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Complete genome sequence of *Nitratireductor* sp. strain OM-1: A lipid-producing bacterium with potential use in wastewater treatment

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ABSTRACT

Reducing CO_2 emissions is necessary to alleviate rising global temperature. Renewable sources of energy are becoming an increasingly important substitute for fossil fuels. An important step in this direction is the isolation of novel, technologically relevant microorganisms. *Nitratireductor* sp. strain OM-1 can convert volatile short-chain fatty acids in wastewater into 2-butenoic acid and its ester and can accumulate intracellularly esterified compounds up to 50% of its dried cell weight under nitrogen-depleted conditions. It is believed that a novel fatty acid biosynthesis pathway including an esterifying enzyme is encoded in its genome. In this study, we report the whole-genome sequence (4.8 Mb) of OM-1, which comprises a chromosome (3,977,827 bp) and a megaplasmid (857,937 bp). This sequence information provides insight into the genome organization and biochemical pathways of OM-1. In addition, we identified lipid biosynthesis pathways in OM-1, paving the way to a better understanding of its biochemical characterization.

1. Introduction

Global warming is one of the most problematic issues facing human civilization today, and mitigation of CO_2 emissions is necessary in order to maintain the rise in temperature within acceptable limits. Burning of fossil fuels is a major factor contributing to CO_2 emissions. Therefore, renewable sources of energy are becoming an increasingly important substitute for fossil fuels. Biodiesel fuel (BDF) is one of the most suitable renewable sources of energy because it can be obtained from biomass and is considered carbon neutral. Therefore, BDF production is expected to develop within the next few years. An important step in this direction is the isolation of novel technologically relevant microorganisms.

Nitratireductor sp. strain OM-1 (subsequently referred to as OM-1) has been isolated from seawater in Seto Inland Sea, Hiroshima, Japan, and studies have assessed it as a propionate-assimilating bacterium [1]. OM-1 can convert volatile short-chain fatty acids (FAs) (e.g., acetate, propionate, butyrate, and valerate) into 2-butenoic acid and its ester. In addition, it can accumulate esterified compounds up to 50% of its dried cell weight under nitrogen-depleted conditions [1]. OM-1 has also been used in trial wastewater treatment after methane fermentation [2]. The methane fermentation residue initially contained 711.7 mg/L of acetic acid, 96.4 mg/L of propionic acid and 113.8 mg/L of butyric acid, with a chemical oxygen demand (COD) of 6339 mg/L, and co-cultivation of OM-1 with microbes in the wastewater reduced COD by 91.1%, organic acids were completely consumed under aerobic conditions for 7 days, and excess sludge production decreased to 60% [2]. These findings show that OM-1 has application in wastewater treatment and biofuel production from wastewater residues.

These phenotypic characteristics show that the OM-1 genome encodes (i) proteins belonging to a novel FA biosynthesis

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pathway for short-chain ester production and (ii) a short-chain FA-esterifying enzyme. OM-1 can also perceive the nitrogen concentration and regulate the expression of genes belonging to the FA biosynthesis pathway. Therefore, in this study, we sequenced the whole genome of OM-1, which provided insight into the genome organization and biochemical pathways of OM-1. In addition, we identified a lipid biosynthesis pathway and a hypothetical biosynthesis pathway of 2-butenoic acid ester, which is an important milestone in biochemical characterization of OM-1.

2. Materials & methods

2.1. Bacterial strain and culture conditions

Nitratireductor sp. strain OM-1 was used to determine the lipid composition, its whole genome sequence and gene expression involved in lipid synthesis. To determine OM-1′s whole genome sequence, OM-1 was grown in original American Type Culture Collection (ATCC) Medium 1409 [3] and incubated at 28 °C with shaking at 100 rpm under aerobic conditions for 1 week for lipid-non-producing conditions. While investigating lipid production, the concentration of ammonium chloride added in the medium was either 10 or 0 mM, and that of carbon sources acetic acid and propionic acid was 10 mM each, that C/N ratio is 4.3 [1].

2.2. Electron microscopy

OM-1 cells grown in both conditions until the stationary phase were collected and washed with Milli-Q (Millipore Corporation, Burlington, MA, USA). Subsequently, they were mounted on 50-mesh copper grids coated with collodion (Nisshin EM Co., Ltd., Tokyo, Japan), and dried in the electric dryer at 65 °C for 2 days. Then, the specimens were sputtered by platinum. Scanning electron microscopy (SEM) was performed using field emission scanning electron microscope (FE-SEM) S-5200 (Hitachi High-Technologies, Tokyo, Japan).

2.3. Lipid extraction and determination

OM-1 cells grown in both conditions were lyophilized by lyophilizer (FDU-1200, EYELA, Tokyo, Japan). Total lipids were extracted from dried cells (3-mL culture) with 500 μL of

Table 1
Primer sets for real-time PCR.

Gene name	Primer name	Primer sequence	Product length (bp)
FabI	NispOM1_01264-157F	gccgagaaagtgaactccaa	221
	NispOM1_01264-377R	tccgtgaaggaatagcagga	
FabB	NispOM1_01265-266F	aggaaggcgacatcaccaac	218
	NispOM1_01265-483R	ggccgacgagatcgagtagt	
FabA	NispOM1_01266-144F	tgagttcggaaagggctatg	242
	NispOM1_01266-385R	tgccgtattcgaccttcttc	
FabI	NispOM1_02078-4F	acaagtggcaatggcctgat	247
	NispOM1_02078-250R	tctcgagcttctcgaacacc	
FabD	NispOM1_02313-120F	gcagaagctttccagcatca	216
	NispOM1_02313-335R	gtatcggccagcgagaacat	
FabG	NispOM1_02314-217F	gatcgcagtgaagtcaagca	234
	NispOM1_02314-450R	cacggaggtgatgttgatga	
FabF	NispOM1_02317-56F	ttgaacatgggtggaagagc	194
	NispOM1_02317-249R	agcaatgccgtaggtgatga	
DesA	NispOM1_02462-156F	agtcgctgttgcaaatggat	223
	NispOM1_02462-378R	gttacctgccgtagcgtgat	
FabH	NispOM1_02523-175F	atgatcattctggccacgtc	228
	NispOM1_02523-402R	gtcgttccagtcgagaatgc	
FabF2	NispOM1_02717-15F	cagctttggaagacccgtta	160
	NispOM1_02717-174F	aaccagatcggcaagctcat	
FabF2	NispOM1_02718-28F	ggtatgggcatcgtttcctc	250
	NispOM1_02718-277R	cctcgtcacccttcatgtcg	
FabZ	NispOM1_02824-35F	aaagcctcgacatcatggac	241
	NispOM1_02824-275R	ctctcatcacccgcattgtt	
AccC	NispOM1_03058-64F	aaggagctcggcatccagac	249
	NispOM1_03058-312R	gccgatgaaggtgagattgt	
FabH	NispOM1_03467-18F	gcaagaggcttcagacttcg	248
	NispOM1_03467-265R	cgtcacgctcatagacatgg	
PlsX	NispOM1_02522-94F	gcccgctttcttgtatacgg	189
	NispOM1_02522-282R	atttttcaccgcttcgatgg	
—	NispOM1_02853-4F	aacaccatcctgtgctacgg	228
	NispOM1_02853-231R	atggctcgtcagaagtgtcg	
GlpK	NispOM1_02947-133F	gagcatgatcccgaagacat	215
	NispOM1_02947-347R	tttttcagcgtttcgcacag	
AccA	NispOM1_00687-131F	ccaaggatgcgctcatagaa	232
	NispOM1_00687-362R	gtgtcattgccctttcctg	
TesB	NispOM1_00827-8F	cggccatgcaagacctactc	225
	NispOM1_00827-232R	cctcgtagatgatcggaacg	
TE	NispOM1_00935-97F	cttgcaaaccaggtcgaaat	215
	NispOM1_00935-311R	atcacgatgtcggtgctctc	
AccD	NispOM1_01103-77F	ggatcaaggatcccgagagt	248
	NispOM1_01103-324R	gtcttccatgctggtcttgg	
AccB	NispOM1_03057-20F	gggtcgatcagcaactcatt	208
	NispOM1_03057-227R	gggctcttcttggcttcttc	
16S rRNA	8F	agagtttgatcctggctcag	529
	536R	gwattaccgcggckgctg	

chloroform/methanol (2:1, v/v); this process was repeated twice to obtain a total extract volume of 1.5 mL. Then an additional 500 μL of milliQ water was added, and the extract was vortexed and centrifuged at 5000 × *g* for 10 min. Next, the lower chloroform phase was transferred to a test tube and desiccated by nitrogen gas flow at 60 °C, and then, 25 μL of chloroform was added to extract total lipids. The lipid content was analyzed by gas chromatography (GC) using the GC-17A and CBM-102 gas chromatographs (Shimadzu Corp., Kyoto, Japan) using a DB-5HT capillary GC column (Agilent Technologies, Santa Clara, CA, USA). Crotonic acid (2-Butenoic acid, TCI Chemistry, Tokyo, Japan), Palmitic acid (Wako Pure Chemical Industries, Ltd. Osaka, Japan), Squalene (Sigma-Aldrich, Missouri, USA) and Triolein (Sigma-Aldrich, Missouri, USA) were used for standards and the peaks in chromatograms were identified by comparison with the retention times of the standards. Analytical conditions were as described previously [1]. The lipids were identified by gas chromatography–mass spectrometry (GC–MS) with the JMS-T100GCV (AccuTOF GCv 4 G) (JEOL Ltd., Tokyo, Japan) using a DB-5HT capillary GC column (Agilent Technologies) was used to identify the lipids. Helium (purity ≥99.999%) was used as a carrier gas at a flow rate of 1.2 ml/min. A 1-μl sample was injected at split ratio 10:1 at an injection temperature of 300 °C. The oven temperature was programmed to increase from an initial temperature of 100 °C to 340 °C at 10 °C/min. The mass spectrometer was operated in EI mode at 70 eV in a scan range of *m/z* 29–800, and FI mode in a scan range of *m/z* 50–800 when obtaining molecular ions. MassCenter, version 2.6.4 (JEOL Ltd.) and Mass Spectral Library (NIST11, National Institute of Standard and Technology), were used for the analysis.

2.4. Whole genome sequencing and annotation

Genomic DNA was isolated using proteinase K and phenol following the standard protocol [4]. The quality and quantity of the isolated genomic DNA were determined using the Qubit double-stranded DNA (dsDNA) HS kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA purity was estimated to be 1.78 (280/260) using spectrophotometry, and it passed the final quality control criteria assessed by Macrogen Corp., Kyoto, Japan.

Whole-genome sequencing was performed with a PacBio RSII sequencer using single-molecule real-time (SMRT) cell 8Pac V3 (Pacific Biosciences, Menlo Park, CA, USA) and a DNA polymerase–binding kit (Pacific Biosciences). The initial de novo assembly was performed using the Hierarchical Genome Assembly Process (HGAP v3.0). As a result of mapping reads against assembled contigs and error correction using Quiver, the consensus sequence with higher quality was generated and two circular contigs were obtained. Gene finding and annotation were performed using PROKKA ver. 1.11 [5] and the Rapid Annotation using Subsystem Technology (RAST) database [6]. We performed an additional annotation of the protein-encoding genes identified with PROKKA using eggNOG ver. 4.5 [7]. The origin of replication was identified using ORI-Finder [8]. Assignment of proteins to the Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiers was done using FOAM software [9], and assignment to KEGG pathways/modules was done using self-written Perl scripts [10]. We calculated the completeness of the KEGG modules using the Metabolic And Physiological potentiaL Evaluator (MAPLE) [11].

Orthologous genes were identified in the genomes of all 11 *Nitratireductor* species (i.e., EBB, NL21, RA22, RR328, UMTGB225, C115, CGMCC, PHT3B, ZZ1, ES061, and OM-1) using SPOCS software with standard parameters [12].

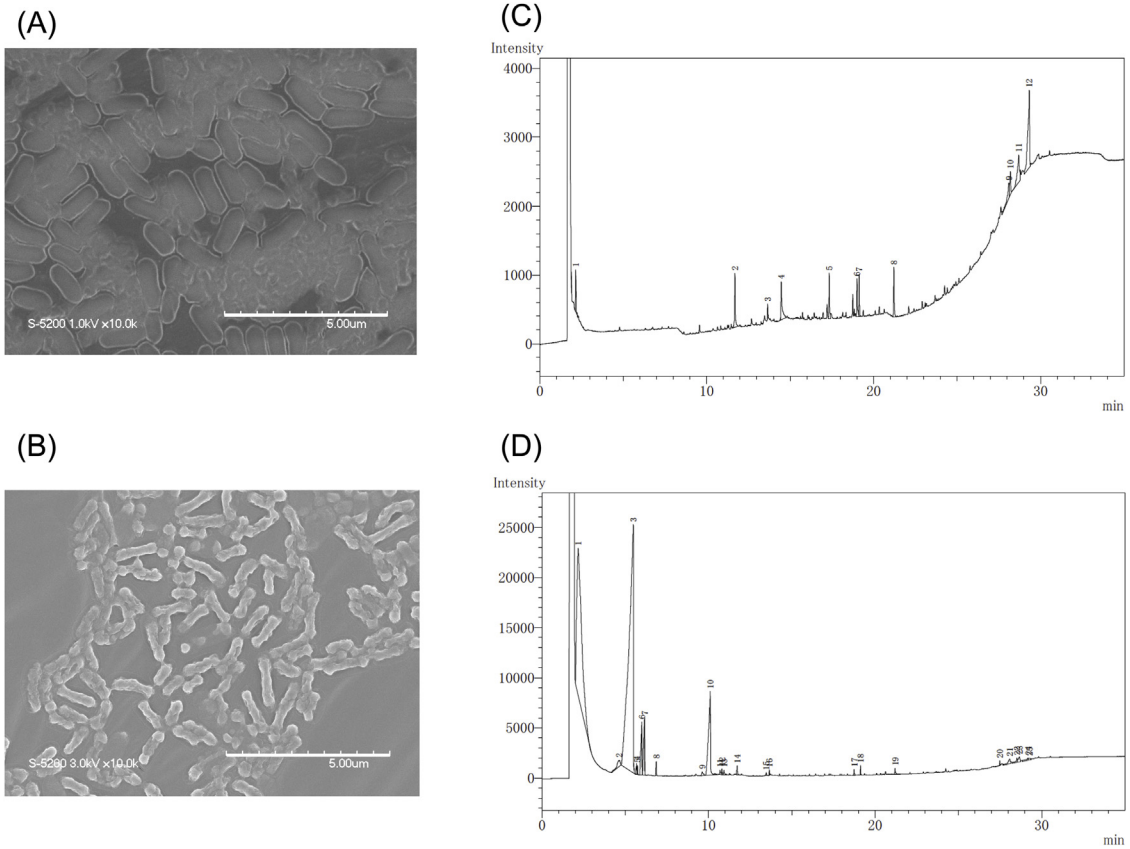

Fig. 1. (A, B) SEM images of OM-1 cells. OM-1 cells were grown with 10 mM (A) and 0 mM (B) ammonium chloride. (C, D) GC chromatogram of total lipid extracts from OM-1 cells grown with 10 mM (B) and 0 mM (C) ammonium chloride. SEM, scanning electron microscopy; ATCC, American Type Culture Collection; GC, gas chromatography.

2.5. Gene expression for fatty acid and 2-butenoic acid synthesis

Total RNA was isolated from the cells grown under the lipid-producing and non-producing conditions using RNAzol® RT (Molecular Research Center, Inc. Ohaio, USA). The total RNA was digested by RNase-free DNase I (Takara Bio, Shiga, Japan) to remove contaminated DNA and purified by using NucleoSpin® RNA Clean-up (Machery-Nagel Inc., Pennsylvania, USA). RNA concentrations were determined by using a Qubit® fluorometer (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) with Qubit® RNA BR Assay Kits (Invitrogen). The cDNAs were synthesized with the total RNA using PrimeScript™ 1 st strand cDNA Synthesis Kit (Takara Bio) and random primers. Real-time quantitative PCR (RT-qPCR) were performed and analyzed with PowerUp™ SYBR® Green Master mix (applied biosysytem/Thermo Fisher Scientific, Austin, TX, USA) and Thermal Cycler Dice Real-Time System II (TP900, Takara Bio). Primer sets for the genes involved in fatty acid and 2-butenoic acid syntheses and 16S rRNA gene as internal control were listed in Table 1. The PCR conditions were 95.0 °C for 5 min and 50 cycles of 95.0 °C for 30 s, 60.0 °C for 30 s and 72.0 °C for 60 s. The relative changes in gene expression were determined by $\Delta\Delta C_T$ method [13].

3. Results & discussion

3.1. Oil accumulation under nitrogen-depleted conditions

When placed under the nitrogen-depleted conditions, the growth of OM-1 was not different from the one with nitrogen, however OM-1 can produce lipid under nitrogen-depleted conditions and oilaccumulation is the most significant characteristic of OM-1. To the best of our knowledge, there are no reports regarding oil production in other *Nitratireductor* species. Fig. 1A and B showed the SEM images of OM-1 grown under the oil accumulation conditions described in a previous study [1] and under nitrogen-supplemented conditions, respectively. Comparing with non-producing cells, the OM-1 cells were bulged by filling oil. Moreover, the oil leaked from the cells because of weight of sputtered platinum and it was observed intercellular spaces. On the other hand, lipid-non-producing cells were shrunk by dehydration.

Fig. 1C and D show the oil composition of OM-1 analyzed by GC and each component was analyzed by GC–MS in order to identify the substances and the molecular mass. Supplemental Table S1 listed the retention times and assignment of peaks obtained by GC, respectively and Supplemental Figure S1 showed GC–MS chromatogram and mass spectra of each peak to predict the substances. The peak at a retention time of 2.15 min represents 2-butenoic acid (Fig. 1C and D). With 10 mM of ammonium chloride in the growth medium, we detected hexadecanoic acid (retention time 11.7 min), octadecanoic acid (retention time 14.47 min) and squalene (retention time 19.0 and 19.1 min.). We also detected retention time > 28 min as presumable TAG; the sum of the TAG peak areas was 53% of the total area. On the contrary, under nitrogen-depleted conditions (i.e., 0 mM of ammonium chloride), the peak at a retention time of 5.49 min was predicted to be 2-butenoic acid 1-methylethyl ester on the basis of the mass determined by GC–MS analysis. We could not confirm this prediction because no standard was present in the database; therefore, we called it 2-butenoic acid

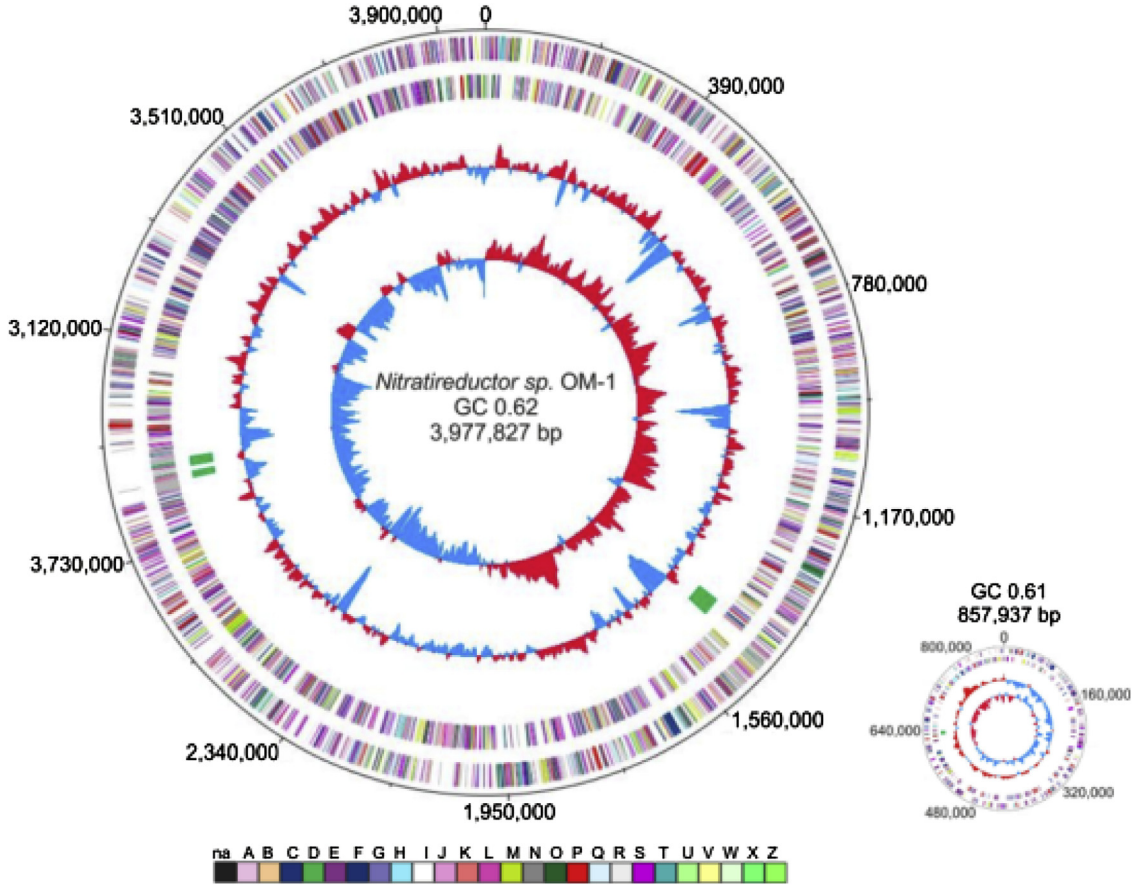

Fig. 2. Circular view of chromosomal and megaplasmid sequences of OM-1. The circular display shows (from outside inward): (a) predicted CDSs localized on forward and reverse strands colored according to the COG classification, (b) genomic regions corresponding to integrated prophages, (c) GC content (%), and (d) GC skew (G + C/G—C). CDS, coding sequence; COG, Clusters of Orthologous Groups; GC, gas chromatography.

ester, and its peak area corresponded to 50% of the total area. The peak area of 2-butenoic acid shared 32.4% of the total area. The peak at a retention time of 10.1 min was pentadecanoic acid, and its peak area shared 7.0% of the total area. These findings suggested that nitrogen-depleted conditions stimulate not only accumulation of lipids but also production of 2-butenoic acid, which is a substrate for ester synthesis and inhibits carbon chain elongation.

Butenoic acid biosynthesis was challenged by Dellomonaco et al. (2011) using engineered reversal of the ß-oxidation cycle in *Escherichia coli* [14]. For engineered reversal of the ß-oxidation cycle, Liu et al. overexpressed thioesterase gene (bTE; Accession # AAO77182) from *Bacteroides thetaiotaomicron* in *E. coli* and achieved 11.4 mg/L of butenoic acid production [15]. OM-1 can naturally produce 64.8 mg/L of 2-butenoic acid under nitrogen-depleted conditions. The 2-Butenoic acid is further converted into its ester via an unknown pathway. The esterifying enzyme involved in this pathway needs to be discovered, because it could represent a relevant target for engineered strains for fuel and chemical synthesis. Therefore, we decided to determine the whole genome sequence of OM-1 in order to identify the genes involved in the FA biosynthesis pathway.

3.2. Features of the OM-1 genome

To obtain a comprehensive overview of the protein-encoding genes of OM-1 and to gain further insight into the genes involved in the FA biosynthesis pathway, we performed whole-genome sequencing at Macrogen Corp. The assembly was completed by August 2017. The OM-1 genome comprised two circular DNA molecules, a chromosome of 3,977,827 bp (depth: 128) and a megaplasmid of 857,937 bp (depth: 71) (Fig. 2). GC contents were 62% in the chromosome and 61% in the megaplasmid. In the chromosomal sequence, we identified an OriC region, while in the megaplasmid, we assigned the replication origin to the maximum of the GC disparity value. Of the total 4615 genes, 4561 were protein-encoding genes, while 60 were RNA genes, including 53 transfer RNAs (tRNAs), 1 transfer-messenger RNA (tmRNA), and 6 ribosomal RNA (rRNA) genes (Table 2).

Comparative genomic analyses of the pan and core genomes of all *Nitratireductor* species revealed that the pan genome comprised 7718 coding regions (CDSs), while the core genome included 1402 CDSs (Supplemental Table S2). In addition, the analysis showed that OM -1 contains 891 strain-specific CDSs. The number of genes assigned to each Clusters of Orthologous Groups (COG) category was calculated using eggNOG (Supplemental Tables S3 and S4). However, the function of 1071 open reading frames (ORFs) (23% of the entire gene content) remains unknown.

3.3. Identification of lipid and hypothetical 2-butenoic acid ester biosynthesis pathways

Analysis of KEGG pathways revealed that the OM-1 genome encodes a complete set of genes for FA and TAG biosynthesis (Fig. 3A and B). FA and TAG production was confirmed under the nitrogen-rich condition in OM-1 (Fig. 1B and [1]). Comparative genome analysis among eleven *Nitratireductor* species suggested the possibility of TAG production in other *Nitratireductor* species. Fig. 3C shows a hypothetical 2-butenoic acid ester biosynthesis pathway. The map was drawn on the basis of the engineered reversal of the ß-oxidation cycle proposed by Liu et al. (2015) [15]. A List of the genes involved in FA, TAG and 2-butenoic acid biosyntheses was shown in Table 3. The presence of genes involved in 2-butenoic acid production was verified in OM-1, and a complete synthesis pathway was identified. The most controversial identification was that of thioesterases, because in the genome, we identified five thioesterase-encoding genes. On the basis of the similarity with bTE utilized by Liu et al. (2015) in engineered *E. coli* strain [15], acyl–coenzyme A (acyl-CoA) thioesterase I was identified as the most probable candidate. TesA and TesB, acyl-CoA thioesterase I and II, utilized in engineered reversal of the ß-oxidation cycle were commonly found in all *Nitratireductor* species. The activity of thioesterase is releasing acyl carrier proteins (ACPs) and generates free fatty acids (FFAs). In this study, But-2-enoyl-ACP was converted into butenoic acid (Fig. 3C). The high activity of thioesterases might be a key factor because OM-1 naturally produces a high yield of 2-butenoic acid under nitrogen-depleted conditions (Fig. 1D). However, OM-1 has another 7 thioesterase super family proteins, so we found 12 thioesterases in OM-1, while other *Nitratireductor* speices had more than 5. We did not find a thioesterase specific to OM-1; however, OM-1 harbors the maximum number of thioesterase genes (Table 4). These genes are candidates for engineered production of 2-butenoic acid. In addition, as shown in Fig. 1C and D, nitrogen is a controlling factor that inhibits 2-butenoic acid production. Expression of the thioesterase gene would be induced directly or indirectly by nitrogen depletion. We attempted to identify other key genes for 2-butenoic acid ester production; however, we could not find the gene predicted to be ester synthase.

Considering all the proteins associated withTAG and wax ester-accumulating machineries in prokaryotes, BLASTp suggested the presence of a long-chain-fatty-acid-CoA ligase and two NADP-dependent malic enzymes. Malic enzymes were associated with TAG biosynthesis in *Streptomyces* [16].However, further manual annotation failed to identify any candidate for ester synthase proteins,such as a CoA-dependent acyltransferase known as wax ester synthase/diacylglycerol acyltransferase (WS/DGAT) [17]. Further research using comparative transcriptome analysis between lipid-producing and lipid-non-producing cells is required to identify new metabolic pathways.

Table 2
Genome statistics. Chr = chromosome; pl = megaplasmid.

Attribute	Value
Genome size (bp)	48,35,764
DNA coding (%)	89.2
G + C content	0.62 (chr); 0.61 (pl)
DNA scaffolds	2
Protein coding genes	4561
RNA genes	60
Genes with function prediction (eggNOG)	4127
Genes assigned to COGs	4127
CRISPR repeats	3

3.4. Effect of nitrogen-depleted conditions on gene expression for lipid biosynthesis

Nitrogen concentration is control factor of lipid production and accumulation in OM-1. Therefore, the transcriptional level of the genes listed in Table 3 was investigated and compared between nitrogen-sufficient and nitrogen-depleted conditions. Table 5 shows the expression ratios when 16S rRNA gene is internal control and relative gene expression is also presented as the mean ratio of the expression ratio of nitrogen-depleted conditions to that of nitrogen-sufficient conditions. All genes of TAG synthesis were down-regulated under nitrogen-depleted conditions. This result supported that OM-1 produced few amount of TAG under nitrogen-depleted conditions. Likewise, almost of the genes in free fatty acid synthesis were down-regulated except for *accA* and *accD* (acetyl-CoA carboxylase carboxyl transferase subunit alpha and beta, respectively) and *fabH* (beta-ketoacyl-acyl carrier protein (ACP) synthase III, also called acetoacetyl-ACP synthase). All the subunits of acetyl-CoA carboxylase (*accA-D*) were identified, however *accB* and *accC*, that probably belong to the same transcriptional unit, showed different transcription pattern from

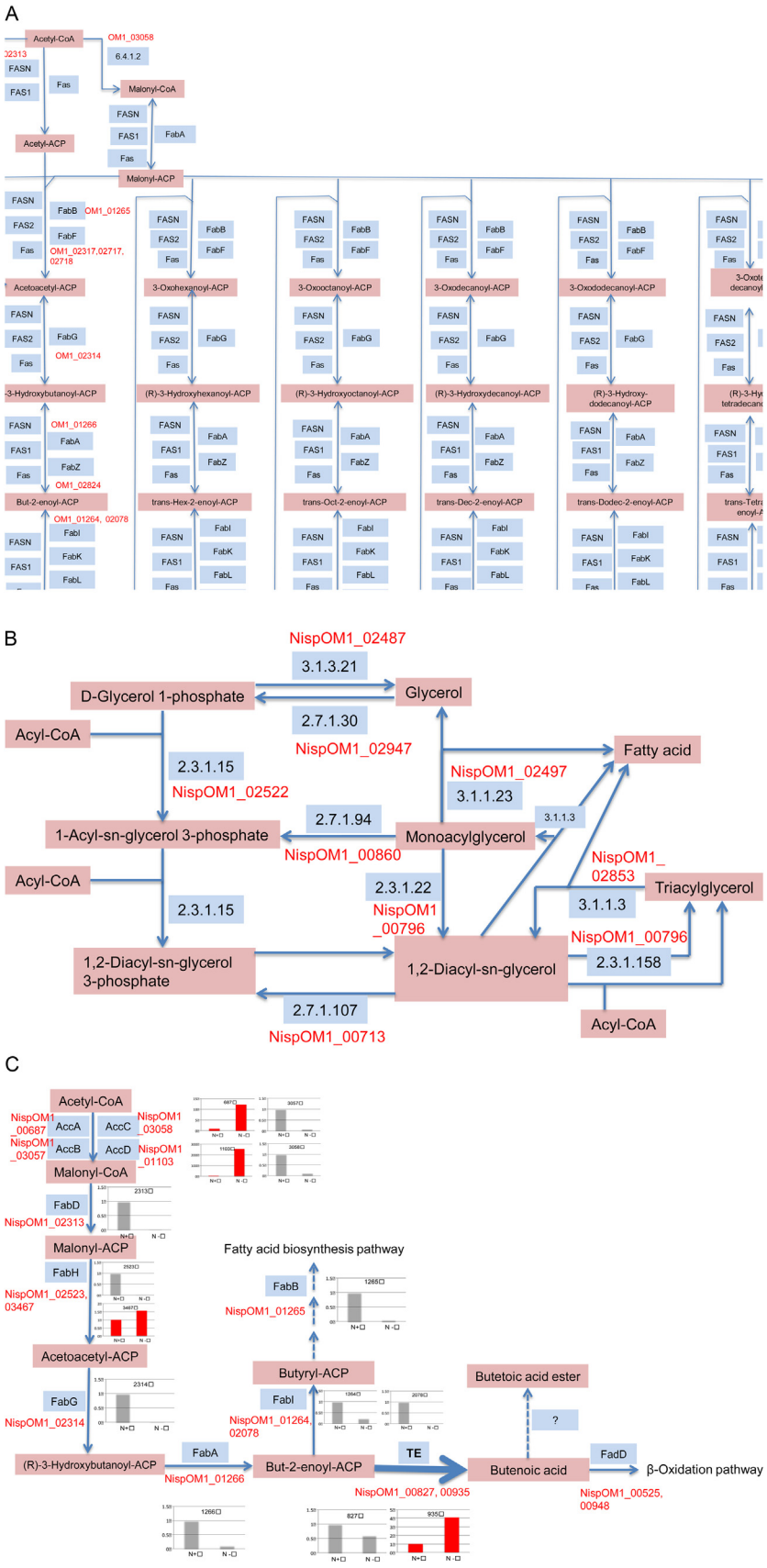

Fig. 3. Predicted metabolic pathways. (A) Fatty acid synthesis, (B) glycerolipid synthesis, and (C) hypothetical biosynthesis pathway for 2-butenoic acid ester. These pathways were drawn on the basis of the KEGG pathways map00061 and map00561 and the schematic pathway proposed by Liu et al. [15]. Pink and light-blue boxes denote compounds and enzymes, respectively. Predicted genes of OM-1 are shown by red letters. Inserted bar graphs showed relative gene expression under the nitrogen-supplemented and nitrogen-depleted conditions. Red and blue bars mean up-regulation under the nitrogen-depleted condition and down-regulation under the nitrogen-supplemented condition, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
List of gene sets found in lipid synthesis pathways.

	predicted_gene	Gene ID	seed_eggNOG_ortholog	seed_ortholog_evalue	eggNOG annotation
Fatty acid synthesis					
	FASN, FabD	NispOM1_02313	266779.Meso_1769	6E-168	malonyl CoA-acyl carrier protein transacylase
	AccC (EC6.4.1.2)	NispOM1_03058	536019.Mesop_4689	1.5E-278	acetyl-CoA carboxylase, biotin carboxylase
	FabB	NispOM1_01265	887898. HMPREF0551_1597	7.1E-163	3-ketoacyl-ACP synthase
	FabF	NispOM1_02317	266779.Meso_1766	4.7E-244	Catalyzes the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons from malonyl-ACP (By similarity)
	FABF2	NispOM1_02717	266779.Meso_1318	1.6E-230	synthase
	FABF2	NispOM1_02718	266779.Meso_1319	1.5E-190	3-oxoacyl-(acyl carrier protein) synthase
	FabH	NispOM1_02523	266835.mlr8424	1E-183	Catalyzes the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons from malonyl-ACP. Catalyzes the first condensation reaction which initiates fatty acid synthesis and may therefore play a role in governing the total rate of fatty acid production. (By similarity)
	FabH	NispOM1_03467	856793.MICA_879	2.5E-116	synthase
	FabG	NispOM1_02314	311402.Avi_1528	4.2E-144	reductase
	FabA	NispOM1_01266	384765. SIAM614_27467	7.1E-106	Catalyzes the dehydration of (3R)-3-hydroxydecanoyl- ACP to E-(2)-decenoyl-ACP and then its isomerization to Z-(3)- decenoyl-ACP. Can catalyze the dehydratase reaction for beta- hydroxyacyl-ACPs with saturated chain lengths up to 16 0, being most active on intermediate chain length (By similarity)
	FabZ	NispOM1_02824	266779.Meso_1390	4.4E-78	Involved in unsaturated fatty acids biosynthesis. Catalyzes the dehydration of short chain beta-hydroxyacyl-ACPs and long chain saturated and unsaturated beta-hydroxyacyl-ACPs (By similarity)
	FabI	NispOM1_01264	266779.Meso_3937	2.5E-158	Enoyl- acyl-carrier-protein reductase NADH
	FabI	NispOM1_02078	266779.Meso_0727	1.4E-162	Enoyl- acyl-carrier-protein reductase NADH
	DesA	NispOM1_02462	287752.SI859A1_03518	4.3E-180	Fatty Acid Desaturase
Triacylglycerol synthesis					
	PlsX (EC2.3.1.15)	NispOM1_02522	266779.Meso_1145	3.3E-198	Catalyzes the reversible formation of acyl-phosphate (acyl-PO(4)) from acyl-acyl-carrier-protein (acyl-ACP). This enzyme utilizes acyl-ACP as fatty acyl donor, but not acyl-CoA (By similarity)
	EC2.3.1.22	NispOM1_00796	266779.Meso_3211	1.3E-151	Phospholipid glycerol acyltransferase
	EC2.3.1.158	NispOM1_00796	266779.Meso_3211	1.3E-151	Phospholipid glycerol acyltransferase
	EC2.7.1.30	NispOM1_02497	266835.mll3568	1.1E-49	Esterase (Lipase)-like protein
	Pgk (EC2.7.1.94)	NispOM1_00860	266779.Meso_3437	3.3E-215	Phosphoglycerate kinase
	EC2.7.1.107	NispOM1_00713	266779.Meso_3102	1.8E-103	Diacylglycerol kinase
	EC3.1.1.3	NispOM1_02853	266779.Meso_1627	1.7E-97	Lipolytic protein, GDSL
	GppA (EC3.1.1.21)	NispOM1_02487	266779.Meso_1120	7.1E-255	ppx gppa phosphatase
	GlpK (EC3.1.1.23)	NispOM1_02947	266835.mll0700	8.5E-265	Key enzyme in the regulation of glycerol uptake and metabolism (By similarity)
Butenoic acid synthesis					
	AccA	NispOM1_00687	266779.Meso_3061	2.6E-179	Component of the acetyl coenzyme A carboxylase (ACC) complex. First, biotin carboxylase catalyzes the carboxylation of biotin on its carrier protein (BCCP) and then the CO(2) group is transferred by the carboxyltransferase to acetyl-CoA to form malonyl-CoA (By similarity)
	AccB	NispOM1_03057	765698.Mesci_3558	7.9E-63	Acetyl-CoA carboxylase, biotin carboxyl carrier protein
*	AccC	NispOM1_03058	536019.Mesop_4689	1.5E-278	acetyl-CoA carboxylase, biotin carboxylase
	AccD	NispOM1_01103	266779.Meso_0663	7.5E-170	Component of the acetyl coenzyme A carboxylase (ACC) complex. Biotin carboxylase (BC) catalyzes the carboxylation of biotin on its carrier protein (BCCP) and then the CO(2) group is transferred by the transcarboxylase to acetyl-CoA to form malonyl- CoA (By similarity)
*	FabD	NispOM1_02313	266779.Meso_1769	6E-168	malonyl CoA-acyl carrier protein transacylase
*	FabH	NispOM1_02523	266835.mlr8424	1E-183	Catalyzes the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons from malonyl-ACP. Catalyzes the first condensation reaction which initiates fatty acid synthesis and may therefore play a role in governing the total rate of fatty acid production. Possesses both acetoacetyl-ACP synthase and acetyl transacylase activities. Its substrate specificity determines the biosynthesis of branched- chain and or straight-chain of fatty acids (By similarity)
*	FabH	NispOM1_03467	856793.MICA_879	2.5E-116	synthase
*	FabG	NispOM1_02314	311402.Avi_1528	4.2E-144	reductase
*	FabA	NispOM1_01266	384765. SIAM614_27467	7.1E-106	Catalyzes the dehydration of (3R)-3-hydroxydecanoyl- ACP to E-(2)-decenoyl-ACP and then its isomerization to Z-(3)- decenoyl-ACP. Can catalyze the dehydratase reaction for beta- hydroxyacyl-ACPs with saturated chain lengths up to 16 0, being most active on intermediate chain length (By similarity)
	TesB	NispOM1_00827	266779.Meso_3238	6.8E-154	acyl-CoA thioesterase
	TE	NispOM1_00935	216596.RL4529	4.7E-80	acyl-CoA thioesterase i

* Genes were listed in both Fig. 3A and C.

Table 4
Comparison of the number of thioesterase Nitratireductor sp. OM-1with other genus Nitratireductor.

Number thioesterase	OM-1	EBB	NL21	RA22	RR328	UMTGB	C115	CGMCC	PHT3B	ZZ-1	ES061
	12	10	10	12	5	9	9	9	10	9	10
Reference strains:											
EBB	*N. aquibiodomus* EBB										
NL21	*N. aquibiodomus* NL21										
RA22	*N. aquibiodomus* RA22										
RR328	*N. basaltis* RR3-28										
UMTGB	*N. basaltis* UMTGB225										
C115	*N. indicus* C115										
CGMCC	*N. indicus* CGMCC										
PHT3B	*N. pacificus* pht-3B										
ZZ1	*N.* soli ZZ-1										
ES061	*Nitratireductor* sp ES061										

Table 5
Gene expression involved in lipid synthesis under the nitrogen-sufficient and nitrogen-depleted conditions.

Gene ID	N (+)			N (-)			ratio
	ΔCt	ΔΔCt	expression ratio	ΔCt	ΔΔCt	expression ratio	N (-)/N (+)
1264	32.70 ± 0.00	17.19 ± 0.60	6.68797E-06	28.83 ± 0.37	19.29 ± 0.42	1.56544E-06	0.23
1265	26.64 ± 0.23	11.13 ± 0.83	0.000446207	24.66 ± 0.17	15.12 ± 0.22	2.81794E-05	0.06
1266	33.98 ± 0.09	18.47 ± 0.69	2.75408E-06	31.12 ± 0.08	21.58 ± 0.13	3.20094E-07	0.12
2078	43.17 ± 1.71	27.66 ± 2.31	4.73168E-09	43.56 ± 0.10	34.02 ± 0.15	5.76056E-11	0.01
2313	30.35 ± 0.08	14.84 ± 0.68	3.42153E-05	30.02 ± 0.22	20.48 ± 0.27	6.83763E-07	0.02
2314	46.77 ± 0.42	31.26 ± 1.02	3.88868E-10	46.92 ± 0.27	37.38 ± 0.32	5.61053E-12	0.01
2317	36.81 ± 0.05	21.30 ± 0.65	3.87312E-07	35.17 ± 0.86	25.63 ± 0.91	1.92576E-08	0.05
2462	41.16 ± 1.18	25.65 ± 1.78	1.90584E-08	42.08 ± 0.15	32.54 ± 0.20	1.6069E-10	0.01
2523	35.94 ± 0.54	20.43 ± 1.14	7.07876E-07	36.82 ± 0.09	27.28 ± 0.14	6.13623E-09	0.01
2717	35.74 ± 0.27	20.23 ± 0.87	8.13136E-07	34.88 ± 0.13	25.34 ± 0.18	2.36268E-08	0.03
2718	33.42 ± 013	17.91 ± 0.73	4.06025E-06	34.22 ± 0.18	24.68 ± 0.23	3.73323E-08	0.01
16S(control)	15.51 ± 0.60	0	1	9.54 ± 0.05	0	1	1.00
2824	31.65 ± 0.76	15.22 ± 2.28	2.62013E-05	33.58 ± 0.12	19.10 ± 0.61	1.77962E-06	0.07
3058	29.36 ± 0.16	12.94 ± 1.68	0.000127696	30.46 ± 0.03	15.98 ± 0.52	1.55255E-05	0.12
3467	30.82 ± 0.24	14.39 ± 1.77	4.67394E-05	28.22 ± 0.39	13.74 ± 0.88	7.3342E-05	1.57
2522	35.14 ± 0.66	18.71 ± 2.18	2.332E-06	35.06 ± 0.61	20.58 ± 1.10	6.37974E-07	0.27
2853	29.87 ± 0.23	13.44 ± 1.75	9.02946E-05	32.25 ± 0.03	17.77 ± 0.52	4.47401E-06	0.05
2947	44.03 ± 1.18	27.60 ± 2.7	4.93262E-09	44.63 ± 0.41	30.15 ± 0.90	8.39355E-10	0.17
687	47.54 ± 0.61	31.11 ± 2.13	4.32974E-10	41.97 ± 0.60	27.49 ± 1.09	5.32342E-09	12.30
827	34.74 ± 1.25	18.31 ± 2.13	3.08778E-06	33.50 ± 1.27	19.02 ± 1.76	1.88109E-06	0.61
935	39.11 ± 0.86	22.68 ± 2.39	1.49329E-07	35.13 ± 0.71	20.65 ± 1.20	6.07758E-07	4.07
1103	43.69 ± 0.04	27.26 ± 1.56	6.22189E-09	33.75 ± 0.23	19.27 ± 0.72	1.5818E-06	254.23
3057	36.00 ± 0.66	19.57 ± 2.19	1.28928E-06	37.45 ± 0.52	22.97 ± 1.01	1.21714E-07	0.09
16S(control)	16.43 ± 1.52	0	1	14.48 ± 0.49	0	1	1.00

Real-time PCR was performed in duplicate tubes.
expression ratio = 2^-ΔΔCt

accA and *accD*. Two different genes encoding beta-ketoacyl-ACP synthase III (*fabH*) were identified in the genome, however NispOM1_03467 was up-regulated but NispOM1_02523 was not. Malonyl-CoA-ACP transacylase (*fabD*) encoding gene is present in the same operon with β-ketoacyl-ACP reductase (*fabG*). Two different enoyl-ACP reductase (*fabI*) genes were also found. One of the two *fabI* genes is probably encoded in the same operon with *fabA* and *fabB*. These genes were down-regulated under the nitrogen-depleted conditions and consistent with free fatty acid synthesis under at 10 mM of ammonium chloride. For butenoic acid synthesis, NispOM1_00935 which is the most probable candidate showing homology with bTE (AAO77182) utilized in the report by Liu et al [15], was up-regulated by nitrogen-depleted conditions. Relative gene expression of each gene for butenoic acid synthesis between nitrogen-sufficient and nitrogen-depleted conditions was inserted in Fig. 3C. It was confirmed that some of the genes were inducible by nitrogen depletion. This result suggests that further transcriptome analysis between lipid-producing and lipid-non-producing cells could illuminate another up-regulated genes involved in butenoic acid synthesis such as ester synthase.

3.5. Accession number

The 4.8 Mb complete genome of *Nitratireductor* sp. strain OM-1 has been deposited at GenBank/ENA/DDBJ under the accession number SAMN09011615.

Authors' contributions

Y.O and I.A conceived this study and designed the experiments. S.Y, S.N, K.W and H.T performed microbiological and biochemical experiments. L.T and S.C performed genome annotation and analysis. T.A., Y.M. and Y.N. interpreted the data. Y.O and L.T wrote the manuscript and all authors read and approved the final manuscript.

Competing interests

Hiroshima University has filed a patent application related to the technology using strain OM-1 to the Japan Patent Office. Y.O., T.A., T.M. and Y.N. are listed as inventors of the patent. No one receive personal

or institutional revenue associated with the patent application. The authors declare that they have no competing interests.

Declaration of Competing Interest

There are no conflicts of interest to declare.

All authors have approved the manuscript and agree with submission to *Biotechnology Reports*.

This manuscript has not been published elsewhere and is not under consideration by another journal.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00366.

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