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Short Communication

Construction of a *URA5.3* deletion strain of the unicellular red alga *Cyanidioschyzon merolae*: A backgroundless host strain for transformation experiments

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Keiko Taki,[1,2] Toshiyuki Sone,[1,2] Yuki Kobayashi,[1] Satoru Watanabe,[2,3] Sousuke Imamura,[1,2] and Kan Tanaka[1,2,*]

[1] *Chemical Resources Laboratory, Tokyo Institute of Technology, 4259-R1 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan*
[2] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tokyo 102-0075, Japan*
[3] *Department of Bioscience, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan*

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Abbreviations: bp, base pair(s); DIG, digoxigenin; 5-FOA, 5-fluoroorotic acid; ORF, open reading frame; PEG, polyethylene glycol

Development of transformable host strains for molecular genetic analysis permits basic biological and applied investigation of microorganisms. The ability to introduce DNA into the cell is essential; as is a selectable marker system, both for genome-independent procedures such as the use of plasmid vectors, and genome manipulation with DNA recombination. *Cyanidioschyzon merolae* is a unicellular red alga living in sulfate-rich, acidic hot springs. It has been used to elucidate aspects of eukaryotic cell biology such as the evolutionary origins and function of organelles, and the effects of compact genomes, and it was the first alga for which the complete genome sequence was produced and annotated (Matsuzaki et al., 2004; Misumi et al., 2005; Nozaki et al., 2007). Because the cell contains a minimal set of organelles, one nucleus, one mitochondrion, one chloroplast and one peroxisome, *C. merolae* is an ideal model organism, particularly for studying inter-organelle interactions. In a previous study, we isolated a uracil auxotroph mutant (M4), by selecting a spontaneously mutated 5-fluoroorotic acid (5-FOA)-resistant clone (Minoda et al., 2004), in which one adenine base was inserted in the *CMK046C* (*URA5.3*) gene coding region for UMP synthase (http://merolae.biol.s.u-tokyo.ac.jp/), resulting in a translational frameshift in the 3′ half of the ORF. The transformants can thus be selected using the uracil synthase gene as a marker for uracil prototrophy. However, because the mutation in the M4 strain resulted from a slippage in a series of adenines, spontaneous reversion mutations frequently occur, which is problematic for the genetic selection of uracil prototrophs.

In this paper, we report the isolation of a mutant in which the *URA5.3* gene is completely deleted, and which has been successfully used for backgroundless selection of transformants. It has previously been shown in *C. merolae* that the nuclear genome can be modified via homologous recombination with exogenously introduced DNA (Fujiwara et al., 2013; Imamura et al., 2009). Here, we isolated a *URA5.3* deletion mutant, by transforming with a *URA5.3*-deleted DNA fragment and directly selecting 5-FOA-resistant clones.

The *URA5.3*-deleted fragment (URA5.3D fragment, Fig. 1a) was generated using three steps of the PCR. The first PCR amplified a chromosomal fragment, from the 5′ upstream position –2708 to +1415, with respect to the *URA5.3* ORF initiation site. This used primers A (5′-TCTCGGCTGAGAGGCCTGTTCG-3′); and B (5′-cgcttgcttctgcccattaggaattaAGTCTTACAACAGTA-CTCAGATCGTTG-3′), in which uppercase letters indicate the sequence upstream of the deleted region, underlining indicates the altered base that prevents the occurrence of non-native translation, and lowercase letters indicate the sequence adjacent to the 3′ end of the *URA5.3* ORF. Primer B spanned a 2286-bp deleted region in its sequence. This PCR was carried out using KOD-Plus-Neo DNA polymerase, following the manufacturer's instructions (Toyobo Co., Ltd., Osaka, Japan). Similarly, a fragment from position –924 to +3352 was PCR-amplified by primers C (5′-CAACGATCTGAGTACTGTTGTAAGA-CTtaattcctaatgggcagaagcaagcg-3′), and D (5′-CTAT-GTCTGCTTTCACCAGGGTGG-3′). These two frag-

*Corresponding author: Kan Tanaka, Chemical Resources Laboratory, Tokyo Institute of Technology, 4259-R1 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan.
Tel: +81-45-924-5274 Fax: +81-45-924-5274 E-mail: kntanaka@res.titech.ac.jp

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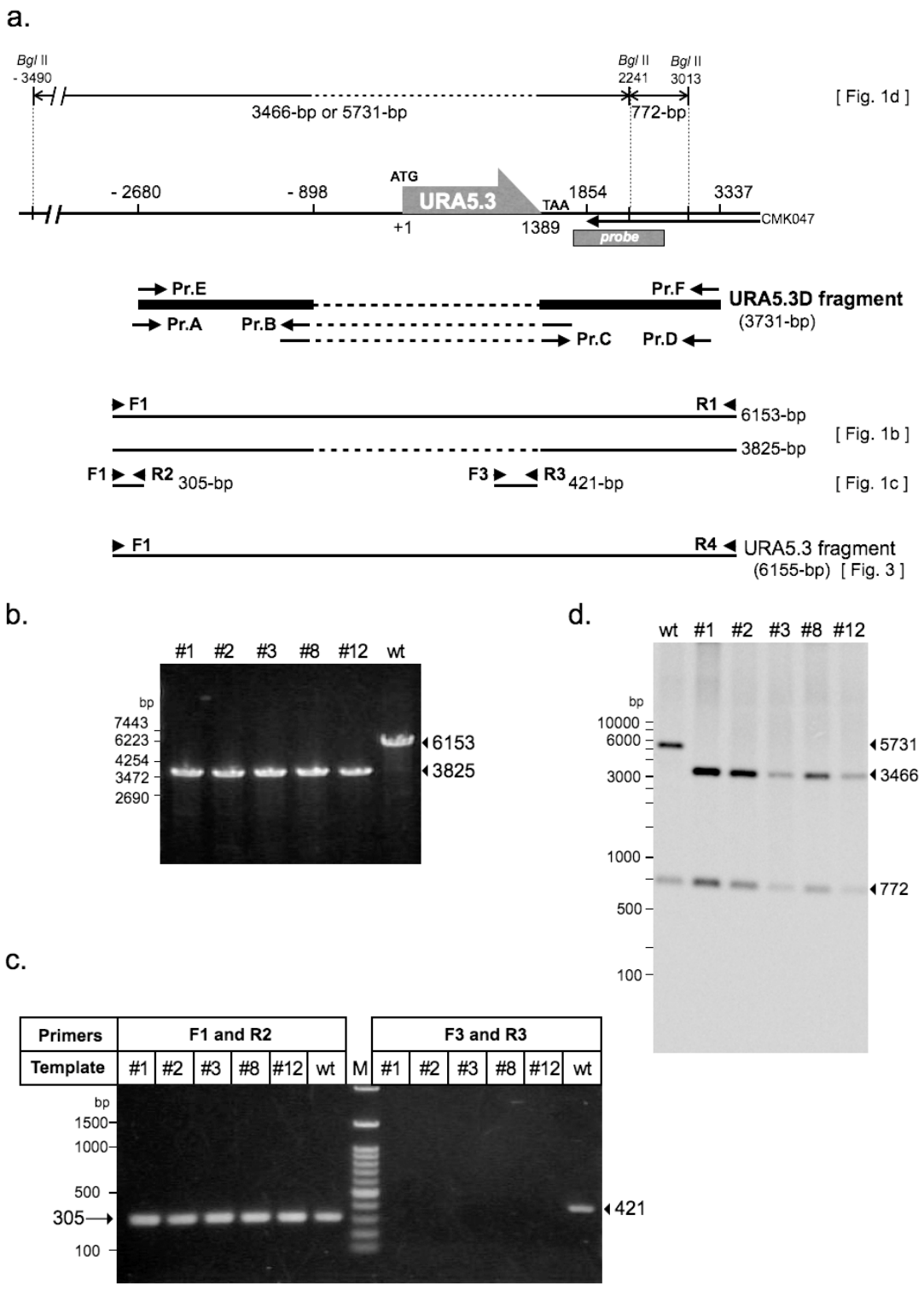

Fig. 1 Experimental design for creating the UMP synthase null mutant, T1.

a. Schematic of the genomic *URA5.3* gene locus, including the position of primers used in mutant construction and validation. Dotted lines show the deleted region. Primers A and B, C and D (Pr.A, Pr.B, *et seq*.), were used to amplify the upstream and downstream parts, respectively, of the fragment. Primers E and F were used to amplify the URA5.3D fragment, which was introduced to make the null mutant. Primers F1 and R1 were located outside the products of primers AB and CD (for b). Primer sets, (F1 and R2) and (F3 and R3), were used to check for the presence of the upstream and the deleted regions, respectively (for c). Primers F1 and R4 were used to amplify the URA5.3 fragment, which was introduced to restore the uracil autotrophic phenotype (for Fig. 3). The box marked as probe indicates the position (from +1668 to +2609) of the DIG-labeled probe for genomic Southern hybridization (for d). Sizes of the hybridized fragments are shown above the schematic of the genomic locus. b, c. Validation of genomic deletion for the *URA5.3* gene null mutant. The fragments amplified with primers F1 × R1 were 6153-bp from wild type *C. merolae* (lane wt; panel b) and 3825-bp from deletion clones (lanes #1, 2, 3, 8, 12; panel b). No fragments were amplified by primers F3 × R3 in clones #1 to #12, indicating the absence of *URA5.3* (lanes #1, 2, 3, 8, 12; right of panel c). A 421-bp fragment was amplified when wild type genomic DNA was used as a template (lane wt, right of panel c). All lanes amplified using F1 × R2 show 305-bp fragments (left of panel c). d. Genomic Southern hybridization of the *URA5.3* deletion strain. Total DNAs isolated from the parental wild type and the five transformants were digested with *Bgl*II and fractionated by 0.8% agarose gel electrophoresis. Expected sizes of the hybridizing signals are shown on the right.

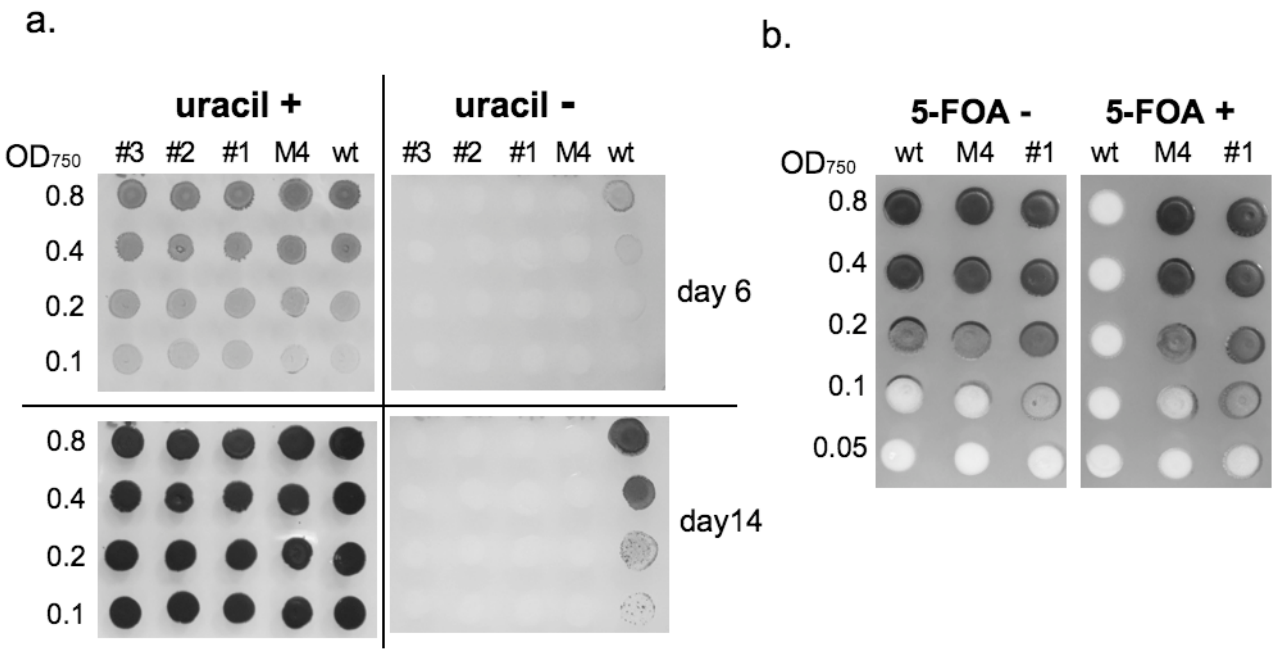

Fig. 2 Characterization of isolated mutants.

a. Cells of wild type (wt), M4 mutant (M4), and the three isolated mutants (#1–3) were grown on MA2 plates, or MA2 plates containing uracil (500 μg ml^{-1}). Spots of 8 μl of cell culture, with a 750-nm optical density (OD_{750}) of 0.8, 0.4, 0.2 or 0.1, were added to the plates. M4 and the isolated mutants required uracil for growth. b. Cells were grown on MA2 containing uracil (500 μg ml^{-1}), in the presence or absence of 5-FOA (800 μg ml^{-1}). M4 and the isolated strain #1 were resistant to 5-FOA, demonstrating the loss of *URA5.3* function.

ments were electrophoresed and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega K.K., Tokyo, Japan), and joined by the second PCR reaction (Pont-Kingdon, 1997, with some modifications). The second PCR was performed as a 50-μl reaction using KOD-Plus-Neo DNA polymerase, with 600–800 ng each of the first two PCR products (as both templates and primers) under the following conditions: 94°C for 2 min, followed by 10 cycles of 15 s at 98°C, 15 s at 55°C, 3 min at 72°C, and a final extension of 7 min at 72°C. The resultant 3774-bp fragment from the completed second PCR was used directly as a template for the third PCR. For the third PCR, the fragment was further amplified using primers E (5′-CCCCTGTGGCGCACGTGCCG-3′) and F (5′-CCAGGGTGGTGCATCTTTATTTTCTG-3′); this was purified with the Wizard® SV Gel and PCR Clean-Up System.

The resultant 3731-bp URA5.3D fragment was used in PEG-mediated transformation of the wild type cell, using the methods described by Ohnuma et al. (2008), except that the included DNAs were 30 μg of the URA5.3D fragment and 550 μg of carrier DNA (salmon sperm DNA, Commercial, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Selection of 5-FOA-resistant clones was performed on a cornstarch bed as described previously (Minoda et al., 2004), except that the concentration of 5-FOA (5-Fluoroorotic acid monohydrate, Wako Pure Chemical Industries, Ltd.) was 400 μg ml^{-1}. Independent clones were isolated after 1.5 months of incubation under continuous white light of 35–50 μmol photons m^{-2} s^{-1}, in an Anaero Pouch with a AnaeroPack® CO_2-generating agent (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). Isolates were screened by colony PCR using the primers F1 (5′-GCCCACCACGAGCGTACTG-3′) and R1 (5′-CCGAATCCTGGGTGAGAGC-3′), which were designed to match the outside of the URA5.3D fragment with its *URA5.3* deletion. Isolates were then cultured separately, and further PCR analyses were performed to confirm the chromosomal *URA5.3* deletion, using DNA extracted from the isolates:

1) PCR with F1 × R1, where a 3867-bp fragment is expected from the deletion mutant and a 6153-bp fragment is expected from the parental strain (Figs. 1a and b).

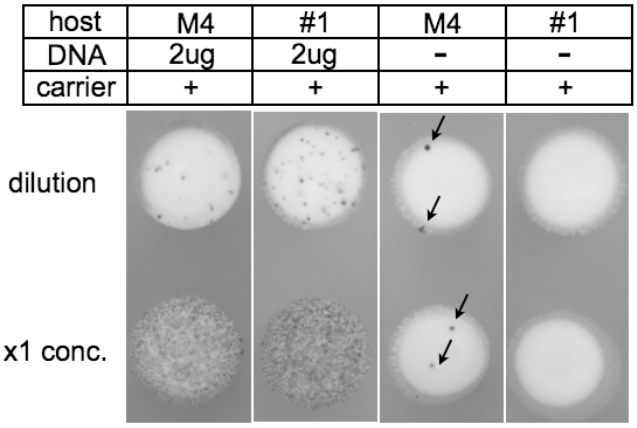

Fig. 3 Complementation of uracil requirement for growth by transformation with the *URA5.3* fragment.

The *URA5.3* fragment (see Fig. 1) was introduced into M4 or isolated mutant cells, and transformants were selected as uracil prototrophs growing on the MA2 plates. It was shown that the uracil prototroph colonies appeared by the transformation of the *URA5.3* fragment. When only salmon sperm DNA was introduced into M4, some uracil prototroph colonies appeared (both of the 1/8 dilution and ×1) as indicated by arrows. No uracil prototroph colonies grew in the isolated null mutant sample (both of the 1/8 dilution and ×1).

2) PCR with F1 × R2 (5′-GTACGGCGTTCGAAAAATAGCG-3′), where the same 305-bp fragment is expected from both the deletion mutant and the parental strain (Figs. 1a and c).

3) PCR with F3 (5′-CAGTTTCATGCTTTTTGAGGATCG-3′) × R3 (5′-ACCCTTGCGCTTCAGCAGATAC-3′), where no amplification is expected from the deletion strain, and a 421-bp fragment is expected from the parental strain (Figs. 1a and c).

As shown in Fig. 1, the results indicated that the *URA5.3* region was deleted as expected in the 5-FOA resistant clones. To confirm that the DNA integration only occurred at the *URA5.3* locus, genomic Southern hybridization analysis was subsequently performed. Total DNA was isolated from the parental wild type and five randomly chosen transformants (#1, #2, #3, #8 and #12), digested with *Bgl*II and the Southern hybridization experiment was performed under standard conditions (Cold Spring Harbor Protocols, 2015). For the probe preparation, a specific DNA region (Fig. 1a) was amplified by primers, 5′-TAGAGCTGGATCCTATGGTTTCTTG-3′ and 5′-GAGATAGCTCTTCGGTCTGAATTTG-3′, and *C. merolae* total DNA as the template, and labeled with a DIG random primed DNA labeling system (Roche Diagnostics K.K., Tokyo, Japan). As shown in Fig. 1d, only signals of the expected sizes, 5731-bp and 772-bp for the wild type and 3466-bp and 772-bp for all transformants, were detected, indicating the single integration at the *URA5.3* locus.

The uracil auxotrophic phenotype of the isolated clones was characterized as shown in Fig. 2. The first selection of the 5-FOA-resistant clones was performed on gellangum plates containing both 5-FOA (400 μg ml^{-1}) and uracil (500 μg ml^{-1}), as 5-FOA-resistant clones should also be uracil auxotrophs. The requirement for uracil is shown in Fig. 2a, confirming successful deletion and that the isolates are, in fact, deficient of the *URA5.3* gene. While the growth of the wild type cells appeared greatly improved by the presence of uracil, the prototrophic characteristics were unaffected by the medium. The 5-FOA resistance of the #1 strain was also confirmed in Fig. 2b.

Next, to evaluate whether the isolated *URA5.3* deletion strain can be used as a host strain for transformation, we chose the #1 strain as the representative and examined this further. When only salmon sperm DNA (which is included as the carrier DNA in the transformation experiments) was included, no uracil prototroph colonies appeared after 12 days incubation (Fig. 3).

Transformation with the URA5.3 fragment containing the *URA5.3* gene (derived from PCR F1 × R4 (5′-CCCCGAggCCTGGGTGAGAGC-3′) where lowercase letters indicate an artificial sequence making a *Stu*I restriction site, see Fig. 1) resulted in the growth of numerous colonies (Fig. 3), indicating DNA had been incorporated into the cells and had recombined with chromosomal DNA. It is currently not confirmed whether only the expected recombination event took place. However, given that a non-homologous recombination event has never been identified in this organism throughout previous molecular genetic analyses, it is quite unlikely that the complementation occurred by unexpected integration. These results show that the #1 strain is functionally useful for transformation experiments, and is referred to as strain T1. The absence of spontaneous uracil prototroph revertants shows that T1 has significant advantages over the frameshift M4 mutant as a host strain (Fig. 3).

In conclusion, we have successfully isolated a *URA5.3* deletion strain, T1, of *C. merolae*, which is more useful for transformation experiments than the previously-isolated M4 strain. Use of this strain will facilitate the development of more sophisticated molecular genetic experimental systems.

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