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Degradation of 1,4-Dioxane by Xanthobacter sp. YN2

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Abstract

1,4-Dioxane is a highly toxic and carcinogenic pollutant found worldwide in groundwater and soil environments. Several microorganisms have been isolated by their ability to grow on 1,4-dioxane; however, low 1,4-dioxane tolerance and slow degradation kinetics remain obstacles for their use in 1,4-dioxane bioremediation. We report here the isolation and characterization of a new strain, *Xanthobacter* sp. YN2, capable of highly efficient 1,4-dioxane degradation. High degradation efficiency and high tolerance to 1,4-dioxane make this new strain an ideal candidate for the biodegradation of 1,4-dioxane in various treatment facilities. The maximum degradation rate of 1,4-dioxane was found to be 1.10 mg-1,4-dioxane/h mg-protein. Furthermore, *Xanthobacter* sp. YN2 was shown to grow in the presence of higher than 3000 mg/L 1,4-dioxane with little to no degradation inhibition. In addition, *Xanthobacter* sp. YN2 could grow on and degrade 1,4-dioxane at pH ranges 5 to 8 and temperatures between 20 and 40 °C. *Xanthobacter* sp. YN2 was also found to be able to grow on a variety of other substrates including several analogs of 1,4-dioxane. Genome sequence analyses revealed the presence of two soluble di-iron monooxygenase (SDIMO) gene clusters, and regulation studies determined that all of the genes in these two clusters were upregulated in the presence of 1,4-dioxane. This study provides insights into the bacterial stress response and the highly efficient biodegradation of 1,4-dioxane as well as the identification of a novel Group-2 SDIMO.

Introduction

1,4-Dioxane has been listed as a Group 2B carcinogen since 1999 by the International Agency for Research on Cancer (IARC). 1,4-Dioxane is highly water soluble while its volatilization from water occurs slowly [1, 2]. This high level of water solubility has led to its wide use as an industrial solvent. 1,4-Dioxane is also an unwanted byproduct in the industrial manufacture of food, medicine, and personal care products [3]. High-industrial use has led to 1,4-dioxane pollution of many aquatic environments [4]. High solubility and low evaporation rates complicate remediation efforts to remove 1,4-dioxane from these environments, thereby increasing the threat to public safety [2, 5–9]. One of the main sources of 1,4-dioxane entering the environment is via industrial sewage. Wastewater treatment plants (WWTPs) employing the use of biodegradation processes would be a very desirable and cost-effective method to deal with 1,4-dioxane contamination [10]. A major drawback in the use of bioremediation, in this instance, is the lack of highly efficient 1,4-dioxane microbial degraders being suitable for application.

Kinetic studies of the isolates have demonstrated that they may not be ideal for environmental applications. The total cell yields of Gram-positive 1,4-dioxane degraders tended to be quite low when compared to Gram negatives, thereby leading to lower rates of 1,4-dioxane degradation. To date, several microorganisms capable of growing on 1,4-dioxane have been reported, yet the number of Gram-negative isolates is quite small [11–16]. To accelerate growth rates and increase cell yields, additional nutrients or analogs of 1,4-dioxane are necessities in bioremediation applications using these strains [16–19]. These additives have the undesirable effect of increasing costs and the possibility of introducing additional environmental pollution if they are not completely mineralized [16, 17, 20]. Therefore, investigations into the isolation and characterization of 1,4-dioxane degraders with higher growth rates and cell yields are critical to efficient and safe biodegradation of 1,4-dioxane.

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Soluble di-iron monooxygenase (SDIMO) has been shown to be the key genes to the degradation of 1,4-dioxane [21]. Enrichment of SDIMO genes at 1,4-dioxane-contaminated sites has been shown to be directly related to bacterial 1,4-dioxane degradation activity [22, 23]. To date, only two SDIMOs have been confirmed to be involved in metabolic degradation of 1,4-dioxane: Group-5 SDIMO gene cluster *thmADBC* [24] and Group-6 SDIMO gene cluster *prmABCD* [25, 26]. Thus, to determine the importance of SDIMOs in 1,4-dioxane, biodegradation requires further research.

In this study, *Xanthobacter* sp. YN2 was isolated from sludge of a municipal WWTP for its ability to grow on 1,4-dioxane. Bacterial characteristics and kinetics of degradation and growth in the presence of various concentrations of 1,4-dioxane as well as under various culture conditions were investigated. Genomic analysis revealed a gene cluster on the chromosome that was predicted to encode a novel Group-2 SDIMO, which was phylogenetically distant from other previously reported SDIMOs involved in 1,4-dioxane degradation. Regulation studies showed that all the genes in this new SDIMO gene cluster were constitutive.

Materials and Methods

Reagents and Culture Media

1,4-Dioxane, 1,3-dioxane, tetrahydrofuran, ethanol, n-hexane, cyclohexane, toluene, ethyl acetate, and methanol were of analytical grade (J&K Scientific Ltd.). Basal salts medium (BSM) and ammonium mineral salts medium (AMS) were prepared by the method of Parales et al. [18]. For solid media, 1.8% (wt/vol) Nobel agar was added. All experiments that involved culture media supplemented with 1,4-dioxane were carried out in headspace bottles sealed with Teflon to limit volatilization.

Analysis of 1,4-Dioxane and Total Oxidizable Carbon (TOC)

Cultures were filtered through nylon filters (0.45 μm pore size), and 1 μL of each sample was analyzed with an Agilent 7890 gas chromatograph (GC) equipped with an INNOWH4 column (30 m × 0.53 mm × 1.0 μm) and a flame ionization detector (FID). The initial column temperature was 45 °C, which was maintained for 3 min, then raised to 70 °C at a rate of 15 °C /min. The temperatures of the inlet and the detector were 250 °C and 300 °C, respectively. The carrier gas was hydrogen/air (400/40), and the flow rate was 6 mL/min. Total oxidizable carbon (TOC) of filtered samples was analyzed with an Analytikjena Multi N/S 2100S TOC analyzer.

Enrichment, Isolation, and Identification of Xanthobacter sp. YN2

The enrichment was started with active sludge from a secondary sedimentation basin of a WWTP in Harbin, China. Sludge (5 g) was added to 100 mL BSM containing 200 mg/L 1,4-dioxane and incubated at 30 °C. Dilutions were carried out weekly by transferring 20% of each culture into fresh BSM with 200 mg/L 1,4-dioxane until 1,4-dioxane degradation was observed (approximately 3 months). Once the 1,4-dioxane was depleted, dilutions into fresh AMS containing 200 mg/L 1,4-dioxane were performed, approximately every few days. When the rate of 1,4-dioxane degradation stabilized, the culture was diluted and spread onto AMS plates containing 200 mg/L 1,4-dioxane to isolate 1,4-dioxane degrading single colonies.

The isolate was identified by 16S rRNA gene sequence analysis. The sequence of the 16S rRNA gene was determined by Sangon Biotech Co., Ltd. (Shanghai, China). Bacteria genome was extracted using Ezup column bacteria genomic DNA purification kit (SK8255) and used as the template for PCR. Primer 7F (5′-CAGAGTTTG ATCCTGGCT-3′) and primer 1540R (5′-AGGAGGTGA TCCAGCCGCA-3′) were used for sequencing. The thermal profile was as follow: initial denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 45 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. The 16 S rRNA gene sequence was deposited in GenBank under accession number MK256301.

Determination of Optimal Growth Conditions

Four different factors involved in growth and degradation of 1,4-dioxane were tested: temperature, pH, aeration, and initial OD_{660} (optical density at 660 nm). All experiments were carried out in 50 mL Teflon sealed vials filled with 10 mL of AMS containing 200 mg/L 1,4-dioxane. Experiments were carried out in duplicate and repeated three times.

Unless otherwise stated, the following experiments were carried out at 30 °C, pH 7.0, with initial OD660 = 0.007 and shaken at 160 rpm. To determine the optimum growth temperature, cultures were incubated at 10, 20, 30, or 40 °C. The optimum pH for growth was determined by growth as described above except that the pH of the media was adjusted to 5.0, 6.0, 7.0, or 8.0 with 1 M HCl or 1 M NaOH if necessary before inoculation, and the pH of each culture was checked at the end of growth. Optimum aeration was determined by growing cultures as above with

varying shaking speeds (120, 140, 160, or 180 rpm). To determine the optimum initial OD_{660} for degradation and growth, cultures were grown as above but started with an initial OD_{660} of 0.001, 0.004, 0.007, or 0.011.

1,4-Dioxane Tolerance

In order to examine tolerance levels for 1,4-dioxane, *Xanthobacter* sp. YN2 was cultivated in AMS containing 1000 mg/L 1,4-dioxane until mid-exponential phase. This culture was then used to inoculate fresh AMS containing various 1,4-dioxane concentrations (3, 5, 8, 10, 20, 30, 40, 50, and 100 g/L) and grown under the optimum conditions determined above. Three replicate cultures were grown for each concentration and uninoculated vials served as negative controls. After 14 days, viability was determined by plating onto AMS plates containing 1,000 mg/L 1,4-dioxane.

Growth on and Degradation of 1,4-Dioxane

Cultures were carried out in duplicate 50 mL headspace vials, filled with 10 mL of AMS containing 1,4-dioxane under optimum growth conditions. In addition, three sets of controls were performed. To determine the effect of volatilization, culture vials with no added bacteria were used. To test for bacterial growth on possible contaminants, cultures with no carbon source were used. To eliminate the effect of bacterial absorption of 1,4-dioxane, autoclaved bacteria ($OD_{660} \approx 0.3$) were also used as controls. All of the growth cultures and controls were set up in duplicate and repeated three times. OD_{660}, 1,4-dioxane concentration and TOC were monitored during incubation.

Preparation of Resting Cells

Cells were cultivated in AMS with 1000 mg/L 1,4-dioxane and harvested by centrifugation when cell growth reached mid-exponential phase. Harvested cells were washed twice with 0.02 M phosphate buffer (PBS, pH 7) and then resuspended in 0.02 M PBS (pH 7) to an $OD_{660} \approx 0.2$.

Utilization Experiments for Various Substrates

Resting cells (0.5 mL) of *Xanthobacter* sp. YN2 were inoculated into 20 mL AMS and grown as above in the presence of 200 mg/L 1,4–dioxane, 1,3-dioxane, tetrahydrofuran, ethanol, n-hexane, cyclohexane, toluene, ethyl acetate, or methanol. Utilization of substrates was determined by growth (measurement of OD_{660}). Generation times were determined from the slope of OD_{660} plotted against time (semi-log) during exponential phase. All experiments were carried out in duplicate. Growth on a variety of other carbon sources was investigated using GEN3 microplates (Biolog) and analyzed using Biolog's Microbial Identification System software.

Total DNA Extraction and Genome Sequencing

Xanthobacter sp. YN2 was grown as above on 1,4-dioxane. Cells were harvested by centrifugation and total DNA was extracted using the DNA extraction kit for bacteria (Shanghai Lifefeng Biotechnology Co., Ltd). The *Xanthobacter* sp. YN2 genome was sequenced by Woosen Biotechnology (China) on the PacBio platform. A de novo assembly was generated using Canu 1.8 [27]. The resulting contigs were then submitted to Genbank under accession number CP063362-CP063366 and annotated using RAST 2.0 [28].

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from cultures grown at 30 °C on liquid AMS with 5 mM succinate or 1000 mg/L 1,4-dioxane as the exclusive carbon source.

RNA was extracted using the Maxigen HiPure Total RNA Mini Kit according to the manufacturer's instructions (including the optional DNase treatment). Reverse transcription was performed with FastQuant RT Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions.

Quantitative real-time reverse transcriptase-PCR (RT-qPCR) analyses were performed by amplification of the cDNA samples from above using the Bestar SybrGreen qPCR Mastermix, according to the manufacturer's instructions. Primer sequences are listed in Table S1. Thermocycling conditions were as follows: 2 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 1 min at 60 °C, followed by melting curve analysis. Expression of the 16S rRNA gene was used as the reference gene to normalize tested genes. The ΔΔCt method with the 16S rRNA gene as the reference was used to determine relative abundance of target transcripts.

Accession Numbers

The 16S rRNA gene sequence has been deposited in GenBank under the accession number of MK256301. The genome sequence has been submitted to GenBank under accession numbers CP063362-CP063366. The strain, *Xanthobacter* sp. YN2, has been deposited in the China General Microbiological Culture Collection Center (CGMCC) under the number CGMCC No. 14610. The strain is also being deposited at the American Type Culture Collection (ATCC), but a culture number is not available at this time.

Table 1 Growth rates for YN2 on various substrates

Substrate	Generation time (h)	Standard deviation
1,4-Dioxane	13.67	0.61
1,3-Dioxane	20.64	1.47
Tetrahydrofuran	11.15	1.53
Ethanol	11.44	0.80
Methanol	8.49	0.35
Ethyl acetate	5.08	1.41

Results

Evaluation of 1,4-Dioxane-Degrading Strain

Using standard enrichment techniques, we were able to obtain a single isolate capable of growing on 1,4-dioxane as sole carbon source. No growth or degradation occurred in autoclaved controls or carbon-free controls, and no decrease of 1,4-dioxane or increase in OD_{660} occurred in abiotic controls (data not shown). Blast result based on the 16S rRNA gene showed that the isolate is most related to *Xanthobacter* sp., and as such was labeled *Xanthobacter* sp. YN2. Experiments were carried out to determine optimum growth conditions for *Xanthobacter* sp. YN2 (Fig. 1). The optimum growth conditions were determined to be 30 °C, pH 7.0, and a shaking speed of 180 rpm. An initial OD_{660} of approximately 0.007 was shown to result in the shortest lag phase (Fig. 1). The growth of YN2 on 1,4-dioxane as the sole carbon source under the optimum conditions is as demonstrated in Fig. 2.

Substrate Range of YN2

YN2 demonstrated the ability to grow on many substrates. Generation times of YN2 grown on different substrates are shown in Table 1 to display growth rates. YN2 grew on analogs of 1,4-dioxane, including 1,4-dioxene, 1,3-dioxane, and tetrahydrofuran and also on ethanol, methanol, and ethyl acetate. However, YN2 was unable to grow on 1,4-dioxanediol, n-hexane, cyclohexane, or toluene. The absence of a lag phase in both growth and degradation of 1,4-dioxane after growth on non-inducing substrates such as succinate, pyruvate, acetate, and citrate indicated that enzymes involved in degradation of 1,4-dioxane are constitutive.

Growth Kinetics

YN2 was capable of growth in the presence of more than 3000 mg/L of 1,4-dioxane and remained viable even when the concentration approached 50 g/L; however, growth rates at these higher concentrations were much slower (data not shown).

Growth kinetics of YN2 on 1,4-dioxane were determined. Cells of YN2 were cultured with 1000 mg/L 1,4-dioxane until mid-exponential phase. This culture was then used to inoculate fresh AMS supplemented with different concentrations of 1,4-dioxane and cultivated under optimum growth conditions. The OD_{660} of the cultures were monitored every 4 h. The growth kinetics of YN2 were well described by Monod kinetics as seen in Fig. 3a. The maximum specific growth rate (μ_{max}) was calculated to be 0.025/h

with the highest cell yield determined as 0.27 mg-protein/mg-1,4-dioxane.

No growth inhibition was seen when YN2 was grown with high 1,4-dioxane concentrations up to 1000 mg/L of 1,4-dioxane. When grown at these high 1,4-dioxane levels, the specific growth rate continued to increase with the substrate concentration; however, when the concentration of 1,4-dioxane was above 1000 mg/L, the specific growth rate of YN2 continued to increase but at a reduced level.

Degradation Kinetics

The relationship between the degradation rate and the concentration of 1,4-dioxane was studied using resting cells. As expected, no increase in biomass was observed throughout these experiments. In order to compare with other reported 1,4-dioxane degraders, the experiments were performed at concentrations between 100 and 1000 mg/L. The degradation kinetics for YN2 were well described by Monod kinetics. The maximum specific degradation rate (k_{max}) and half-saturation concentration (K_s) were calculated as 1.10 mg-1,4-dioxane/h mg-protein and 410.91 mg/L, respectively (Fig. 3b).

Genome Sequence Analysis

The genome of YN2 was found to have a size of 6,650,818 bp with a 67.95% G + C content spread over 6 contigs. N50 and N90 values were 5,964,455 bp and 312,888 bp, respectively. The genome consisted of 5 replicons, including the chromosome (6.2 Mb), plasmid 1 (circular, 147 kb), plasmid 2 (circular, 144 kb), plasmid 3 (circular, 51 kb), and plasmid 4 (linear, 30 kb). Automated annotation by RAST identified 6,585 protein-coding sequences and 58 RNA genes in the YN2 genome. The genome annotation revealed two SDIMO gene clusters (Fig. 4) that may be involved in 1,4-dioxane metabolism, both located in the chromosome, each encoding six protein components: a monooxygenase α, β and γ subunit, a ferredoxin, a coupling/effector protein, and a reductase. The sequences of the two clusters had extremely high-sequence homology to each other and are located distantly in the chromosome. Blast results showed that the protein sequences of the two gene clusters had high similarity with toluene monooxygenase genes of *Sinirhodobacter hungdaonensis* (84%) [29], *Bradyrhizobium* sp. ORS 375 (84%) [30] and *Pseudooceanicola lipolyticus* (84%) [31]. However, YN2 was not capable of utilizing toluene, but it could grow on tetrahydrofuran. Thus, the YN2 open-reading frames were designated as *thmABCDEF*.

Upregulation of thmABCDEF by 1,4-dioxane

The SDIMO gene clusters were shown to be expressed when cells were grown on either 1,4-dioxane or succinate, indicating that the genes are constitutively expressed. In addition, all six genes were significantly upregulated by 1,4-dioxane when compared to succinate (Fig. 5). Since the sequences of the two gene clusters are highly similar to each other, little to no sequence differences existed between the two gene clusters; therefore, their expression products were not distinguishable at present.

Previous studies have grouped SDIMOs into six groups based on sequence similarity and substrate range [32–34]. Sequence comparisons based on pairwise identity place the SDIMOs of YN2 into Group-2. According to the phylogenetic tree of amino acid sequences of α subunits of reported SDIMOs (Fig. 4), ThmA of YN2 is distant from PrmA of *Mycobacterium sp*. PH-06 and ThmA of *Pseudonocardia dioxanivorans* CB1190. Among SDIMOs of Group-2, ThmABCDEF is the first that appears to be involved in the metabolism of 1,4-dioxane.

Discussion

The 1,4-dioxane-specific degradation rate of YN2 (1.1 mg-1,4-dioxane/h mg-protein) is the highest of all Gram-negative 1,4-dioxane-degrading bacteria reported to date. It is only second to *P. dioxanivorans* CB1190 (0.92–1.98 mg-1,4-dioxane/h mg-protein), the highest of Gram-positive 1,4-dioxane degraders so far reported (Table 2). However, the half-saturation constant of YN2 is also very high, suggesting that the strain prefers higher concentrations of 1,4-dioxane and may not perform well at low dioxane concentrations. At a 1,4-dioxane concentration of 100 mg/L, there are 4 degraders showing higher specific degradation rates than YN2; with 1,4-dioxane concentrations higher than 100 mg/L, only CB1190 and PH-06 possess higher specific degradation rates than YN2 (Fig. S1). Nonetheless, the actual performance of CB1190 and PH-06 is severely hindered by their low cell yield [18, 25, 35]. Comparatively, the growth rate of YN2 (as measured by cell yield) is relatively high (Table 2). Cell yield may play a major factor in the biotreatment of pollutants [12]. Utilization of 1,4-dioxane is generally concomitant with growth of degraders [36, 37]. Typically, Gram-negative organisms are easier to grow and have higher cell yields than those of Gram-positive organisms as shown in Table 2. Among all reported 1,4-dioxane degrading isolates, Gram-positive CB1190 has the highest k_{max} of 0.92–1.98 mg-1,4-dioxane/h mg-protein [18, 38]. However, the cell yield of CB1190 is relatively low (0.09 mg-protein/mg-1,4-dioxane) [38]. To achieve

higher cell yield, additional substrates are often required to enhance the growth of degraders, which increases the cost of bioremediation efforts [15]. *Acinetobacter baumannii* DD1 is reported to have a high cell yield, but its degradation performance is relatively poor (11 days to degrade concentrations of 1000 mg/L 1,4-dioxane starting with low biomass) [13]. To date, YN2 is the only 1,4-dioxane-degrading isolate with both a high cell yield and a high 1,4-dioxane degradation rate, demonstrating that YN2 is a good choice for 1,4-dioxane bioremediation.

YN2 was shown to have the unique ability to maintain its degradation rate at extremely high concentration levels of 1,4-dioxane. For example, YN2 was shown to completely degrade 1000 mg/L 1,4-dioxane in 40 h, whereas in similar experiments, *Pseudonocardia* sp. N23 takes 108 h and *Mycobacterium* sp. PH-06 takes 15 days to achieve the same result (which also started with 1000 mg/L 1,4-dioxane and relatively low biomass) [35, 39]. This is unique among previously reported 1,4-dioxane degrading isolates. The specific growth rate of YN2 was positively correlated to 1,4-dioxane concentrations up to 3000 mg/L, which is already much higher than the tolerance of most reported degraders (Table 2). Since transformation products of 1,4-dioxane may

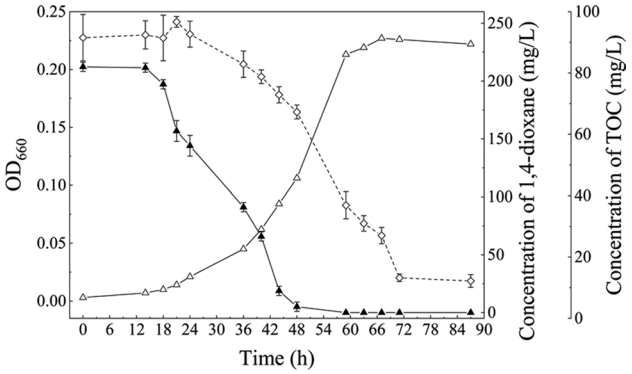

Fig. 2 Removal of 1,4-dioxane during growth of YN2. Open triangles represent OD_{660}; solid triangles represent 1,4-dioxane concentration; open diamonds represent total organic carbon concentration. Created using ORIGIN 2018. The error bars represent the range of triplicates

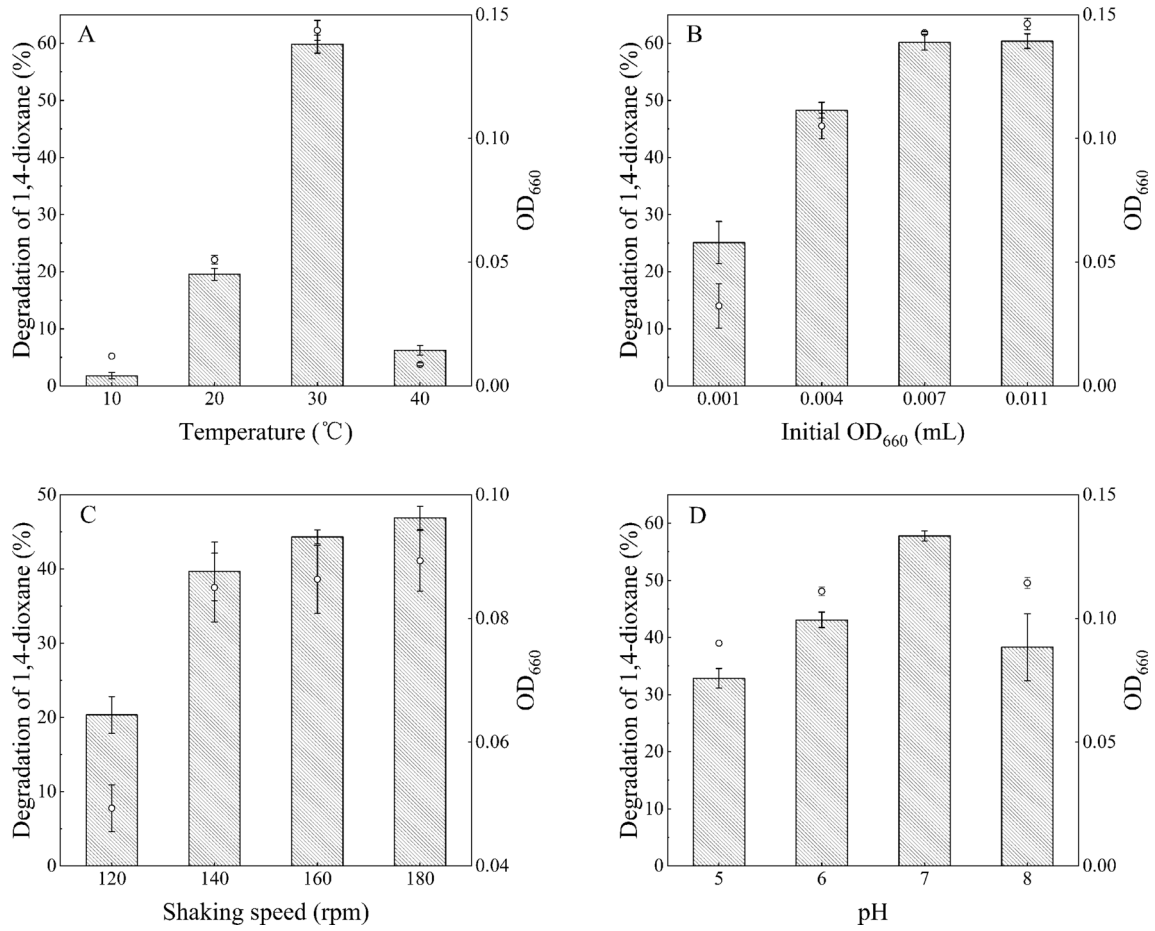

Fig. 1 Effects of incubating temperature (**a**), inoculation amount (**b**), rotation speed of shaking (**c**), and pH (**d**) on growth and degradation of YN2 with 1,4-dioxane as sole carbon source. Columns represent degradation ratio of 1,4-dioxane; open circles represent OD_{660}. Created using ORIGIN 2018. The error bars represent the range of triplicates

Table 2 Kinetic parameters of 1,4-dioxane biodegradation by degraders

		Cell yield (mg-protein/mg-1,4-dioxane)	k_{max} (mg-1,4-dioxane/h mg-protein)	K_s (mg/L)	Tolerance (mg/L)	Degradation rate description
Gram-negative degraders	YN2	0.27	1.10	410.91	≥50,000	1000 mg/L in about 40 h starting with very low biomass
	Xanthobacter flavus DT8 [14]	0.35	0.43	17.5	≥1200	100 mg/L in 48 h starting with very low biomass
	Afipia sp. D1 [12]	0.185	0.263	25.8	≥1000	450 mg/L in about 150 h starting with very low biomass
	Acinetobacter baumannii DD1 [13]	0.414	–	–	1000	100 mg/L in 42 h, 1000 mg/L in 11 days starting with very low biomass
	Flavobacterium sp. [16]	–[a]	–	–	–	100 mg/L in about 16 days (with the existence of 100 mg/L tetrahydrofuran, unknown initial biomass)
	Rhodanobacter sp. AYS5 [15]	Very low	–	–	–	95% of 1000 mg/L in 10 days starting with biomass of a protein content approaching 30 μg/L
	Mean value (YN2 not included)	0.32	0.35	21.65		

Table 2 (continued)

			Cell yield (mg-protein/mg-1,4-dioxane)	k_{max} (mg-1,4-dioxane/h mg-protein)	K_s (mg/L)	Tolerance (mg/L)	Degradation rate description
Gram-positive degraders	*Pseudonocardia* spp.	*Pseudonocardia dioxanivorans* CB1190 [18, 38]	0.02–0.09	0.92–1.98	159 ± 44	>1000	61.6 mg/L in about 75 h starting with very low biomass
		Pseudonocardia sp. BERK-1 [53]	–	–	–	1000	–
		Pseudonocardia sp. N23 [39]	0.323	0.23	79.9	≥1000	1000 mg/L to 5.1 mg/L in 108 h starting with biomass of a dry cell weight concentration approaching 300 mg/L
		Pseudonocardia sp. D17 [12]	0.223	0.096	59.7	–	5.0 mg-1,4-dioxane/h·L at maximum
	Mycobacterium spp.	*Mycobacterium sp.* PH-06 [25, 35]	0.16	–	78 ± 10	1000	1000 mg/L in 15 days starting with very low biomass
		Mycobacterium sp. D6 [12]	0.185	0.139	20.6	–	8.5 mg-1,4-dioxane/h·L at maximum
		Mycobacterium sp. D11 [12]	0.179	0.052	69.8	–	4.5 mg-1,4-dioxane/h·L at maximum
	Rhodococcus spp.	*Rhodococcus* sp. 219 [54]	–	–	–	–	880 mg/L in about 120 h with initial OD_{578} of culture a little higher than 4
		Rhodococcus sp. JCM 14,343 [55]	0.031 (with 1,117 mg/L 1,4-dioxane)	0.0073	56.9	>1100 mg/L	90 mg/L in 124 h, 74–92.2% of 1100 mg/L in 34 days, initial biomass unknown
		Mean value	0.17	0.42	82.03		
Fungus		*Cordyceps sinensis* [37]	–	–	–	7929 mg/L	90% of 2996 mg/L in about 3 days starting with very low biomass
Mean value			0.25	0.39	51.84		

[a] "-" not reported

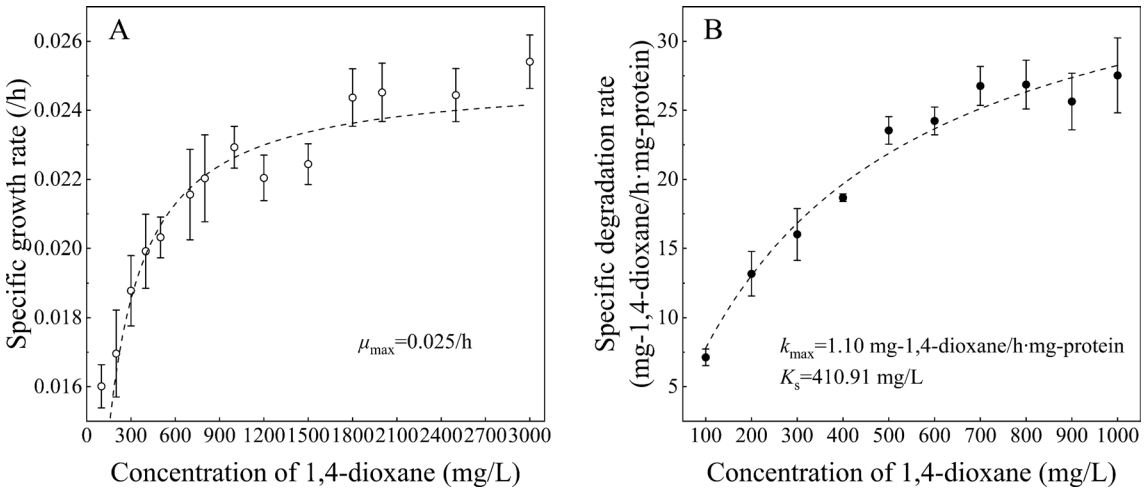

Fig. 3 Growth kinetics (**a**) and degradation kinetics (**b**) of YN2 on 1,4-dioxane. Open circles represent specific growth rates; solid circles represent specific degradation rates; the dotted line represents nonlinear curve fitting result of specific growth rates (**a**) or specific degradation rates (**b**) at each concentration using Monod equation by minimizing the least absolute residuals. Created using ORIGIN 2018. The error bars represent the range of triplicates

be toxic to the monooxygenase enzyme and/or the cells [38], this indicates that YN2 may produce non-toxic intermediates or not accumulate them at high enough levels to be toxic. The mechanisms underlying the unique high tolerance of YN2 to 1,4-dioxane is unknown at this time and will require further research. Possessing an extremely high tolerance to high levels of 1,4-dioxane makes YN2 an appropriate choice for field applications in bioremediation of large scale industrial spills.

Genomic analysis of YN2 indicated it belongs to the genus *Xanthobacter*. Another 1,4-dioxane degrading *Xanthobacter*, *X. flavus* DT8, was also isolated in China [36]. Both were isolated from wastewater treatment plants located about 1,700 km away from each other, suggesting that *Xanthobacter* may be an important 1,4-dioxane degrading genus present in wastewater treatment plants. Interestingly, although both strains belong to the *Xanthobacter* genus, YN2 could grow at extremely high 1,4-dioxane levels with no apparent decrease in growth rate, whereas the growth rate of *X. flavus* DT8 decreases significantly as the concentration of 1,4-dioxane was raised above 50 mg/L due to serious substrate inhibition [36]. 1,4-Dioxene is a product of metabolic degradation of 1,4-dioxane by DT8 [36]. YN2 was unable to grow on 1,4-dioxene suggesting that YN2 has a different dioxane degradation pathway than DT8.

We report here the substrate specificity of YN2 which is found to be similar but not identical to other 1,4-dioxane degrading strains (Table 3). Monooxygenases have been confirmed to participate in degradation of 1,4-dioxane in *P. dioxanivorans* CB1190 [24] and *Mycobacterium sp*. PH-06 [25, 40], which are both Gram-positive organisms. However, the detailed functions of monooxygenases and other related enzymes are still uncertain. 1,4-Dioxene was detected as a product during metabolic degradation of 1,4-dioxane by *X. flavus* DT8 [36], but it is not found in cultures of any other 1,4-dioxane degrader, and it could not be utilized by YN2. In addition, 1,4-dioxane-ol has been reported to be a degradation product of some strains [35, 41]. YN2 was shown not to grow on 1,4-dioxane-ol, suggesting that this compound is not an intermediate of YN2.

As mentioned above, monooxygenases have been reported to play an essential role in 1,4-dioxane degradation by metabolism in *P. dioxanivorans* CB1190 [41] and *Mycobacterium sp*. PH-06 [35]. The genome sequence of YN2 revealed the unique presence of two chromosomally encoded soluble di-iron monooxygenase (SDIMO) gene clusters of Group-2. Group-2 SDIMOs not only predominantly function as aromatic monooxygenases but also exhibit great variance in substrate range [42]. Currently, Group-2 SDIMOs include phenol, toluene, benzene and alkene monooxygenases [32, 43–45]. A great majority of Group-2 members presently reported are toluene monooxygenases, as is shown in Fig. 4, and most of them are able to degrade 1,4-dioxane by cometabolism. Group-2 toluene monooxygenases of *Azoarcus* sp. DD4 [45–47], *Pseudomonas mendocina* KR1, *Pseudomonas pickettii* PKO1, and *Burkholderia cepacia* AA1 are able to oxidize 1,4-dioxane after induction by toluene [38]. Similarly, Group-1 toluene monooxygenase of *Burkholderia cepacia* G4 [38] can also oxidize 1,4-dioxane. In spite of being similar to toluene monooxygenases of DD4, KR1, and G4, the Group-2 toluene monooxygenase of *Pseudomonas stutzeri* OX1 cannot oxidize 1,4-dioxane [45].

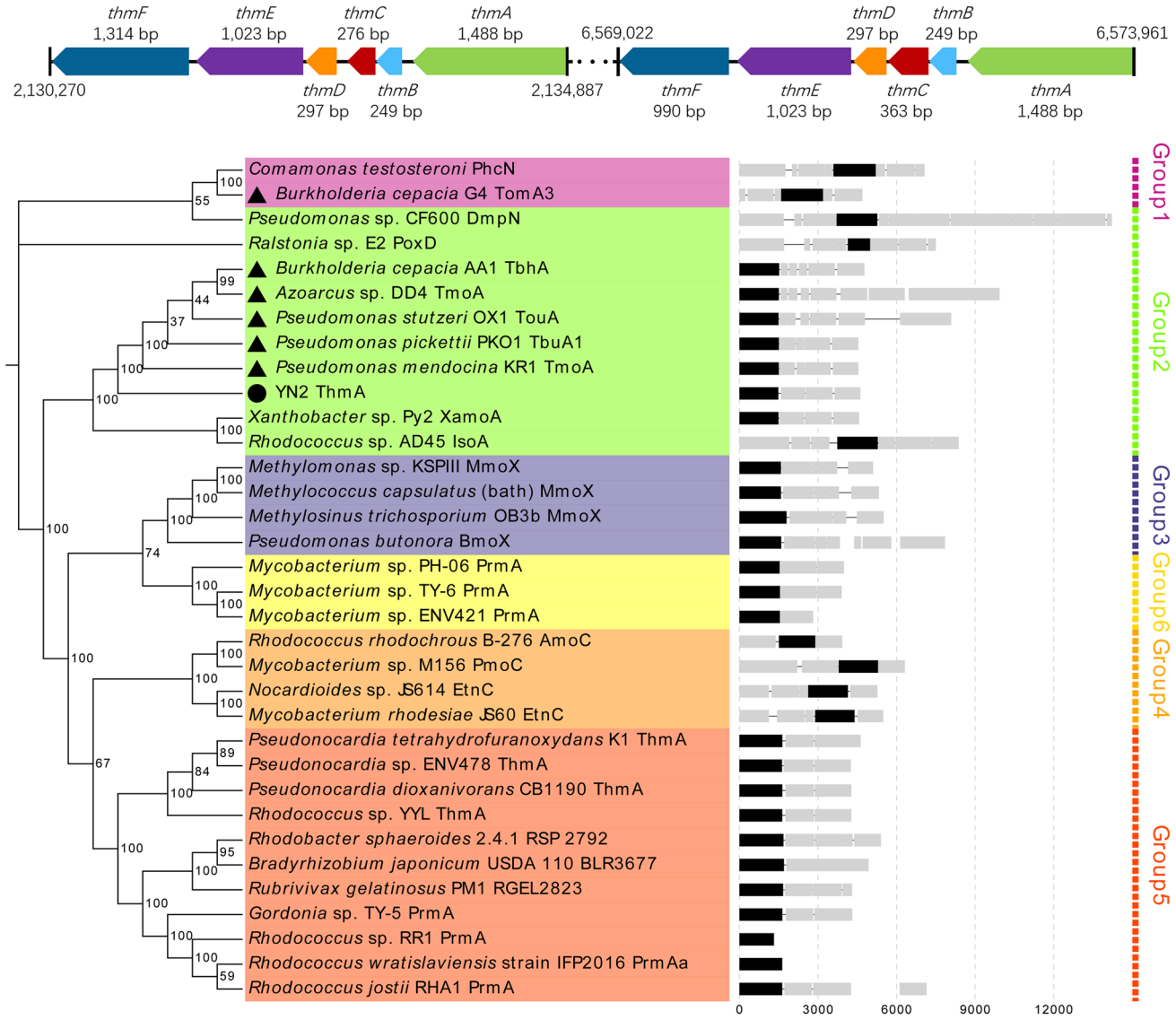

Fig. 4 Two SDIMO gene clusters of YN2 and phylogenetic tree of ThmA of YN2 with α subunit genes of other SDIMOs and their classification. Numbers below gene designations indicate the gene lengths in bp. Numbers above and below hash marks at the terminals of the clusters represent the locations within the chromosome of YN2. Phylogenetic tree of ThmA of YN2 with α subunit genes of other SDIMOs and their classification. Each group is marked with a different color, with their names listed on the right. Blocks on the right side are schemes of the whole clusters encoding each α subunit, with black blocks representing α subunit genes, and gray blocks for the remaining genes. The numbers below the blocks indicate the lengths (bp) of the genes. YN2 ThmA is marked with the black circle; toluene monooxygenases are marked with black triangles. Created using EVOLVIEW v2 [52]

Although SDIMOs groups are defined more by operon structure and composition than by the substrate range or the phylogenetic classification of the respective bacteria [32], there seems to be a phylogenetic pattern of characteristics among the strains. For example, *Xanthobacter* sp. Py2 belongs to the same genus of YN2, and it expresses a Group-2 SDIMO that can metabolize alkene [44]. Similarly as YN2, Py2 does not grow on toluene [44]. Interestingly, another strain of the same genus of YN2, *Xanthobacter* sp. Strain ENV481, is not able to grow on or degrade 1,4-dioxane, but it can grow on a common product of 1,4-dioxane degradation, 2-hydroxyethoxyacetic acid (2HEAA) [24, 48]. For other species, a strain may possess several SDIMOs of the same or different groups [25, 45, 49]. It is not rare to find more than one SDIMO of the same group in the genome of a strain with high pairwise sequence identity [32]. To our knowledge, ThmABCDEF of YN2 is the first Group-2 SDIMO reported to hypothetically degrade 1,4-dioxane by metabolism.

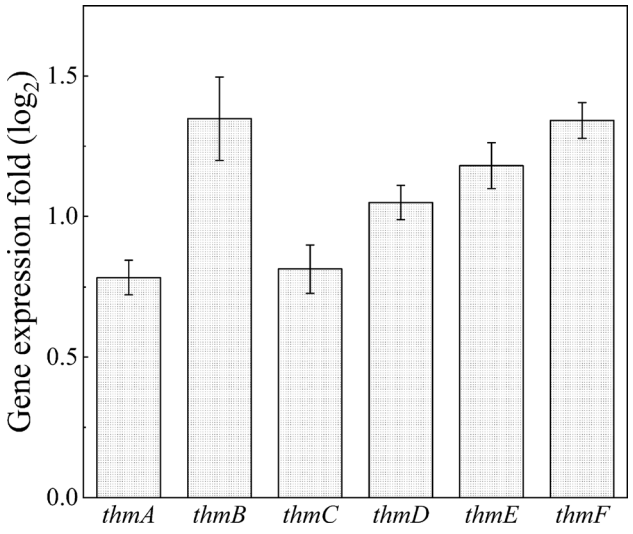

Fig. 5 Upregulation of *thmABCDEF* in a pure culture of YN2 grown on 1,4-dioxane relative to succinate. The 16S rRNA gene was used as the housekeeping gene for error control. Created using ORIGIN 2018

Group-2 SDIMOs play a very important role in bioremediation for 1,4-dioxane [38, 45–47]. The process can happen cometabolically or metabolically, depending on the SDIMOs involved. 1,4-Dioxane degradation by Group-2 SDIMOs via cometabolism is the most reported [38, 45–47]. However, cells cannot benefit from the reaction of cometabolism, and competition against growth substrates can hinder cell growth, which reduces the efficiency of cometabolic degraders with more favorable kinetic properties and higher affinity for the substrate [50]. The expression of SDIMOs can be constitutive or inducible. It is essential to maintain the activity of desired enzyme during bioremediation [42], so amendment of toluene or other analogs are unavoidable in application of Group-2 SDIMOs that are cometabolic or inducible [45]. RNA analyses demonstrate that expression of the YN2 SDIMO gene clusters is constitutive. It is in agreement with the observation that YN2 did not need to be pregrown on 1,4-dioxane to have maximum 1,4-dioxane degrading activity. Therefore, ThmABCDEF of YN2 as a constitutively expressed SDIMO is the optimum for bioremediation of 1,4-dioxane. Also, many SDIMO gene clusters in other 1,4-dioxane degrading organisms are found on plasmids, which leads to higher risk of gene loss [25, 51]. In comparison, *thmABCDEF* is located in the chromosome, indicating that degradation ability loss caused by plasmid curing would not happen during field application of YN2.

This study demonstrates that the unique characteristics of YN2 (high growth rate on 1,4-dioxane, high 1,4-dioxane degradation rate, and tolerance to high concentrations of 1,4-dioxane) make it an ideal candidate for use in the bioremediation of 1,4-dioxane. This study also sets a solid foundation for future studies exploring the elucidation of the entire 1,4-dioxane degradation pathway in YN2 and other 1,4-dioxane degrading organisms.

Conclusion

YN2 has the ability to grow on and degrade 1,4-dioxane at higher concentrations than 3000 mg/L with no substrate inhibition and is also capable of degrading several 1,4-dioxane analogs metabolically. Of particular note is that the tolerance of YN2 to 1,4-dioxane (50 g/L) far exceeds those of all other reported degraders, and is six times higher than the highest tolerance reported previously. Furthermore, both degradation and growth kinetics of YN2 are well described by Monod kinetics, and the strain exhibits higher

Table 3 Substrate range of 1,4-dioxane degraders

Substrates	YN2	*Xanthobacter flavus* DT8 [14]	*Acinetobacter baumannii* DD1 [13]	*Pseudonocardia dioxanivorans* CB1190 [18]	*Mycobacterium sp.* PH-06 [35, 40]
1,4-Dioxane	+[a]	+	+	+	+
1,3-Dioxane	+	+	+	+	+*[c]
Tetrahydrofuran	+	+	+	+	+*
Ethanol	+	+	+	+	/
n-Hexane	–[b]	–	+	/	/
Cyclohexane	–	–	/	–	+*
Toluene	–	–	+	–	/
Ethyl acetate	+	+	/	/	/
Succinate	+	/[d]	/	–	+

[a]"+" able to degrade the substrate

[b]"–" unable to degrade the substrate

[c]"+*" induced by pre-growing on 1,4-dioxane

[d]"/" not reported

degradation ability compared to other reported 1,4-dioxane degraders, by means of its high cell yield (0.27 mg-protein/mg-1,4-dioxane) and maximum specific 1,4-dioxane degradation rates (1.10 mg-1,4-dioxane/h·mg-protein), indicating that YN2 is suitable for bioremediation applications. This is also the first report of constitutive Group-2 SDIMO gene clusters upregulated by 1,4-dioxane.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human and Animal Participants This research does not involve any human participants and/or animals.

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