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Short Genome Communications

Complete genome sequence of the dissimilatory azo reducing thermophilic bacterium *Novibacillus thermophiles* SG-1

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ABSTRACT

With the isolation and identification of efficient azo-dye degradation bacteria, bioaugmentation with specific microbial strains has now become an effective strategy to promote the bioremediation of azo dye. However, Azo dye wastewater discharged at high temperature restricted the extensive application of the known mesophilic azoreducing microorganisms. Here we present the complete genome sequence of a bacterium capable of reducing azo dye under thermophilic condition, *Novibacillus thermophiles* SG-1 (=KCTC 33118^T =CGMCC 1.12363^T). The complete genome of strain SG-1 contains a circular chromosome of 3,629,225 bp with a G + C content of 50.44%. Genome analysis revealed that strain SG-1 possessed genes encoding riboflavin biosynthesis protein that would secrete riboflavin, which could act as electron shuttles to transport the electrons to extracellular azo dye in decolorization process. HPLC analysis showed that the concentration of riboflavin increased from 0.01 μM to 0.255 μM with the growth of strain SG-1 under azo dye reduction. Quantitative real-time PCR analysis further demonstrated that the gene encoding riboflavin biosynthesis protein would be involved in the azo dye decolorization. The results from this study would be beneficial to research the mechanism of anaerobic reduction of azo dye under thermophilic conditions.

Azo dyes, which are aromatic compounds containing one or more −N═N− groups, are extensively used in dyeing industry, and more than 50% of them end up in darkly colored wastewater (Fernando et al., 2014). As the initial step for treatment of this wastewater, azo dye decolorization is facilitated by anaerobic conditions, resulting in the reductive cleavage of azo linkages and the formation of aromatic amines (Zhang et al., 2010). Under anaerobic conditions, bacterial decolorization might be attributable to specific reactions catalyzed by cytoplasmic enzymes that are only effective when using cell extract (Russ et al., 2000). On the other hand, increasing evidences have proved that bacterial azo reduction is a non-specific reaction mediated by extracellular electron transport system (Hong et al., 2007). In addition, it was found that the addition of redox mediators or electron shuttles such as quinone compoumds or humics could accelerate bacterial decolorization (Rau et al., 2002; Liu et al., 2009; Liu et al., 2011). Azo reduction driven by nonspecific reactions might be related to the electron transport chain on the cellular membrane, in which azo dye acts as the terminal electron acceptor supplied by carriers (Brigé et al., 2008; Yu et al., 2015). In nature, the capability of azo reduction may be found in a broad range of anaerobic and facultatively anaerobic bacteria, most of which are mesophilic (e.g. *Shewanella decolorationis S12* (Xu et al., 2005), *Shewanella oneidensis* MR-1 (Brigé et al., 2008), *Sphingomonas* sp.BN6 (Kudlich et al., 1997), *Citrobacter* sp. CK3 (Wang et al., 2009), and *Planococcus* sp. MC01 (Ma et al., 2013)). However, dye wastewater is normally discharged at high temperature (50–80 °C), which could hamper the application of these mesophilic bacteria in the process of wastewater treatment. Therefore, identifying thermophilic or thermotolerant microorganisms that stimulate azo dye decolorization would be beneficial to industrial applicability in dye wastewater treatment.

Recently, a thermophilic, facultatively anaerobic bacterium strain SG-1, was isolated from a saline soil sample collected from the southern tip of Guangdong Province, China (Yang et al., 2015). It was characterized by polyphasic taxonomic analysis, and was proposed as a novel species of anew genus with the name of *Novibacillus thermophilus*. Cell growth occurred optimally at 50 °C, pH 7.5–8.0 and with 5.0–7.0%

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Table 1
Genome features of *Novibacillus thermophilus* SG-1.

Feature	Chromosome
Genome size (bp)	3,629,225
G + C content (mol%)	50.44
Total genes	3532
Protein coding genes	3254
tRNAs	51
rRNAs	12
ncRNAs	4
Pseudo genes	211
Genes with predicted functions	1827
GenBank accession	CP019699.1

NaCl. Strain SG-1 was able to anaerobically decolorize azo dye Orange I by utilizing a large variety of organic substrates as electron donors (Yu et al., 2015). These findings suggested its potential application in treatment of dye-containing wastewater discharged at high temperature. In this study, we further determined the ability of strain SG-1 to reduce azo dyes after adding anthraquinone-2,6-disulfonate (AQDS) as the redox mediator. Furthermore, in order to obtain detailed insights into the genomic features of strain SG-1, the complete genome sequence was detected and several kinds of genes responsible for azo reduction were discovered.

For genome sequencing, the strain was cultured in LB medium at 50 °C and pH 7.5, and genomic DNA was extracted using a DNA extraction kit (Aidlab). The genome of strain SG-1 was sequenced by PacBio SMRT high-throughput sequencing technologies. Sequencing was performed at the Beijing Novogene Bioinformatics Technology Co.,

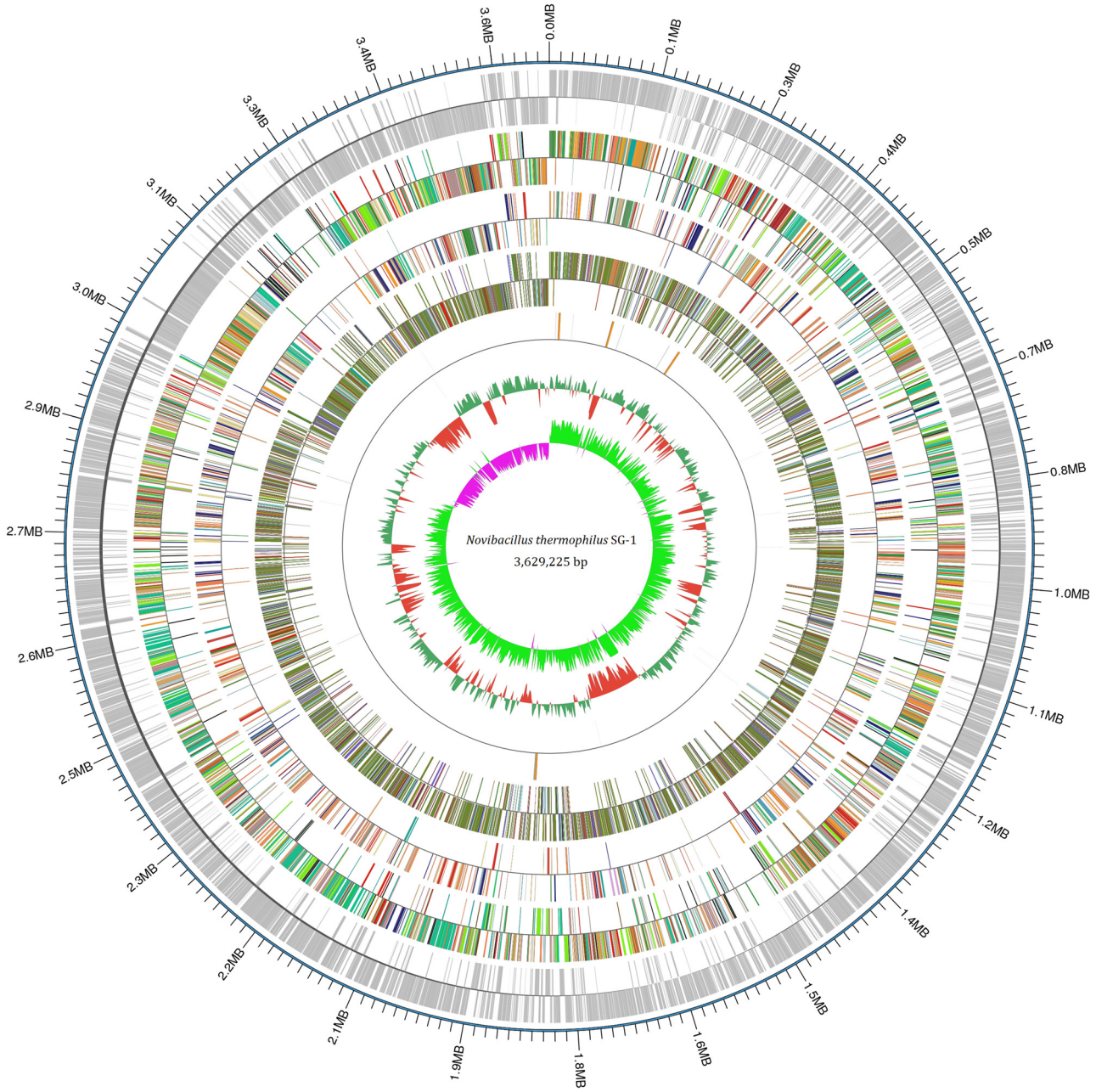

Fig. 1. Circular genome map of the chromosome of *N. thermophilus* SG-1. From inside to outside, circle 1 illustrates the GC skew. Circle 2 shows GC content (peaks outside or inside the circle indicate values higher or lower than the average G + C content). Circle 3 denotes ncRNA genes. Circles 4, 5 and 6 indicate the CDSs, colored according to COG, KEGG and GO categories, respectively. Circle 7 demonstrates the predicted protein-coding sequences.

Table 2
COG functional categories in the sequenced genome of *N. thermophilus* SG-1.

Code	Value	% of total[a]	Class_description
[B]	1	0.04	Chromatin structure and dynamics
[C]	167	5.96	Energy production and conversion
[D]	46	1.64	Cell cycle control, cell division, chromosome partitioning
[E]	285	10.17	Amino acid transport and metabolism
[F]	74	2.64	Nucleotide transport and metabolism
[G]	317	11.31	Carbohydrate transport and metabolism
[H]	137	4.89	Coenzyme transport and metabolism
[I]	108	3.85	Lipid transport and metabolism
[J]	193	6.89	Translation, ribosomal structure and biogenesis
[K]	205	7.32	Transcription
[L]	96	3.43	Replication, recombination and repair
[M]	138	4.93	Cell wall/membrane/envelope biogenesis
[N]	40	1.43	Cell motility
[O]	105	3.75	Posttranslational modification, protein turnover, chaperones
[P]	153	5.46	Inorganic ion transport and metabolism
[Q]	41	1.46	Secondary metabolites biosynthesis, transport and catabolism
[T]	117	4.18	Signal transduction mechanisms
[U]	40	1.43	Intracellular trafficking, secretion, and vesicular transport
[V]	73	2.61	Defense mechanisms
[W]	8	0.29	Extracellular structures
[X]	88	3.14	Mobilome: prophages, transposons
[Z]	1	0.04	Cytoskeleton
	369	13.17	Unknown

[a] The total is based on the total number of protein coding genes in the annotated genome.

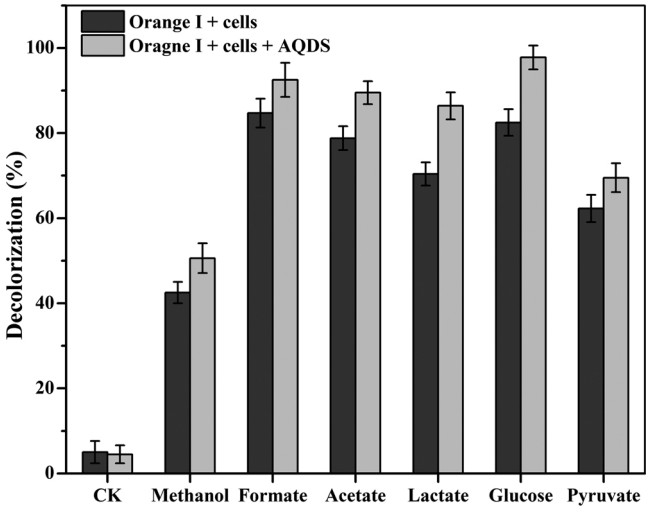

Fig. 2. Effect of AQDS on decolorization of Orange I by strain *N. thermophilus* SG-1 fed by various electron donors.

Ltd. using SMRT Analysis 2.3.0 to filter low quality reads, and the filtered reads were assembled into a circular contig with the genomic coverage of 291-fold. The complete genome of strain SG-1 was annotated by NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP, http://www.ncbi.nlm.nih.gov/books/NBK174280). *N. thermophiles* SG-1 has a circular chromosome of 3,629,225 bp with a G + C content of 50.44 mol%. The genome totally contains 3532 genes, which include 3254 protein coding genes, 211 pseudo genes and 67 RNA genes (12 rRNAgenes, 51 tRNA genes and 4ncRNA genes) (details can be seen in Table 1 and Fig. 1). The most abundant groups of genes with predicted functions were those are related to carbonhydrate transport and metabolism (COG category G [11.31%]), amino acid transport and metabolism (COG category E [10.17%]), transcription (COG category K [7.32%]), and translation, ribosomal structure and biogenesis (COG category J [6.89%]) (Table 2).

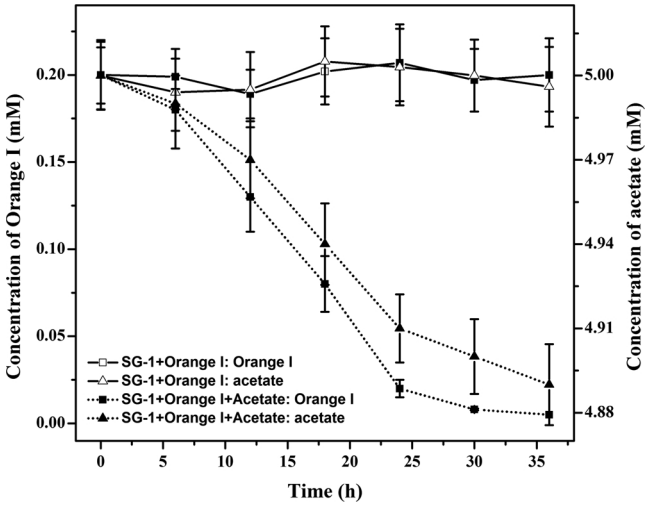

Fig. 3. The concentration of Orange I detected by HPLC in treatments of SG-1 + Orange I + Acetate (5 mM acetate as the electron donor, 0.2 mM Orange I as the electron acceptor) and SG-1 + Orange I.

Our previous study has proposed *N. thermophilus* SG-1 as a novel genus and species, which can couple Orange I reduction with acetate oxidation and conserve energy for cell growth under thermophilic conditions (Yang et al., 2015). To investigate the azo reducing ability of strain SG-1 using AQDS as redox mediator, experiments were carried out with 20 ml basal medium containing 0.2 mM Orange I served as electron acceptor, 5 mM various electron donors (methanol, formate, acetate, alcohol, glucose, lactate and pyruvate), 0.5 mM AQDS served as redox mediator and 1.0 ml cell suspension (1.0×10^7 CFU/mL). The cultures without additional AQDS were set as the control. The incubation occurred at 50 °C in 25 mL serum bottles sealed with butyl rubber stoppers in an anaerobic station (Sheldon Manufacturing Inc.) for 24 h. The concentration of Orange I was determined by using a UV/vis spectrophotometer (TU1800-PC, Beijing) at the absorbance wavelength of 480 nm (Ma et al., 2013). After 24 h incubation, decolorization extent reached 50.6, 92.5, 89.5, 86.4, 97.8, and 69.5% for methanol, formate, acetate, lactate, glucose and pyruvate serving as the electron donor, respectively (Fig. 2). For each electron donor, the decolorization extent of the treatments amended with AQDS were significant higher than those in the treatments without AQDS, suggesting that redox mediators could facilitate the reduction of Orange I by strain SG-1. To verify the degradation of Orange I, the concentration of Orange I in treatment with acetate as electron donor and the control was further measured by HPLC (Shimadzu LC-15, Japan). HPLC analysis were performed using a diode array detector (473 nm) with a C18 column (250 mm × 2.0 mm) and a gradient elution with a mobile phase composed of acetonitrile-ammonium acetate 0.1 mM (5/95, v/v to 100/0 in 30 min) (Bianco et al., 2008). As shown in Fig. 3, with the acetate degradation, the concentration of Orange I decreased from 0.2 mM to 0.02 mM after 24 h incubation. These results demonstrated that the addition of redox mediators or electron shuttles, such as AQDS, could facilitate azo dye reduction by some bacteria, which is similar to the results from the reports of Kudlich et al. (1997); Liu et al. (2009) and Rau et al. (2002).

Azo reduction could be manipulated by some specific cytoplasmic enzymes, such as flavin-containing (Bürger and Stolz, 2010) or flavin-free azoreductases (Chen et al., 2010), or attributable to combined function of the multihaem cytochromes as MtrA, OmcA and OmcB that consisted the complicated extracellular electron transport chain (Hong et al., 2007; Brigé et al., 2008). As shown in the genome of *N. thermophilus* SG-1, however, the strain did not possess the genes encoding cytoplasmic azoreductase or cytochromes able to transfer the electrons

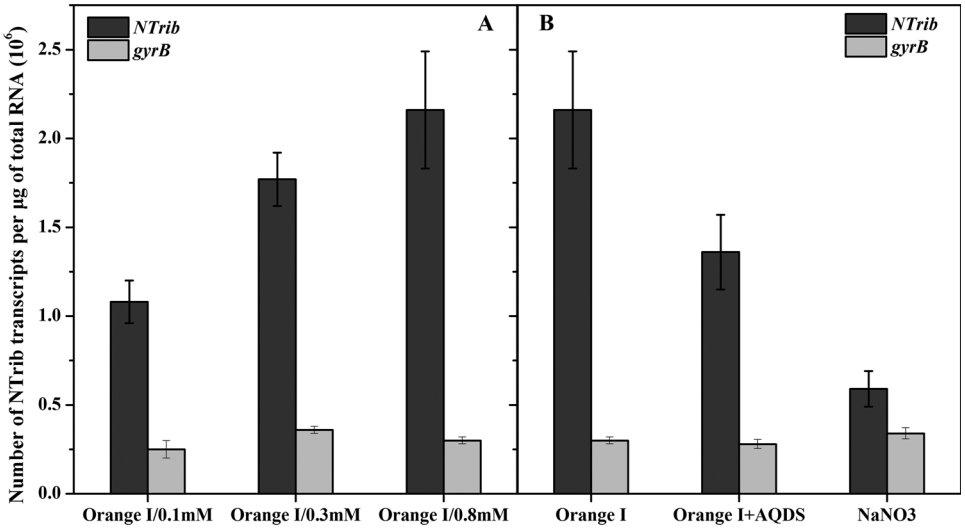

Fig. 4. Numbers of *NTrib* and *gyrB* transcrits expressed by *N. thermophiles* grown in different concentrations of Orange I (A) and in different electron acceptors (B).

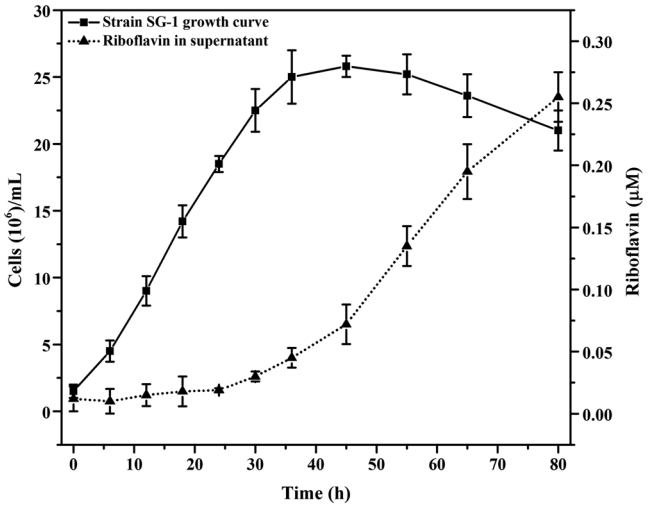

Fig. 5. The concentration of riboflavin with the growth of strain SG-1 in the culture of azo dye reduction.

to extracellular azo dye. We noticed that *N. thermophilus* SG-1 harbored the genes encoding the riboflavin biosynthesis protein (WP_077720713.1 in NCBI) that exhibited 47% similarity to RibC in strain *Paenibacillus* sp. FSL R5-192 (Moreno Switt et al., 2014) and would secret riboflavin. As a known electron shuttle, riboflavin could mediate the extracellular electron transport to the terminal electron acceptors (Covington et al., 2010).

To confirm the function of genes encoding riboflavin biosynthesis protein, we determined the number of transcripts under different incubation circumstances through quantitative real-time PCR method using the *gryB* housekeeping gene as a normalizer (CristóBal et al., 2016). The treatments were set as different concentrations of Orange I (0.1 mM, 0.3 mM, and 0.8 mM), and different electron acceptors (NaNO3, Orange I, and Orange I + AQDS), in which acetate was the sole electron donor. RNA was extracted from all samples at mid-log phase using Bacterial RNA Kit (Omega bio-tek, USA) and further purified with MicroElute RNA Clean Up Kit (Omega bio-tek, USA) following the manufacturer's instructions. Primer was designed for qPCR analysis according to the Primer Premier 5.0 (Premier Biosoft) from the genome of *N. thermophiles*: *NTribF* (5' CTTTCCTACAGCGAACCTCC 3') and NTribR (5' GCTCGGCTGAGTCGAATTTA 3'). The qPCR parameters consisted of an initial denaturation step of 95 °C for 5 min; 5 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s, with a ramp rate of 0.1 °C/s from the annealing to the extension temperature; 40 cycles of 95 °C for 30 s, 58 °C for 45 s, 72 °C for 30 s; and a final extension at 72 °C for 10 min. The qPCR results showed that the number of transcripts went up with the increasing concentration of Orange I (Fig. 4A). The number of *NTribF* transcripts was two times higher in the culture that was grown at 0.8 mM Orange I than in 0.1 mM Orange I. The level of *NTribF* transcripts was significantly higher in the treatments of Orange I and Orange I + AQDS than in the treatment with $NaNO_3$ as electron acceptors (Fig. 4B). In contrast, the levels of transcripts for expressed housekeeping gene *gyrB* remained relatively stable in all of culture conditions. After 80 h incubation amended with 0.8 mM Orange I, we further detected the concentration of riboflavin of the supernatant through HPLC equipped with a FAD detector (Ex/Em: 450/516 nm) and a C18 column under gradient elution of a mobile phase composed with methanol-water-acetate 650 mM (9/90/1, v/v/v, to 89/10/1 in 8 min, to 9/90/1 in 11.1 min, to 9/90/1 in 15 min) (Stahmann et al., 2000). Fig. 5 presented the concentration of riboflavin with the cell growth under Orange I reduction, which revealed that with the cell growth of strain SG-1 due to the azo dye reduction, concentration of riboflavin increased from 0.01 μM to 0.255 μM after 80 h incubation. These results suggested that the concentration of riboflavin and levels of *NTribF* transcripts were related to the rates and extents of decolorization by *N. thermophilus* SG-1, which demonstrated that decolorization by *N. thermophilus* SG-1 would be an extracellular reduction process, in which strain SG-1 secreted riboflavin that might transport electrons to extracellular azo dye.

In summary, this study provides insight into the azo dye reduction in *N. thermophilus* SG-1. The genome sequence reported would be helpful for us to study the mechanism of azo decolorization under thermophilic condition.

Nucleotide sequence accession number

The complete genome sequence of *N. thermophilus* SG-1 was deposted at GenBank with accession number CP019699.1. This strain had been deposited at the Korean Collection for Type Cultures (=KCTC 33118^T), and China General Microbiological Culture Collection Center (=CGMCC 1.12363^T).

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