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Enhanced heterologous production of eicosapentaenoic acid in *Escherichia coli* cells that coexpress eicosapentaenoic acid biosynthesis *pfa* genes and foreign DNA fragments including a high-performance catalase gene, *vktA*

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Abstract

Cellular eicosapentaenoic acid (EPA) makes up approximately 3% of total fatty acids in *Escherichia coli* DH5 α , a strain that carries EPA biosynthesis genes (pEPA Δ 1). EPA was increased to 12% of total fatty acids when the host cell coexpressed the vector pGBM3::sa1(*vktA*), which carried the high-performance catalase gene, *vktA*. Where this vector was coexpressed, the transformant accumulated a large amount of VktA protein. However, the EPA production of cells carrying the vector that included the insert lacking almost the entire *vktA* gene was approximately 6%. This suggests that the retention of a large DNA insert in the vector and the accumulation of the resulting protein, but not the catalytic activity of VktA catalase, would potentially be able to increase the content of EPA.

Introduction

Bacteria that are able to produce eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are a potential source of *n*-3 long chain polyunsaturated fatty acids (LC-PUFAs) for commercial use. In bacteria, EPA and DHA are synthesized *de novo* by polyunsaturated fatty acid synthase *pfa* genes following the polyketide biosynthesis pathway (Metz et al. 2001; Orikasa et al. 2004; 2006b), rather than by chain elongation and oxygen-dependent desaturation of existing fatty acids. The bacterial polyketide biosynthesis pathway has some benefits for the production of *n*-3 LC-PUFAs compared with the fatty acid elongation and desaturation processes, which are summarized in some reviews (Ratledge 2004; Bergé and Barnathan 2005; Okuyama et al. 2007).

Various techniques have been presented in order to enhance metabolically the production of *n*-3 LC-PUFAs by bacteria that inherently synthesize them and by host bacteria transformed with *pfa* genes. The production of EPA and DHA in *Shewanella marinintestina*

IK-1 and *Moritella marina* MP-1, respectively, was significantly enhanced by the addition of cerulenin (Morita et al. 2005), an inhibitor of the *de novo* biosynthesis of fatty acids (up to C18 fatty acids). A similar result was obtained using an EPA-producing deep-sea bacterium, *Photobacterium profundum* SS9 (Allen et al. 1999). The enhancement of EPA production was also reported in cells of *Shewanella baltica* that had been mutated with transposon Tn5 (Amiri-Jami et al. 2006).

It has been reported that EPA (and probably DHA) reduces the antioxidative stress caused by exogenously added hydrogen peroxide (H₂O₂) (Nishida et al. 2006a; 2006b). These results suggest that there might be some physiological relationship between EPA and H₂O₂ (oxidative stresses), or between EPA and H₂O₂-decomposing reactions. In the present study, the enhanced production of EPA was observed in recombinant systems consisting of *E. coli* that carried both EPA biosynthesis genes and a 4.9 kbp fragment of foreign DNA that included the 1.5 kbp high-performance catalase gene, *vktA* (Ichise et al. 2000). 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 LC-PUFAs using recombinant systems.

Materials and methods

Bacterial strains and culture conditions

E. coli strain DH5 α (Takara Bio, Kyoto, Japan) was used throughout the work. Luria-Bertani medium supplemented with 50 μ g ampicillin ml⁻¹ was used for the culture of *E. coli* DH5 α cells that had been transformed with the EPA biosynthesis *pfa* genes (pEPA Δ 1). Cultivation was carried out on a shaker (180 rpm) at 20 °C for 30–40 h. Cells carrying pGBM3 (Manen et al.

1997), pKT230 (Bagdasarian et al. 1981), or their derivatives were grown in the presence of 30 $\mu\text{g streptomycin ml}^{-1}$.

Growth was monitored by measuring the optical density of cultures at 660 nm (OD_{660}) and the cell dry weight per milliliter of cultures (mg ml^{-1}). Cells of *E. coli* DH5 α and its transformants were harvested when the OD_{660} was approximately 1.0.

Construction of vectors carrying the EPA biosynthesis genes or high-performance catalase gene

The pEPA Δ 1 is a cosmid vector that carries a 35 kbp DNA fragment including all five genes necessary for the production of EPA (*pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*) (Orikasa et al. 2004; 2006a).

The high-performance catalase gene, pBSsa1 (Ichise et al. 2000) was used. pBSsa1 is a derivative of pBluescript II SK+ that includes a *vktA*-containing DNA fragment, sa1 [presented as sa1(*vktA*) in this study], consisting of 4904 bp cloned from the H_2O_2 -resistant bacterium *Vibrio rumoiensis* S-1 (Yumoto et al. 1999). The restriction maps of pGBM3 vector and inserts are shown in Fig. 1.

The gene pBSsa1 was treated with *XhoI* and *BamHI*, and the resulting sa1(*vktA*)-containing fragment was integrated into the *XhoI*–*BamHI* site of pBBR1MCS-2 (5144 bp) (Kovach et al. 1995). The constructed vector, pBBR1MCS-2::sa1(*vktA*), was digested with *BamHI* and *XhoI*, and the insert fragment sa1(*vktA*) was ligated into the plasmids pGBM3 and pKT230, which had been digested with the same restriction enzymes. The resulting clones were designated pGBM3::sa1(*vktA*) and pKT230::sa1(*vktA*).

To generate a clone without *vktA*, pBBR1MCS::sa1(*vktA*) was constructed by integrating the *XhoI* and *BamHI*-containing fragment from pBSsa1 into pBBR1MCS (Kovach et al. 1994). Vector pBBR1MCS::sa1(*vktA*) was digested with *PstI* to remove the 1594 bp region (between two *PstI* sites), including most of *vktA* and a partial sequence (51 bp) derived from

pBluescript II SK+. The pBBR1MCS vector from which *vktA* had been removed [pBBR1MCS::*sa1*(Δ *vktA*)] was joined into a circle. Vector pBBR1MCS::*sa1*(Δ *vktA*) was digested with *Xho*I and *Bam*HI, and the *vktA*-deleted *sa1* clone (from *Xho*I to *Bam*HI; 3.4 kbp) was integrated into the *Xho*I-*Bam*HI sites of pGBM3. The resulting vector was designated pGBM3::*sa1*(Δ *vktA*). The *sa1*(Δ *vktA*) clone included all the sequence upstream from the ATG initiation codon of *vktA*, and only a remnant of *vktA* from the initiation codon to the 85th nucleotide of *vktA* (the *Pst*I site; see Fig. 1B) remained. This was followed by the 16-nucleotide sequence derived from pBluescript II SK+. When pBBR1MCS::*sa1*(Δ *vktA*) was treated with *Pst*I and *Sal*I, no *vktA* gene was detected using gel electrophoresis (data not shown). *E. coli* JM109 transformed with pBBR1MCS::*sa1*(Δ *vktA*) had only base levels of catalase activity (data not shown).

Determination of catalase activity, SDS-PAGE and gas-liquid chromatography (GLC)

Catalase activity was measured spectrophotometrically using cell-free extracts by monitoring the decrease in absorbance at 240 nm resulting from the elimination of H₂O₂ (Yumoto et al. 2000). The enzyme activity unit (U) was defined as the amount of enzyme activity that decomposed 1 μ mol H₂O₂ min⁻¹.

SDS-PAGE of cell-free extracts was carried out as described previously (Orikasa et al. 2006a). Protein was estimated by the method of Bradford using bovine serum albumin as a standard.

Fatty acids were analyzed as their methyl esters by GLC as described previously (Orikasa et al. 2006a).

Results and discussion

Growth of *E. coli* DH5 α transformants

E. coli DH5 α transformants carrying pEPA Δ 1, pEPA Δ 1 plus pGBM3, and pEPA Δ 1 plus pGBM3::sa1(*vktA*) were designated DH5 α (pEPA Δ 1), DH5 α (pEPA Δ 1)(pGBM3), and DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)], respectively. *E. coli* DH5 α cells carrying pEPA Δ 1 plus pKT230 and pEPA Δ 1 plus pKT230::sa1(*vktA*) were similarly named. The *E. coli* DH5 α transformant carrying pEPA Δ 1 plus pGBM3::sa1(Δ *vktA*) was named DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)].

E. coli DH5 α carrying no vector needed approximately 30 h to reach the late log phase of growth (an OD₆₆₀ of approximately 1.0) at 20 °C (Fig. 2). Transformation with pEPA Δ 1 or pEPA Δ 1 plus another vector delayed the growth of the transformants by 2–10 h. Generally, transformants carrying pGBM3 with a larger insert grew more slowly. Approximately 40 h was needed for DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)] to reach an OD₆₆₀ of 1.0. The cell concentration of any culture with the OD₆₆₀ of 1.0 was in the range of 0.54–61 mg ml⁻¹.

Fatty acid composition and production of EPA in *E. coli* DH5 α transformants

Introduction of pEPA Δ 1 to the host cell led to the production of EPA (approximately 3% of total fatty acids; Table 1). The production of EPA in host organisms carrying pEPA Δ 1 was increased to 12% of total fatty acids by the introduction of the sa1(*vktA*) insert that included the high-performance catalase gene, *vktA*, in pGBM3. The empty pGBM3 had no effect on EPA production. In DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)], EPA made up 6% of total fatty acids. The increase in EPA production in DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)] was accompanied by a decrease in the proportions of palmitoleic acid [16:1(9)] (Table 1). When pGBM3 and

pGBM3::sa1(*vktA*) were replaced in the *E. coli* transformants with pKT230 and pKT230::sa1(*vktA*), respectively, similar trends were observed (data not shown).

The yield of EPA per culture was approximately 1.5 $\mu\text{g ml}^{-1}$ for DH5 α (pEPA Δ 1) and DH5 α (pEPA Δ 1)(pGBM3). It increased to 7.3 $\mu\text{g ml}^{-1}$ for DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)]. The yield of EPA from DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)] was 3.3 $\mu\text{g ml}^{-1}$ (Table 1).

Catalase activity and catalase protein accumulation in *E. coli* DH5 α transformants

E. coli DH5 α has an inherent catalase activity of 2–3 U mg protein $^{-1}$ (Nishida et al. 2006a). The plasmid pEPA Δ 1 had no effect on the catalase activity of the host cells. Catalase activity was increased to 535 U mg protein $^{-1}$ for DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)]. However, there was no enhancement of catalase activity in DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)] (Table 2).

Fig. 3 shows the profiles of proteins prepared from various *E. coli* DH5 α transformants using SDS-PAGE. A significant amount of protein in the VktA band, i.e., 57 kDa, was detected only for DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)]. No notable novel band was observed in DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)] or in any of the other transformants.

It is evident that bacterial EPA or DHA is synthesized by the polyketide biosynthesis pathway (Metz et al. 2001; Orikasa et al. 2006b), 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 (Allen and Bartlett 2002; Morita et al. 2005) 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; Valentine and Valentine 2004). All of these findings suggest that the metabolic regulation of host organisms carrying *pfa* genes responsible for EPA or DHA biosynthesis could potentially be used commercially to enhance the production of these LC-PUFAs.

In this study, the enhanced production of EPA in *E. coli* DH5 α expressing pEPA Δ 1 has been achieved by introducing another 4.9 kbp foreign DNA fragment [sa1(*vktA*)] that included the high-performance catalase gene, *vktA* (ORF a, Fig. 1B). The VktA belongs to the bacterial clade 3 catalase (Ichise 2003; Chelikani et al. 2004) and was cloned from *V. rumoiensis* S-1, a strain with an extraordinarily high catalase activity (Ichise et al. 2000). This bacterium accumulates high levels of VktA protein, of which amount is calculated approximately 2% of total soluble proteins (Yumoto et al. 2000). As well as *V. rumoiensis* S-1, a significant accumulation of VktA was observed in DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)] (Fig. 3). However, the fact that a slight increase in EPA production was also observed in DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ *vktA*)] excludes the possibility that the catalytic activity of VktA protein *per se* was involved in this increased EPA production. Although the sa1(*vktA*) DNA fragment includes one other open reading frame (ORF b) in addition to the *vktA* gene (Fig. 1B), protein product from this ORF would not increase the production of EPA as the ORF b remains in the sa1(Δ *vktA*) fragment.

At present, the mechanism for the enhanced production of EPA in *E. coli* recombinant systems carrying DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)] (Table 1) and DH5 α (pEPA Δ 1)[pKT230::sa1(*vktA*)] (data not shown) is unknown. One possibility is that the increase in production of EPA is a response against intracellular stress. The growth of *E. coli* DH5 α recombinants was delayed (Fig. 2), which would be due to their retention of the second vector, pGBM3, or its derivatives. The size of the insert was 0 bp (4066 bp as vector), 4955 bp (9021 bp), and 3361 bp (7427 bp) for pGBM3, pGBM3::sa1(*vktA*), and pGBM3::sa1(Δ *vktA*), respectively. The slowing of growth was positively correlated with the size of the pGBM3 insert

(Fig. 2). Additionally, DH5 α (pEPA Δ 1)[pGBM3::sa1(*vktA*)] accumulated a large amount of VktA protein, which may have increased the stress for the host cell. This would have delayed their growth.

Recently, Nishida et al. (2006a; 2006b) provided evidence that cellular EPA has an antioxidative function against extracellular H₂O₂ 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 were not treated with H₂O₂. That is, cellular EPA may exert an antioxidative effect on reactive oxygen species produced intracellularly (Nishida et al. 2006a). 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 and Pedersen 1995). However, to our knowledge, instances where the expression of one foreign gene (DNA) induces the expression of another foreign gene(s) have never been reported. Clarification of the mechanism of increased EPA (and probably DHA) biosynthesis and the combined use of this technique with others described above would provide the possibility of greater production of these useful polyunsaturated fatty acids.

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Table 1 Fatty acid composition of *E. coli* DH5 α and its various transformants and recovered amount of EPA from cultures.

Strains ^a	Fatty acid ^b (% total)					Content of EPA ($\mu\text{g ml}^{-1}$)
	16:0	16:1(9)	18:1(11)	EPA	Others ^c	
<i>E. coli</i> DH5 α	36.0 \pm 1.0	29.6 \pm 0.7	22.0 \pm 0.6	0.0	12.5 \pm 1.4	0.0
<i>E. coli</i> DH5 α (pEPA Δ 1)	35.6 \pm 0.9	26.9 \pm 1.5	21.8 \pm 0.9	2.5 \pm 0.2	13.2 \pm 2.7	1.7 \pm 0.1
<i>E. coli</i> DH5 α (pEPA Δ 1)(pGBM3)	38.6 \pm 1.8	28.2 \pm 0.6	20.8 \pm 0.3	3.2 \pm 1.7	9.2 \pm 1.1	1.5 \pm 1.3
<i>E. coli</i> DH5 α (pEPA Δ 1)[pGBM3::sa1(<i>vktA</i>)]	35.9 \pm 3.1	18.5 \pm 0.4	22.9 \pm 1.9	12.3 \pm 0.7	10.3 \pm 0.8	7.3 \pm 1.2
<i>E. coli</i> DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ <i>vktA</i>)]	34.0 \pm 0.7	26.7 \pm 0.2	24.1 \pm 1.2	5.9 \pm 0.2	9.2 \pm 1.7	3.3 \pm 0.2

^a The cells were grown at 20 °C until the culture had an OD₆₆₀ of 1.0.

^b Fatty acids are denoted as number of carbon atoms:number of double bond. The Δ -position of double bond is presented in parenthesis.

^c Others include 12:0, 14:0, 18:0, and 3-hydroxyl 14:0.

Table 2 Catalase activity of *E. coli* DH5 α and its various transformants.

Strains ^a	Catalase activity (U mg protein ⁻¹)
<i>E. coli</i> DH5 α (pEPA Δ 1)	3.3
<i>E. coli</i> DH5 α (pEPA Δ 1)(pGBM3)	3.2
<i>E. coli</i> DH5 α (pEPA Δ 1)[pGBM3::sa1(<i>vktA</i>)]	535.1
<i>E. coli</i> DH5 α (pEPA Δ 1)[pGBM3::sa1(Δ <i>vktA</i>)]	1.7

^a Cells were washed three times with phosphate buffer (pH 7.5) by centrifugation at $3000 \times g$ for 15 min, and they were then suspended in 0.2 ml of 60 mM potassium phosphate buffer (pH 7.0). Cells were disrupted by sonic oscillation using a Sonifier Cell Disruptor (model W185; Branson Ultrasonic Corp., Danbury, CT) for 40 s in an ice bath. Supernatants were removed after the centrifugation of cell lysates at $20\,000 \times g$ for 60 min and were used as cell-free extracts.

Figure legends

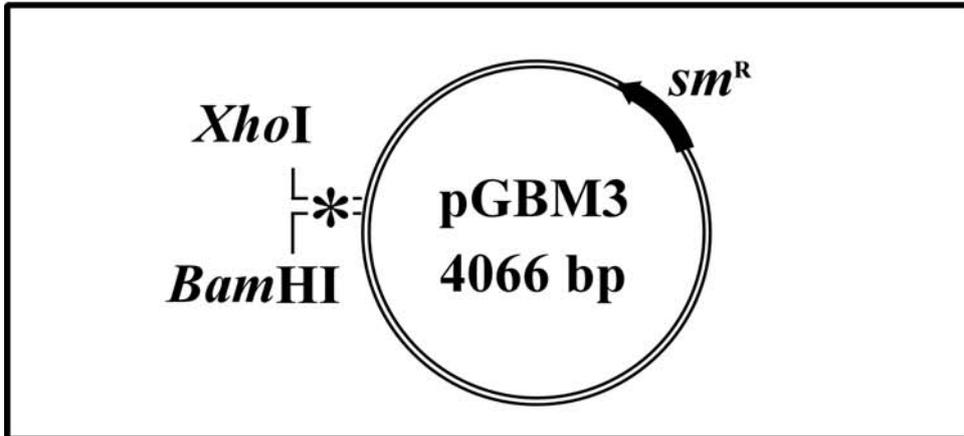
Fig. 1 Restriction map of pGBM3 (A) and the inserts *sa1(vktA)* (B, upper panel) and *sa1(ΔvktA)* (B, lower panel). Arrow indicates the relative position and size of ORF. a, *vktA*; b, unidentified ORF.

Fig. 2 Growth profiles of *Escherichia coli* DH5α and its various transformants at 20 °C. ○, *E. coli* DH5α carrying no vector; ●, *E. coli* DH5α carrying pEPAΔ1; ▲, *E. coli* DH5α carrying pEPAΔ1 plus empty pGBM3; ■, *E. coli* DH5α carrying pEPAΔ1 plus pGBM3::*sa1(vktA)*; □, *E. coli* DH5α carrying pEPAΔ1 plus pGBM3::*sa1(ΔvktA)*. Growth was first monitored by measuring the optical density of cultures at 660 nm (OD₆₆₀). Cells of DH5α and its transformants were harvested when the OD₆₆₀ was approximately 1.0. The harvested cells were washed with deionized water and then freeze-dried and weighed. The relationship between the cell dry weight per ml of culture and the OD₆₆₀ was as follows: 0.53 mg ml⁻¹ at the OD₆₆₀ of 0.982 for DH5α; 0.55 mg ml⁻¹ at 0.915 for DH5α(pEPAΔ1); 0.62 mg ml⁻¹ at 1.009 for DH5α(pEPAΔ1)(pGBM2); 0.40 mg ml⁻¹ at 0.699 for DH5α(pEPAΔ1)[pGBM3::*(vktA)*]; 0.52 mg ml⁻¹ at 0.947 for DH5α(pEPAΔ1)[pGBM3::*sa1(ΔvktA)*]. The growth of each strain was presented by cell dry weight per ml of culture (mg ml⁻¹).

Fig. 3 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::*sa1(vktA)*; lane 4, *E. coli* DH5α carrying pEPAΔ1 plus pGBM3::*sa1(ΔvktA)*. Lane M, molecular marker standard (kDa). Arrow indicates the position of running dye.

Figure 1

A



B

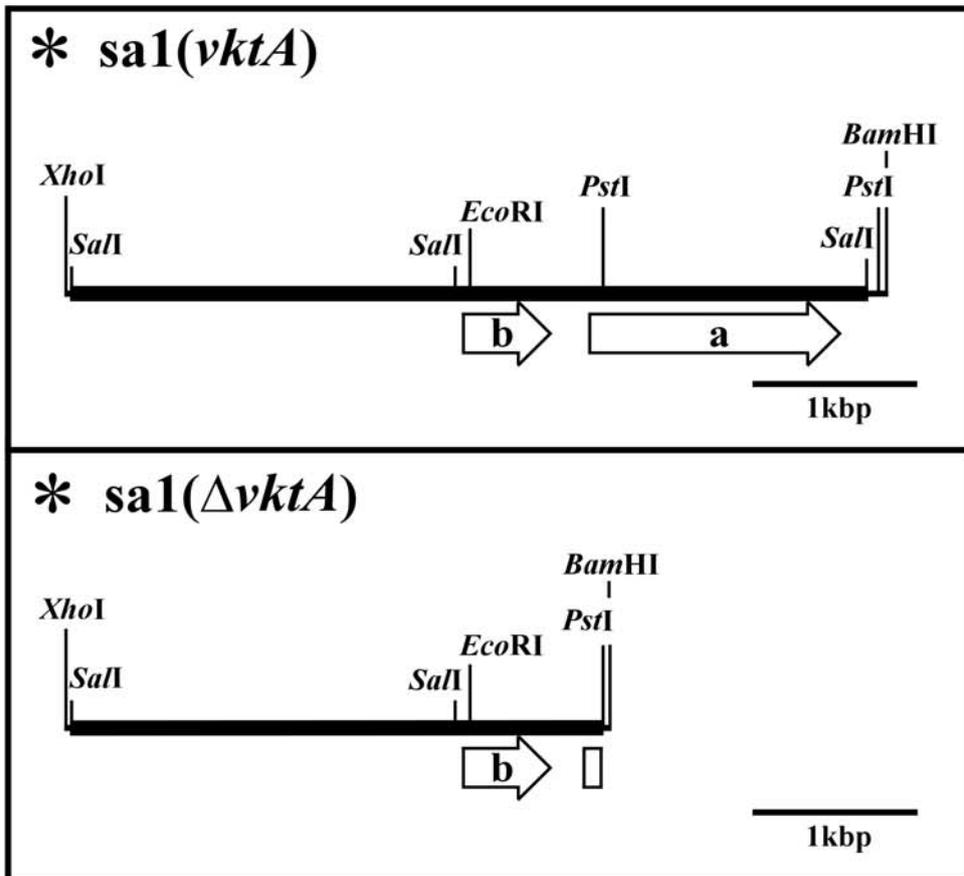


Figure 2

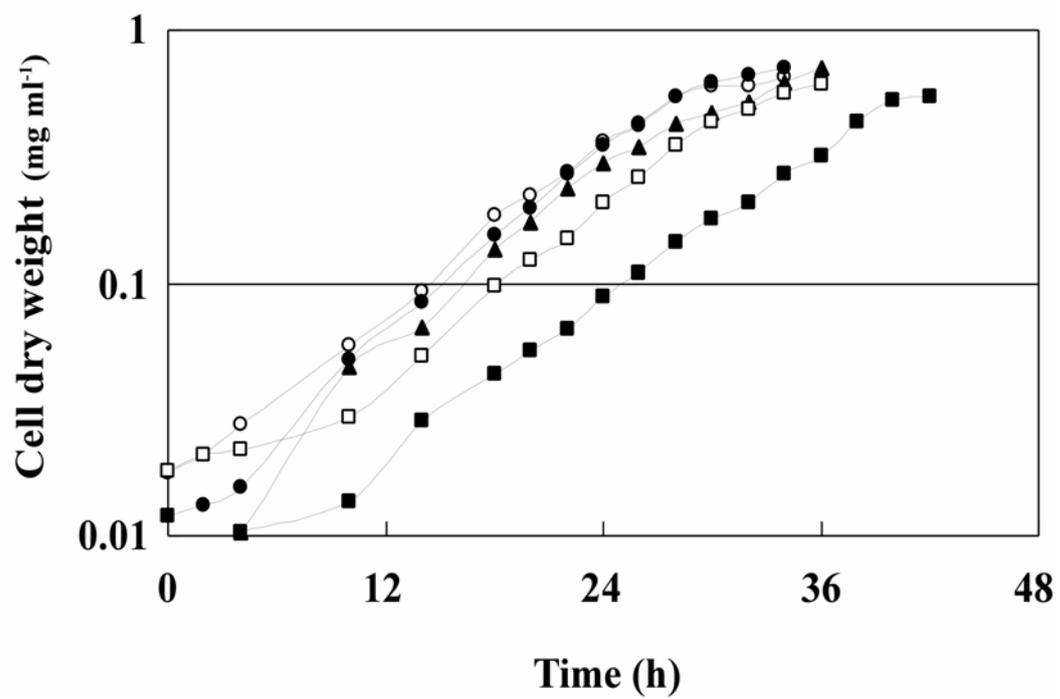


Figure 3

