weak compared with that of the VktA catalase and were not major in the *vktA*-transformant. Additionally, the immunoblot analysis was conducted using polyclonal anti-VktA and a goat anti-rabbit IgG-horseradish peroxidase conjugate as primary and secondary antibodies, respectively. The results indicated that a positive antigen-antibody reaction occurred as a single band between the anti-VktA antiserum and the VktA catalase. These results clearly showed that the efficient production of VktA catalase was responsible for the high catalase activity in the *vktA*-transformant. As described above, since catalase could be mainly responsible for the H_2O_2 tolerance in bacteria and the catalase activities in the rhizobia tested were considerably

lower compared with the other genera tested, the growth of almost all of rhizobia tested was severely repressed even in the presence of 1.5 mM H_2O_2. However, the *vktA*-transformant showed almost the same cell density as the parent in the presence of 10 mM H_2O_2; even with 50 mM H_2O_2, the *vktA*-transformant could grow and the cell density reached levels half that of the parent at 24 h, then almost the same as the parent at 30 h after incubation. These results indicate that the *vktA*-transformant acquired resistance against H_2O_2 through the enhancement of catalase production in the cells.

Cultivation condition	Strain	Nodule number (per plant)	Nodule weight (per dry wt per plant)	ARA [1] (per mg dry wt of nodule)	ARA [1] (per plant)
Seed bag	Parent	129 ± 8.2	34.1 ± 1.6	0.7 ± 0.1	25.6 ± 2.0
	vktA -transformant	111 ± 8.2	31.6 ± 1.8	1.2 ± 0.1 [2]	36.5 ± 2.7 [2]
Pot	Parent	75 ± 11.4	16.6 ± 2.8	0.7 ± 0.1	11.3 ± 2.7
	vktA -transformant	89 ± 12.2	21.9 ± 1.8 [2]	1.6 ± 0.2 [2]	34.3 ± 4.5 [2]

[1] Acetylene reduction activity (μmol of C_2H_2/h).
[2] Significant differences were evaluated by Student's *t* test ($P < 0.01$).

Table 1. Number, weight and acetylene reduction activity (ARA) of nodules formed in the combination of *Phaseolus vulgaris* (L.) cv. Yukitebou and *vktA*-transformed *R. leguminosarum*. Values were obtained from 20 determinants of at least two independent experiments. The values given are the means ± S. D. of 20 different tests. Adapted with permission from Orikasa et al. (2010).

The host plant, *Phaseolus vulgaris* (L.), was inoculated with *vktA*-transformed *R. leguminosarum* cells (10^6 cells per seed) and, after cultivation in a seed bag with Norris and Date medium (Dye, 1980) or in a pot filled with vermiculite, the number, weight, and nitrogenase activity (acetylene reduction activity, ARA) of the nodules were measured (Table 1). For the seed bag, the number and weight of nodules did not show a significant difference between *vktA*-transformant and the parent cells. However, the acetylene reduction activity (ARA) of nodules formed with *vktA*-transformed cells was significantly higher than that formed with the parent cells, and around 1.7 times as many nodules were formed as with the parent cells (around 1.4 times per plant). For the pot, the number and weight of the nodules formed with *vktA*-transformant were larger than those of the parent cells, with around 1.2 and 1.3 times those of the parent, respectively, although these levels tended to be lower than those for seed bag cultivation. Higher levels of ARA in the nodules formed with *vktA*-transformant were also observed and the levels reached around 2.3 times those of the parent (around 3.0 times per plant). Another set of experiments with the combination of *vktA*-transformed *S. fredii* and *Glycine max* (L.) also showed that the production of VktA significantly increased the ARA per nodule or plant weight. These results indicate that enhancing the catalase activity in *Rhizobium* cells significantly increased the nodules' nitrogen-fixing ability.

Next, catalase production in bacteroids of *vktA*-transformed *R. leguminosarum* was measured. Bacteroids were separated immediately after the nodules were detached from the plant roots (Kouchi & Fukai, 1989). The result showed that the *vktA*-transformant maintained an even higher catalase activity compared with the parent (around 150 units per mg protein). Results of western blot analysis using the anti-VktA antiserum showed a single band for VktA catalase, indicating that higher production of VktA catalase resulted

in a high catalase activity even in bacteroids. However, the catalase activity in bacteroids was considerably low as compared with free-living cells. Given that a decrease in the relative amounts of DNA, as well as the dynamic conversion of cellular metabolism such as the repression of sugar degradation, was reported during the differentiation process of bacteroids (Bergersen & Turner, 1967; Verma et al., 1986; Vierny & Laccarino, 1989), the loss of a certain number of the *vktA*-recombinant plasmids and/or the repressive production of VktA catalase might occur through the differentiation to bacteroids in the absence of antibiotics. The localization of the VktA catalase in free-living cells and bacteroids of *vktA*-transformant was studied by immunoelectron microscopy using the polyclonal antiserum against VktA with a secondary anti-rabbit antibody, which was coupled with gold particles. The number of gold particles at the periphery of the free-living cells including periplasm accounted for about 57.4 % of the sum total. For bacteroids, a relatively large number of gold particles (about 52.3% of the sum total), were observed at the periphery of the bacteroids including the symbiosome. These results indicate that the VktA catalase was preferentially distributed at the peripheral part of the cells for both free-living cells and bacteroids. H_2O_2 and leghemoglobin contents in the nodule formed with *vktA*-transformant were also measured. Nodules were detached 35 days after planting and H_2O_2 was extracted by grinding in 1 M $HClO_4$ (Ohwada & Sagisaka, 1987). The H_2O_2 content in the extracts was measured by Quantitative Hydrogen Peroxide Assay (OXIS International, Portland, USA). The extraction and quantification of leghemoglobin components using capillary electrophoresis were carried out according to Sato et al. (1998). The results showed that the H_2O_2 content (nmol/g fresh wt of nodule) in the nodules formed with the parent cells was around 21.0, but this level was decreased to around 15.4 by the production of VktA catalase in the cells. By contrast, the VktA production increased the content of the leghemoglobins (Lba and Lbb) and the levels in the nodules formed with *vktA*-transformant were around 1.2 (Lba) and 2.1 (Lbb) times higher than those with the parent cells.

Considering that ROSs such as H_2O_2 are released from the plant root not only under pathogenic conditions but also during the infection process (Mehdy, 1994; Vasse et al., 1993), it is possible that *Rhizobium* cells with a higher catalase activity are advantageous to the infection process because they decrease the amounts of H_2O_2 around them. This supports the possibility that the VktA catalase is preferentially located near the surface area of the cells, suggesting that they could be effective in decomposing H_2O_2. The peripheral distribution of VktA was also observed in strain S-1 (Ichise et al., 2000). In nodules, lack of the ability to remove H_2O_2 caused the reduction of both nodulation and nitrogen-fixing ability (Bergersen et al., 1973). Given that electron microscopic observation did not seem to reveal any difference in the density of bacteroids inside the nodules between *vktA*-transformant and the parent, it is thought that the enhancement of the ability to decrease H_2O_2 by higher catalase activity is responsible for the increased levels of nitrogen-fixing activity. On the other hand, it was reported that leghemoglobins accumulated in the infected plant cells before nitrogen fixation in order to decrease the partial pressure of oxygen inside the nodule and protect nitrogenase from inactivation by oxygen (Appleby, 1984). Adding leghemoglobin to bacteroid suspensions enhanced the nitrogenase-mediated reactions, and the nitrogenase activity of bacteroids was dependent on the concentration of leghemoglobin (Bergersen et al., 1973). Furthermore, the deficiency of leghemoglobin synthesis in nodules of *Lotus japonicus* using RNAi led to the

absence of symbiotic nitrogen fixation (Ott et al., 2005). Therefore, it is considered that the increase of leghemoglobin content also contributed toward the improvement of nitrogen-fixing ability, although the accelerated mechanism of the leghemoglobin production is still under investigation. It was reported that the effective nodules of white clover and soybean contained higher activity of catalase compared with the ineffective nodules (Francis & Alexander, 1972). It seems that catalase is disadvantageous to protect nitrogenase from the cytotoxic effect of H_2O_2 because oxygen, which represses nitrogenase activity, is generated through the decomposition of H_2O_2. However, considering that a large amount of ATP, which could be supplied by bacteroidal oxidative phosphorylation, is required for the nitrogen-fixing reaction and that the leghemoglobins maintain a high oxygen flux for respiration through the facilitated oxygen diffusion (Ott et al., 2005; Tajima et al., 1986; Wittenberg et al., 1975), it might be possible that the oxygen generated by the catalase reaction could also be useful for energy production. The results here show that an increase in catalase activity reduced H_2O_2 levels in the nodules concomitantly with the enhancement of leghemoglobin contents, followed by improvement of the nitrogen-fixing ability in the nodules. The enhanced nitrogen fixation from the expression of *vktA* in rhizobia would lead to the growth of the host plant with reduced use of chemical nitrogen fertilizer.

4. Enhancement of eicosapentaenoic acid production in *E. coli* through expressing *vktA*

Eicosapentaenoic acid (EPA) is an essential nutrient for humans and animals. Its derivatives, such as eicosanoids, are known as signal compounds in blood and nervous systems. Therefore, the ethyl ester of EPA is used a medicine. Fish oils, which have been the most widely used source of EPA to date, have been recognized as unsuitable because of their low EPA content and their unavoidable contamination with heavy metals from seawater; therefore new sources of EPA have been sought. Bacteria or fungi, which inherently produce EPA, constitute one of such possible source. Another possibility is the heterologous expression of EPA biosynthesis genes or chain elongation/desaturase genes of fatty acids in various types of host organism. This section describes the EPA biosynthesis in *E. coli* transformed only with EPA biosynthesis genes and the enhancement of EPA biosynthesis by coexpression of the *vktA* gene.

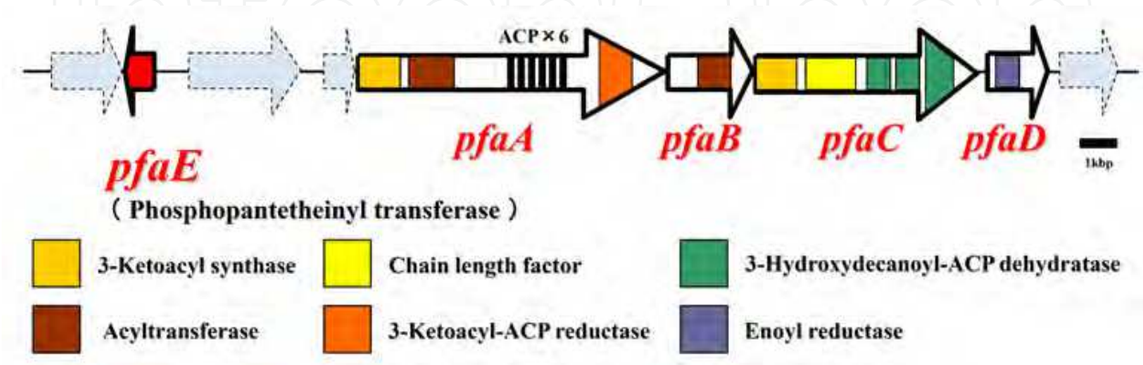

Fig. 5. Domain structure of *pfa* genes responsible for the biosynthesis of EPA from *Shewanella pneumatophori* SCRC-2738.

4.1 Bacterial biosynthesis of EPA

Bacterial species belonging to *Shewanella*, *Vibrio*, *Flexibacter*, and *Halomonas* (Salunkhe et al., 2011) are known to produce EPA as a major long-chain polyunsaturated fatty acid. EPA is synthesized de novo in a polyketide biosynthesis mode by the enzyme complex consisting of PfaA, PfaB, PfaC, PfaD, and PfaE, which are encoded by *pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*, respectively. These five genes (designated as an EPA biosynthesis gene cluster) generally locate in proximity on the chromosome (Fig. 5). PfaA and PfaC are multifunctional proteins and have some functional domains (Fig. 5). Only one functional domain for each of acyltransferase, enoyl reductase, and phosphopantetheinyl transferase is found, in PfaB, PfaD, and PfaE, respectively. Since the EPA gene cluster was first cloned from *Shewanella* sp. SCRC 2738 (*S. pneumatophori* SCRC-2738; Hirota et al., 2005) in 1996 (Yazawa, 1996), much attention has been paid to increasing the content of EPA in *E. coli* host cells and to its heterologous expression of these genes in various organisms, such as bacteria, yeast, and plants (Yazawa, 1996). The EPA gene clusters have been successfully expressed in various types of *E. coli* strains (Orikasa et al., 2004). Furthermore, numerous attempts have been made to express bacterial EPA biosynthesis genes in bacteria other than *E. coli* and in eukaryotic cells. However, to our knowledge, the report by Yu et. al. (2000) is the only one, in which a marine cyanobacterium is used as a host organism to express the EPA gene cluster.

4.2 Enhanced production of EPA by expression of *vktA* in *E. coli* carrying *pfa* genes

The enhanced production of EPA was observed in recombinant systems of *E. coli* that carried both EPA biosynthesis genes (*pfa*) and a *vktA* catalase gene. Although no molecular mechanism has been determined for this enhanced production of EPA, this technique may become another useful method to increase the productivity of EPA using recombinant systems. Docosahexaenoic acid (DHA) can be synthesized also in bacteria using DHA biosynthesis *pfa* genes, because the two *pfa* genes have a very similar structure (Okuyama et al., 2007).

E. coli DH5α transformants carrying pEPAΔ1 that included *pfaA-E* genes to the host cell led to the production of EPA (approximately 3% of total fatty acids; Table 2). The production of EPA in host organisms carrying pEPAΔ1 was increased to 12% of total fatty acids by the introduction of a *vktA* insert in pGBM3 [strain DH5α (pEPAΔ1) (pGBM3::*vktA*)]. The empty pGBM3 had no effect on EPA production. In strain DH5α carrying (pEPAΔ1) and partially deleted *vktA* in pGBM3(pGBM3::Δ*vktA*), EPA made up 6% of total fatty acids. The increase in EPA production in strain DH5α (pEPAΔ1)(pGBM3::*vktA*) was accompanied by a decrease in the proportions of palmitoleic acid [16:1(9)] (Table 2). When pGBM3 and pGBM3::*vktA* were replaced in the *E. coli* transformants with pKT230 and pKT230::*vktA*, respectively, similar trends were observed (data not shown). The yield of EPA per culture was approximately 1.5 μg/ml for DH5α(pEPAΔ1) and DH5α(pEPAΔ1)(pGBM3). It increased to 7.3 μg/ml for DH5α(pEPAΔ1) (pGBM3::*vktA*). The yield of EPA from DH5α (pEPAΔ1) pGBM3::Δ*vktA*) was 3.3 μg/ml (Table 2). *E. coli* DH5α has an inherent catalase activity of 2–3 U/mg protein (Nishida et al., 2006). The plasmid pEPAΔ1 had no effect on the catalase activity of the host cells. Catalase activity was increased to 535 U/mg protein for DH5α(pEPAΔ1)(pGBM3::*vktA*). However, there was no enhancement of catalase activity in DH5α(pEPAΔ1)(pGBM3::Δ*vktA*)

(Table 2). Figure 6 shows the profiles of proteins prepared from various *E. coli* DH5α transformants using SDS-PAGE. A significant amount of protein in the VktA band of 57 kDa, was detected only for DH5α(pEPAΔ1)(pGBM3::*vktA*). No notable novel band was observed in DH5α (pEPAΔ1)(pGBM3::Δ*vktA*) or in any of the other transformants.

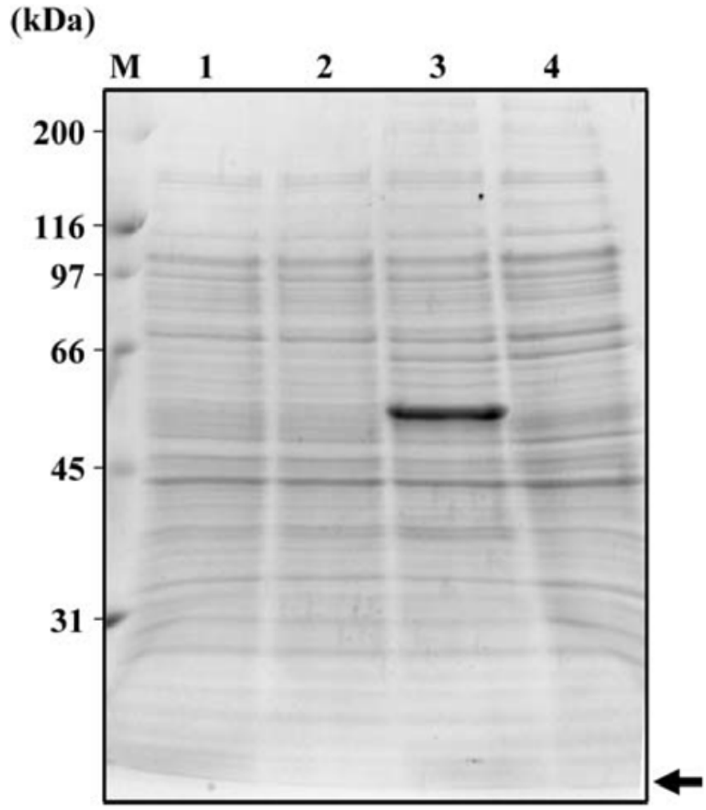

Fig. 6. SDS-PAGE profiles of cell-free extracts from various *Escherichia coli* DH5α transformants. Lane 1, *E. coli* DH5α carrying pEPAΔ1; lane 2, *E. coli* DH5α carrying pEPAΔ1 plus empty pGBM3; lane 3, *E. coli* DH5α carrying pEPAΔ1 plus pGBM3::*vktA*; lane 4, *E. coli* DH5α carrying pEPAΔ1 plus pGBM3::Δ*vktA*. Lane M, molecular marker standard (kDa). Arrow indicates the position of running dye. Adapted with permission from Orikasa et al. (2007).

It is evident that bacterial EPA (and DHA) is synthesized by the polyketide biosynthesis pathway, and that this process operates independently of the de novo biosynthesis of fatty acids up to C16 or C18 (Metz et al., 2001; Morita et al., 2000). However, it is likely that acetyl-CoA would be commonly used as a priming substrate in both processes, as specific inhibition of the de novo synthesis of fatty acids up to C18 by cerulenin enhanced the production of EPA and DHA in bacteria and probably also in *Schizochytrium* (Hauvermale et al., 2006). This is analogous to the situation in the unsaturated fatty acid auxotroph *E. coli fabB*$^-$ that was transformed with bacterial *pfa* genes, where EPA accounted for more than 30% of total fatty acids (Metz et al., 2001; R.C. Valentine & D. L Valentine, 2004). All of these findings suggest that the metabolic regulation of host organisms carrying *pfa* genes responsible for EPA biosynthesis could potentially be used

commercially to enhance the production of EPA. *V. rumoiensis* S-1 accumulates high levels of VktA protein, the amount of which is calculated approximately 2% of total soluble proteins (Yumoto et al., 2000). A significant accumulation of VktA was observed in DH5α(pEPAΔ1)(pGBM3::*vktA*) (Fig. 6). However, the fact that a slight increase in EPA production was also observed in DH5α(pEPAΔ1)(pGBM3::Δ*vktA*) excludes the possibility that the catalytic activity of VktA protein per se was involved in this increased EPA production.

Strains [1]	Fatty acid[2](% total)					Content of EPA (μg/ml)
	16:0	16:1(9)	18:1(11)	EPA	Others[3]	
E. coli DH5α	36.0 ± 1.0	29.6 ± 0.7	22.0 ± 0.6	0	12.5 ± 1.4	0
E. coli DH5α (pEPAΔ1)	35.6 ± 0.9	26.9 ± 1.5	21.8 ± 0.9	2.5 ± 0.2	13.2 ± 2.7	1.7 ± 0.1
E. coli DH5α (pEPAΔ1)(pGEM3)	38.6 ± 1.8	28.2 ± 0.6	20.8 ± 0.3	3.2 ± 1.7	9.2 ± 1.1	1.5 ± 1.3
E. coli DH5α (pEPAΔ1)(pGEM3::*vktA*)	35.9 ± 3.1	18.5 ± 0.4	22.9 ± 1.9	12.3 ± 0.7	10.3 ± 0.8	7.3 ± 1.2
E. coli DH5α (pEPAΔ1)(pGEM3::Δ*vktA*)	34.0 ± 0.7	26.7 ± 0.2	24.1 ± 1.2	5.9 ± 0.2	9.2 ± 1.7	3.3 ± 0.2

[1] The cells were grown at 20˚C until the culture had an OD_{660} of 1.0
[2] Fatty acids are denoted as number of carbon atoms:number of double bond. The Δ-position of double bond is presented in parenthesis
[3] Others incude 12:0, 14:0, 18:0 and 3-hydroxyl 14:0.

Table 2. Fatty acid composition of *E. coli* DH5α and its various transformants and recovered amount of EPA from cultures. Adapted with permission from Orikasa et al. (2007).

At present, the mechanism for the enhanced production of EPA in *E. coli* recombinant systems carrying DH5α(pEPAΔ1)(pGBM3::*vktA*) is unknown. One possibility is that the increase in production of EPA is a response against intracellular stress. DH5α(pEPAΔ1)(pGBM3::*vktA*) accumulated a large amount of VktA protein, which may have increased the stress for the host cells. This would have delayed their growth. Nishida et al. (2006) provided evidence that cellular EPA has an antioxidative function against extracellular H_2O_2 in bacterial recombinant systems expressing EPA biosynthesis (*pfa*) genes. Interestingly, levels of protein carbonyls were much lower in *E. coli* carrying *pfa* genes (with EPA) than in *E. coli* carrying no vector (without EPA), even if they had not been treated with H_2O_2. That is, cellular EPA may exert an antioxidative effect on ROS produced intracellularly (Nishida et al., 2006). A variety of stressful conditions, such as heat shock, osmotic shock, nutrient deprivation, and oxidative stress, are known to induce the synthesis of specific proteins. In *E. coli*, the induction of a protein was elicited in response to the overexpression of foreign proteins (Arora et al., 1995). However, to our knowledge, instances where the expression of one foreign gene (DNA) induces the expression of another foreign gene(s) have not been reported.

Clarification of the mechanism of increased EPA (and probably DHA) biosynthesis and the combined use of this technique with the others described above would create the possibility of greater production of these useful polyunsaturated fatty acids.

5. Conclusions

The VktA catalase is characterized by its high specific activity (Yumoto et al., 1998; 1999; 2000). However, the molecular mechanism of this notable future has not been clarified by its

primary structure of protein. VktA accumulate predominantly in the periplasmic space at a level of approximately 2% of total soluble proteins of strain S-1 cells (Yumoto et al., 2000) and part of it is localized at the surface of cells. Such specific distribution of VktA may protect it from attack by protein-degrading enzymes. We are not able to conclude whether the high specific activity of VktA and/or VktA accumulation in the cell are involved in enhancing the nitrogen-fixing activity in *R. leguminosarum* and the increased production of EPA (and probably DHA) in *E. coli* cells. If the high accumulation of catalase with a significantly high specific activity is essential for metabolic modifications (discussed above) in *vktA*-transformed host cells, it is desirable to use other kinds of catalase that accumulate in the cells and have a high specific activity. Such catalases are the *Exiguobacterium oxidotolerans* catalase *(*EKTA catalase; Hara et al., 2007) and the *Psychrobacter piscatorii catalase (*Kimoto et al., 2008), whose specific activity is comparable to that of VktA, indicating that these could be used instead of VktA.

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