Biotechnology and Bioprocess Engineering **2006**, 11: 127-133

The Growth and EPA Synthesis of *Shewanella oneidensis* MR-1 and Expectation of EPA Biosynthetic Pathway

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Abstract *Shewanella oneidensis* MR-1 has the ability to inhale certain metals and chemical compounds and exhale these materials in an altered state; as a result, this microorganism has been widely applied in bioremediation protocols. However, the relevant characteristics of cell growth and biosynthesis of PuFAs have yet to be thoroughly investigated. Therefore, in this study, we have attempted to characterize the growth and fatty acid profiles of *S. oneidensis* MR-1 under a variety of temperature conditions. The fastest growth of *S. oneidensis* MR-1 was observed at 30°C, with a specific growth rate and doubling time of 0.6885 h^{-1} and 1.007 h. The maximum cell mass of this microorganism was elicited at a temperature of 4°C. The eicosapentaenoic acid (EPA) synthesis of *S. oneidensis* MR-1 was evaluated under these different culture temperatures. *S. oneidensis* MR-1 was found not to synthesize EPA at temperatures in excess of 30°C, but was shown to synthesize EPA at temperatures below 30°C. The EPA content was found to increase with decreases in temperature. We then evaluated the EPA biosynthetic pathway, using a phylogenetic tree predicted on 16s rRNA sequences, and the homology of ORFs between *S. oneidensis* MR-1 and *Shewanella putrefaciens* SCRC-2738, which is known to harbor a polyketide synthase (PKS)-like module. The phylogenetic tree revealed that MR-1 was very closely related to both *Moritella* sp., which is known to synthesize DHA via a PKS-like pathway, and *S. putrefaciens,* which has been reported to synthesize EPA via an identical pathway. The homology between the PKS-like module of *S. putrefaciens* SCRC-2738 and the entire genome of *S. oneidensis* MR-1 was also analyzed, in order to mine the genes associated with the PKS-like pathway in *S. oneidensis* MR-1. A putative PKS-like module for EPA biosynthesis was verified by this analysis, and was also corroborated by the experimental finding that *S. oneidensis* MR-1 was able to synthesize EPA without the expression of dihomo-γ-linoleic acid (DGLA) and arachidonic acid (AA) formed during EPA synthesis via the FAS pathway.

Keywords: *Shewanella oneidensis* MR-1, growth characteristics, eicosapentaenoic acid (EPA), EPA biosynthesis, PKS-like pathway

INTRODUCTION

Polyunsaturated fatty acids (PuFAs), and in particular eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6 ω3), are critical components of the glycolipids and phospholipids comprising the plasma membranes. They also function as both precursors of certain hormones and as signaling molecules [1,2]. Additionally, these fatty acids have been determined to exert beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation, and certain cancer types [3,4].

PuFA synthesis has been shown to be preceded by elongation and aerobic desaturation reactions of fatty acid synthase (FAS) [16]. Recently, a novel alternative pathway for C_{20+} PuFAs biosynthesis was investigated by Jim Metz *et al.* [1]. This pathway employs a polyketide synthase (PKS)-like gene cluster, rather than the multiple desaturase and elongase enzymes, for the synthesis of PuFAs. Therefore, in the polyketide synthase-catalyzed system, the complete cycle of reduction, dehydration, and reduction seen in association with FAS is often abbreviated. However, several rounds of sequential reactions, catalyzed by ketoreductase, dehydratase, and enoyl reductase, result in the synthesis of PuFAs from a primer molecule in the form of acetyl-CoA (Fig. 1) [1,5].

Shewanella oneidensis MR-1 has been identified as a member of the proteobacteria γ-subgroup, according to a phylogenetic classification prediced on a 16s ribosomal RNA gene [6]. It is a gram-negative, facultatively anaerobic proteobacterium, capable of growing under a variety of conditions. It also exhibits several special abilities. Not only can the strain effect the bioremediation of halogenated organic pollutants, but it is also able to reduce

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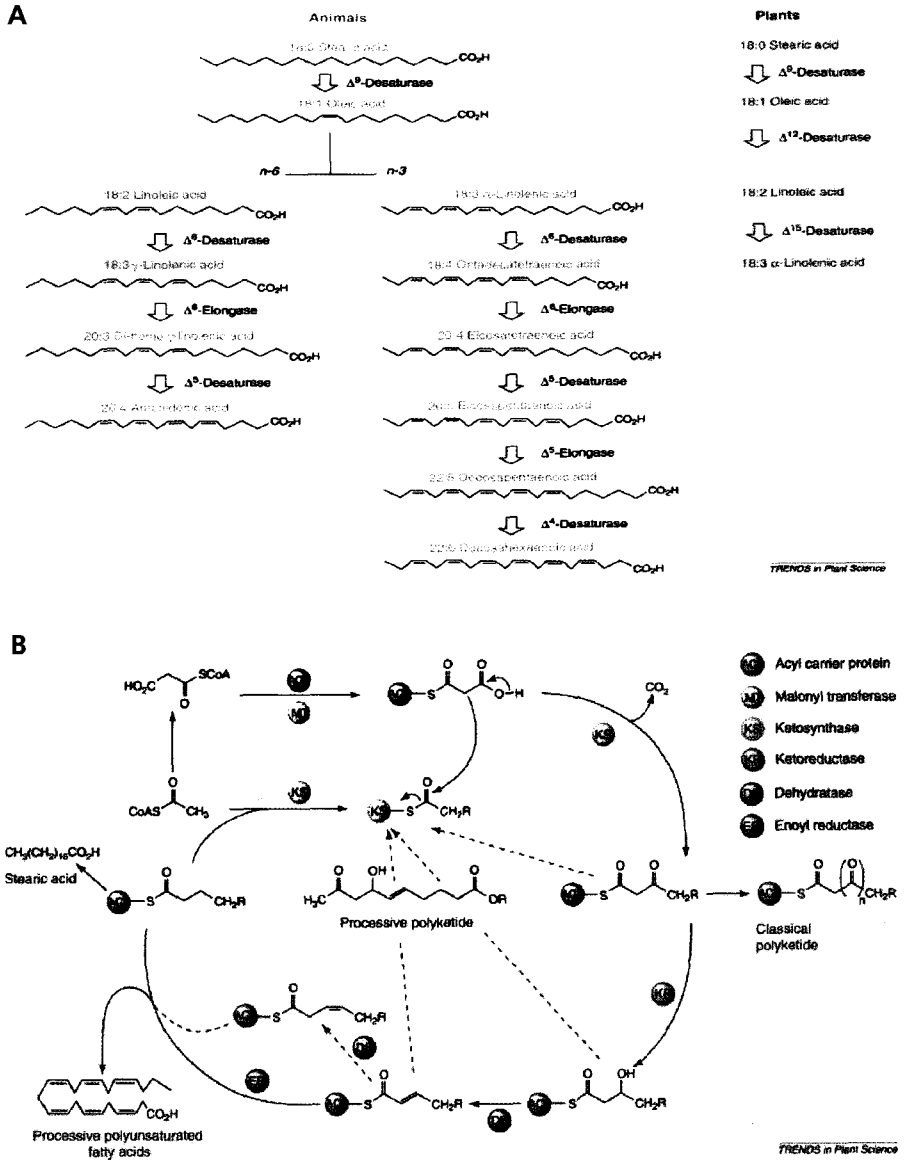

Fig. 1. (A) Pathway scheme in lower eukarotes by an FAS pathway. (B) Proposed scheme for the processive synthesis of PuFAs by a PKS-like system.

and dissolve certain insoluble metal dioxides, including iron and manganese (Fig. 2) [7]. These capabilities have attracted a great deal of attention from researchers concerned with bioremediation, metal leaching, and corrosion [7,8].

S. oneidensis MR-1 is reportedly able of growth at rather low temperatures (3°C). However, it evidences a growth transition at approximately 10°C, and below this temperature exhibits a dramatically different phenotype, with changes in morphology, growth rate, ultrastructure, and protein and lipid composition. The proteins biosynthesized by this strain at 3 and 22°C are noticeably different. Upon analysis of protein expression in cells grown at 3 and 22°C, 17 of the proteins were found to have been overexpressed, and 33 were found to have been significantly underexpressed at 3°C. These different expressions elicited different phospholipid fatty acid profiles, varying with the growth temperature (low-temperature growth). Moreover, the potential characteristics of *S. oneidensis* MR-1 constituted an impetus for the sequencing of the entire genome, and have also spurred interest in the application of this species to bioremediation, as well as the production of several specific materials, including EPA [8].

In this study, the growth and PuFAs synthesis characteristics of *S. oneidensis* MR-1 were investigated under a variety of temperature conditions [17]. The biosynthesis pathways were analyzed in order to predict whether the strain utilized an FAS pathway or a PKS-like pathway, and also to mine for genes associate with EPA synthesis.

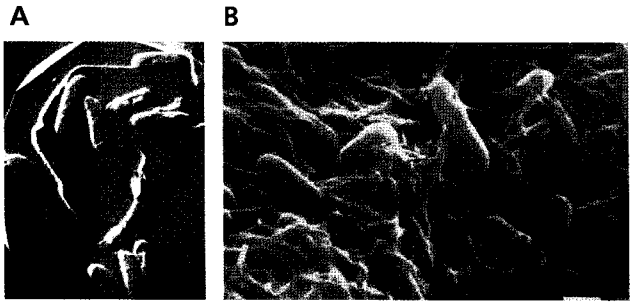

Fig. 2. (A) *S. oneidensis* MR-1 growing on the surface of the iron oxide mineral, hematite. (B) Biofilm formation of *S. oneidensis* MR-1.

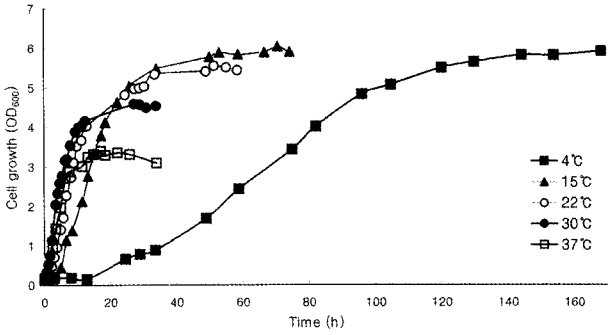

Fig. 3. Growth curve of *S. oneidensis* MR-1 at various temperatures.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism employed in this study was *S. oneidensis* MR-1 (ATCC700550^{TM}), and Luria-Bertani (LB) was utilized as the culture medium. MR-1 was activated in order to obtain colonies on the LB plate via 20 h of streaking at 30°C. The inocula were prepared in 100 mL Erlenmeyer flasks, each containing 20 mL of the liquid medium, and were then grown overnight at 30°C with orbital shaking at 180 rpm in a shaking incubator (JSSKI-1000RL, Johnsam Corp., Korea). The primary cultures were prepared using the 12 h inocula at a rate of 2% (v/v) in 250 mL Erlenmeyer flasks, each containing 50 mL of the medium, over 8 days. The cultivation temperatures were 4, 15, 22, 30, and 37°C, and the rotation speed was set at 180 rpm. For the analyses of growth and fatty acid composition, sampling was conducted according to culture period.

Analytical Methods

The growth of the cells was determined via measurements of the optical densities of each of the culture times, using a UV spectrophotometer (UV-1601, Shimadzu, Japan) at 600 nm. The unsaturated fatty acids were analyzed in the following manner. The samples were obtained once per culture time, then centrifuged for 15 min at 5,000 rpm. The supernatants were removed, after which the cells were washed three times in distilled water and then dried for 2 h at 70°C. The lipids in the dried cells were transformed into fatty acid methyl esters via the Lepage Method [9], and the fatty acid composition was analyzed using a gas chromatography apparatus (Hewlett Packard 6890, USA) equipped with a flame-ionized detector (FID) and a DB23 (30 m × 0.25 mm × 0.26 μm, Agilent Technologies, USA) capillary column. The column temperature was raised from 150 to 270°C (2 min) at 7°C/min.

Phylogenetic Analysis

The 16s ribosomal RNA sequences of *Shewanella* sp., including *S. oneidensis* MR-1, *Moritella* sp., which is known to synthesize DHA, *Escherichia coli* within γ-*proteobacteria, Altermonas, Pseudomonas, Photobacterium,* and *Bacillus* were obtained from the NCBI database (The National Center for Biotechnology, USA). A phylogenetic tree of these species, predicted on 16s ribosomal RNA sequences, was constructed using the ClustalW program (European Bioinformatics Institute, UK).

Mining of Genes Related to the PKS-like Module

The genome sequences of the PKS-like module of *Shewanella putrefaciens* SCRC-2738 and the whole genome sequence of *S. oneidensis* MR-1 were obtained from the NCBI and TIGR databases (The Institute for Genomic Research, USA). The nine genes of *S. oneidensis* MR-1 that evidenced a high degree of homology with the nine ORFs of the PKS-like module of *S. putrefaciens* SCRC-2738 were then searched via the application of the obtained genomic sequences to the BLAST programs in the NCBI database.

RESULTS AND DISCUSSION

Growth Characteristics of *S. oneidensis* MR-1

The cell growth curves are provided for the various culture temperatures (Fig. 3). *S. oneidensis* MR-1 was able to grow even at temperatures as low as 4°C, although the fastest rate of growth was observed at 30°C (Table 1). *S. oneidensis* MR-1 was initially isolated from the sediments of Lake Oneida, in New York [10]. Lake Oneida completely freezes over during winter, while the water temperature begins to rise in May, reaching a maximum of 25°C during mid-summer [11]. Therefore, the growth characteristics of this organism were expected to be closely related to the environment of Lake Oneida, from which *S. oneidensis* MR-1 was isolated. *S. oneidensis* MR-1 appeared pink when cultured in a temperature range from 4 to 30°C, but turned orange at 37°C. The reason for this change in color remains to be elucidated.

The specific growth rate (μ), doubling time (T_d), maximum cell mass, and lag period of *S. oneidensis* MR-

Table 1. Growth characteristics of *S. oneidensis* MR-1 at various temperatures

Temperature (°C)	Specific growth rate (h^{-1})	Doubling time (h)	Maximum cell mass (g/L)	Exponential phase reaching time (h)
4	0.0309	22.432	2.14	12.83
15	0.1179	5.879	2.11	4.92
22	0.2451	2.828	1.93	2.17
30	0.6885	1.007	1.59	0.5
37	0.2802	2.474	1.18	1.0

1 were assessed using the experimental data provided in Fig. 3, and are shown in Table 1. At a culture temperature of 30°C, the specific growth rate and the doubling time were 0.6885 h^{-1} and 1.007 h, respectively, but were 0.0309 h^{-1} and 22.432 h at 4°C. The lag period was 12.83 h at 4°C, and was 0.5 h at 30°C. These findings suggest that the optimum temperature, in terms of the maximum specific growth rate, minimum doubling time, and lag period, was 30°C (Table 1). However, the maximum cell mass was elicited at a temperature of 4°C, although it took a very long time for the cells to reach stationary phase at this temperature (Fig. 3).

EPA Synthesis of *S. oneidensis* MR-1

The polyunsaturated fatty acid biosynthesis characteristics of *S. oneidensis* MR-1 were evaluated according to culture times at a variety of temperatures. *S. oneidensis* MR-1 was initially reported not to biosynthesize EPA. However, it has recently been determined that the species does, indeed, synthesize EPA, albeit only a small amount, and this EPA content can be reduced by a decrease in culture temperature [6,8].

The experimental finding of this study revealed that *S. oneidensis* MR-1 does not synthesize any EPA at temperatures higher than 30°C, and that these EPA contents increase along with decreases in temperature (Fig. 4). EPA contents achieve a maximum level during the exponential phase, and then gradually decrease with advancing culture time. The trend of change in EPA content with temperature observed in this study differs from the observation reported in previous studies [6,8], but was consistent with the trend regarding polyunsaturated fatty acid biosynthesis in other species (Fig. 4) [12]. Therefore, the enzymes associated with EPA biosynthesis are expected to be more active than the enzymes contributing to the synthesis of other types of fatty acids. This phenomenon can also be explained in conjunction with the differences in protein content observed with different temperatures. Previous reports have demonstrated the differentiation between cold-shock proteins and cold-acclimation proteins in a mesophilic gram-positive bacterium [13], and differences in protein expression according to temperature have also been previously investigated. In the case of *S. oneidensis* MR-1, the proteins associated

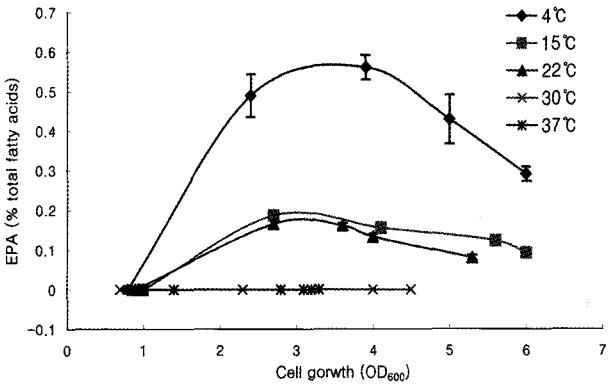

Fig. 4. EPA biosynthetic ability of *S. oneidensis* MR-1 at various temperatures.

with molybdopterin biosynthesis and amino acid metabolism were found to have been overexpressed, but those employed in respiration, transport, amino acid synthesis, and nucleotide synthesis were underexpressed at 3°C, as compared with the levels observed at 22°C [8]. The reason that EPA can be produced more abundantly at lower temperatures is assumed to involve the overexpression of proteins associated with EPA synthesis.

EPA Synthesis Pathway of *S. oneidensis* MR-1

We also attempted to determine whether EPA synthesis was carried out via the FAS pathway or the PKS-like pathway, especially at low temperatures, via the experimental investigation of the EPA synthesis of *S. oneidensis* MR-1. First, a phylogenetic tree was constructed in order to predict the pathway on the basis of the 16s rRNA sequence (Fig. 5). In this case, all *Shewanella* sp. in the NCBI database, as well as *Moritella* sp., which are known to synthesize DHA via a PKS-like pathway, were used, in addition to several procaryotes. The tree indicated that *S. oneidensis* MR-1 was quite closely related to *Moritella* sp. and *S. putrefaciens* from an evolutionary viewpoint. The one is known to synthesize DHA via the PKS-like pathway, and the other has been reported to synthesize EPA via an identical pathway. Therefore, *S. oneidensis* MR-1 can be presumed to form EPA via a PKS-like pathway.

Throustochytrium aureum synthesizes polyunsaturated fatty acids via an iterative elongation and desaturation reaction of elongase and desaturase, such as DGLA (dihomo-γ-linolenic acid) → AA (Arachidonic acid) → EPA (eicosapentaenoic acid) → DPA (docosapentaenoic acid) → DHA (docosahexaenoic acid) [14]. The presence of DGLA, AA, EPA, and DHA in *T. aureum* has previously been confirmed via gas chromatography [13]. If *S. oneidensis* MR-1 synthesizes EPA via an FAS pathway, the precursors DGLA and AA should be detected. However, in the gas chromatography profile of the fatty acids synthesized by *S. oneidensis* MR-1, only EPA was observed, and no DGLA and AA were detected (Fig. 6). Therefore, *S. oneidensis* MR-1 was surmised to utilize a PKS-like pathway, rather than an FAS pathway.

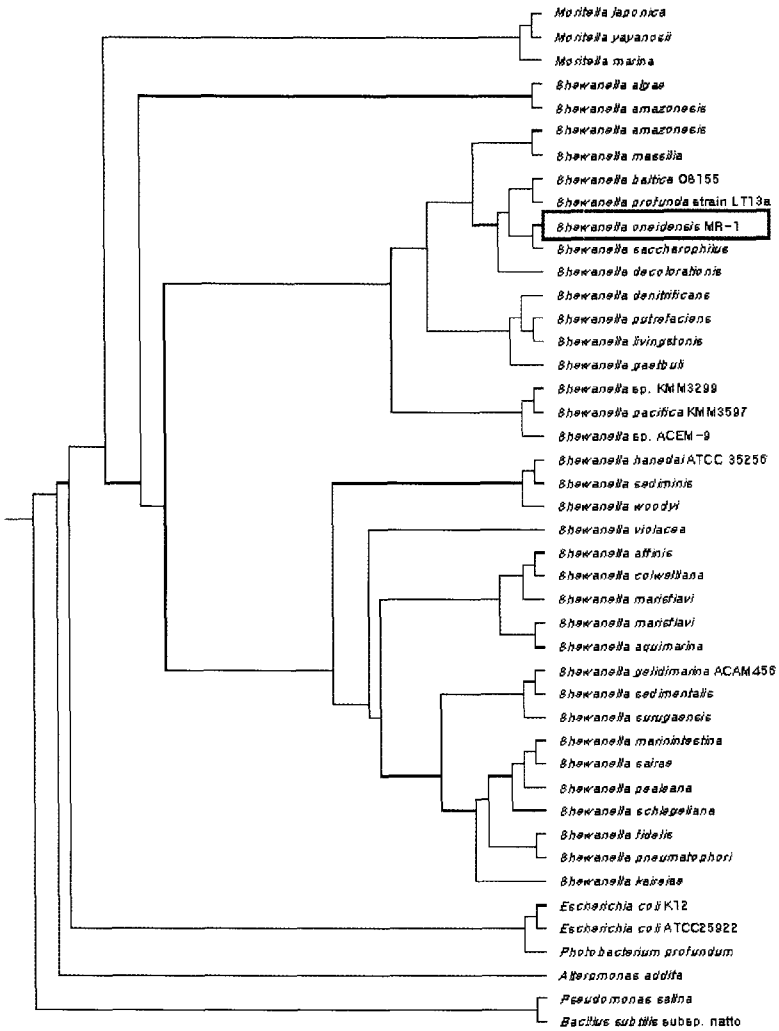

Fig. 5. Phylogenetic tree of *Shewanella* sp.

Mining of the Genes Related to the PKS-like Pathway

As *S. oneidensis* MR-1 was believed to synthesize EPA via a PKS-like pathway, we conducted mining in order to determine whether *S. oneidensis* MR-1 possesses PKS-like pathway-associated genes. Yazawa [15] previously reported that *S. putrefaciens* SCRC-2738 harbors a PKS-like module, which consists of nine ORFs (Open Reading Frames). The genomic sequence of the PKS-like module of *S. putrefaciens* SCRC-2738 was obtained from the NCBI database, and the entire genome sequence was obtained from the TIGR database. The homology between the PKS-like module of *S. putrefaciens* and the entire genome of *S. oneidensis* MR-1 was then analyzed, in order to mine for genes associated with the PKS-like pathway in *S. oneidensis* MR-1. Additionally, a detailed investigation was conducted regarding the process of fatty acid biosynthesis, using the function of each gene.

The sequences of the ORFs, SO1597 to 1606 and SO1309 of *S. oneidensis* MR-1, revealed a minimum 30% homology and a maximum 90% homology, as com-

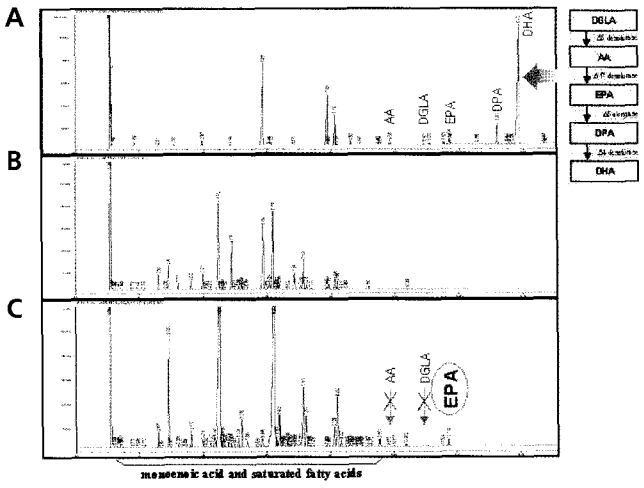

Fig. 6. Comparision of GC profiles between *S. oneidensis* MR-1 and *T. aureum* BK-1. (A) GC profile of *T. aureum* synthesizing PuFAs by FAS pathway, (B) *S. oneidensis* MR-1 at 37°C, (C) *S. oneidensis* MR-1 at 4°C.

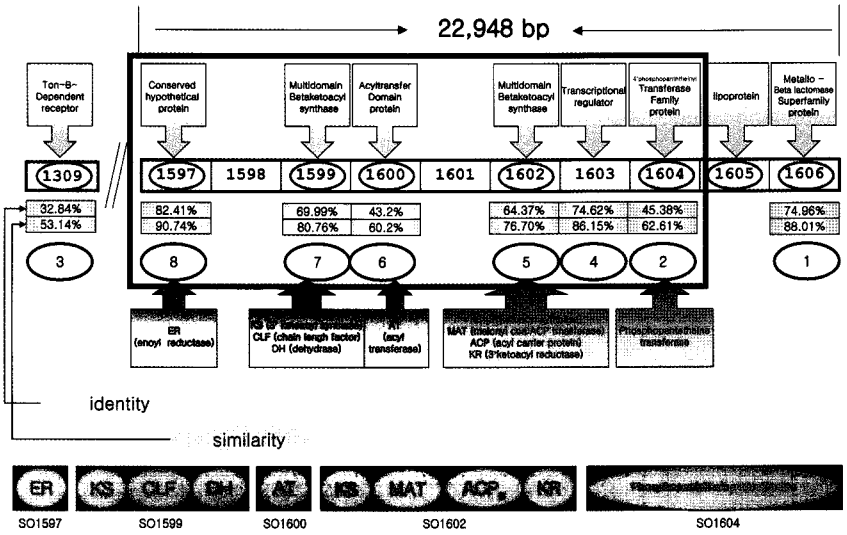

Fig. 7. Mining of the PKS-like module for comparison of the whole genome sequence of *S. oneidensis* MR-1 and the PKS-like module sequence in *S. putrefaciens* SCRC-2738.

pared with the corresponding ORFs of the PKS-like module of *S. putrefaciens* SCRC-2738 (Fig. 7). Moreover, the ORFs of *S. oneidensis* MR-1 exist as one type of module. Therefore, it could be putatively concluded that the ORFs, SO1597 to 1606 and SO1309, are components of a PKS-like module for EPA synthesis in *S. oneidensis* MR-1.

Another putative conclusion derived from the comparison of the sequences of each of the ORFs is that SO1309 is a Ton-B-dependent receptor, SO1597 is enoyl reductase (ER), SO1599 and SO1602 are multi-domain betaketoacyl synthase, SO1600 is acyl transferase (AT), SO1604 is phosphopantetheine transferase, SO1605 is a lipoprotein, and SO1606 is metallo beta lactamase. Two multi-domain betaketoacyl synthases, which play the most important roles in the PKS-like module, were detected, and these were identified as a typical type of polyketide synthase. The locations of the functional domains of each of the ORFs were also mined via *in silico* analyses (Fig. 7).

A putative PKS-like module associated with EPA synthesis was verified via experimental data analysis and a homology analysis of the ORFs between *S. oneidensis* MR-1 and *S. putrefaciens* SCRC-2738, which is known to harbor a PKS-like module.

CONCLUSION

The fastest growth rate of *S. oneidensis* MR-1 was observed at a temperature of 30°C, within a tested range of 4 to 37°C. At 30°C, the specific growth rate and doubling time were 0.6885 h^{-1} and 1.007 h, respectively. The microorganism was able to grow at a temperature as low as 4°C, at which it evidenced a specific growth rate of 0.0309 h^{-1} and a doubling time of 22.432 h. The maximum cell mass was elicited at 4°C, although at this temperature, it took a very long time for the cells to achieve stationary phase.

The EPA synthesis characteristics of *S. oneidensis* MR-1 appeared to depend upon the culture temperature. EPA was not synthesized at all at temperatures in excess of 30°C. However, EPA synthesis was detected at temperatures below 30°C, and the EPA content was shown to increase with a decrease in temperature. The EPA content achieved maximum levels during the exponential phase, and gradually decresed afterwards.

The phylogenetic tree constructed in this study revealed that *S. oneidensis* MR-1 was quite closely related to *Moritella* sp. and *S. putrefaciens*. The first of these is known to synthesize DHA via a PKS-like pathway, and the other has been reported to synthesize EPA via the same pathway. Our experimental results revealed that *S. oneidensis* MR-1 synthesized EPA without expressing DGLA and AA, which are precursors for FAS pathway-mediated EPA synthesis.

The homology between the PKS-like module of *S. putrefaciens* SCRC-2738 and the entire genome of *S. oneidensis* MR-1 was also analyzed, in order to mine for genes related to the PKS-like pathway in *S. oneidensis* MR-1. The sequences of ORFs, SO1597 to 1606 and SO1309 of *S. oneidensis* MR-1, evidenced a minimum 30% homogy and a maximum 90% homology with the corresponding ORFs of the PKS-like module of SCRC-2738. Moreover, the ORFs of *S. oneidensis* MR-1 were demonstrated to exist as one module type. Finally, a putative PKS-like module associated with EPA synthesis was confirmed through experimental data analysis and homology analysis of the ORFs between *S. oneidensis* MR-1 and *S. putrefaciens* SCRC-2738, which is known to harbor a PKS-like module. Furthermore, the exact sequence of PKS-like pathway-mediated PuFA synthesis is expected to be investigated in the future, using *S. oneidensis* MR-1. Additional polyketide antibiotics are also expected from different combinations of ORFs contained

in the PKS-like module of *S. oneidensis* MR-1.

Acknowledgement This work was supported by a Korea Research Foundation Grant (KRF-2004-005-D00006).

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[Received December 25, 2005; accepted March 8, 2006]