

A New Type of YumC-Like Ferredoxin (Flavodoxin) Reductase Is Involved in Ribonucleotide Reduction

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ABSTRACT The *trxB2* gene, which is annotated as a thioredoxin reductase, was found to be essential for growth of *Lactococcus lactis* in the presence of oxygen. The corresponding protein (TrxB2) showed a high similarity with *Bacillus subtilis* YumC (E value = $4.0E-88$), and YumC was able to fully complement the Δ *trxB2* mutant phenotype. YumC represents a novel type of ferredoxin (flavodoxin) reductase (FdR) with hitherto-unknown biological function. We adaptively evolved the Δ *trxB2* mutant under aerobic conditions to find suppressor mutations that could help elucidate the involvement of TrxB2 in aerobic growth. Genome sequencing of two independent isolates, which were able to grow as well as the wild-type strain under aerated conditions, revealed the importance of mutations in *nrdI*, encoding a flavodoxin involved in aerobic ribonucleotide reduction. We suggest a role for TrxB2 in nucleotide metabolism, where the flavodoxin (NrdI) serves as its redox partner, and we support this hypothesis by showing the beneficial effect of deoxynucleosides on aerobic growth of the Δ *trxB2* mutant. Finally, we demonstrate, by heterologous expression, that the TrxB2 protein functionally can substitute for YumC in *B. subtilis* but that the addition of deoxynucleosides cannot compensate for the lethal phenotype displayed by the *B. subtilis* *yumC* knockout mutant.

IMPORTANCE Ferredoxin (flavodoxin) reductase (FdR) is involved in many important reactions in both eukaryotes and prokaryotes, such as photosynthesis, nitrate reduction, etc. The recently identified bacterial YumC-type FdR belongs to a novel type, the biological function of which still remains elusive. We found that the YumC-like FdR (TrxB2) is essential for aerobic growth of *Lactococcus lactis*. We suggest that the YumC-type FdR is involved in the ribonucleotide reduction by the class Ib ribonucleotide reductase, which represents the workhorse for the bioconversion of nucleotides to deoxynucleotides in many prokaryotes and eukaryotic pathogens under aerobic conditions. As the partner of the flavodoxin (NrdI), the key FdR is missing in the current model describing the class Ib system in *Escherichia coli*. With this study, we have established a role for this novel type of FdR and in addition found the missing link needed to explain how ribonucleotide reduction is carried out under aerobic conditions.

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Lactococcus lactis is a Gram-positive aerotolerant anaerobe of particular importance to the dairy industry, where it is annually involved in milk fermentations on a hundred-million-ton scale (1). In these dairy fermentations, the main role of *L. lactis* is to produce lactic acid, through its fermentative metabolism, and to contribute to texture and flavor development (1). As an aerotolerant anaerobe, oxygen normally has little effect on the growth of *L. lactis* (2), but lack of a catalase activity can make it more vulnerable to the oxidative stress caused by reactive oxygen species (ROS) (3, 4). During industrial handling, *L. lactis* is normally exposed to oxygen (5), and since this can lead to oxidative stress and influence performance, a great deal of focus has been on understanding how *L. lactis* responds to this type of stress (4). ROS negatively affect all macromolecules (DNA, lipids, and proteins) (6), and for proteins, one of the most frequent modifications by ROS is formation of disulfide bonds (6). In *L. lactis*, enzymes involved in central metabolism, such as glyceraldehyde 3-phosphate dehydrogenase, have been shown to be vulnerable to inactivation

due to formation of disulfide bonds in the presence of oxygen (7). In bacteria, the glutathione-glutathione reductase (Gsh-GshR) and thioredoxin-thioredoxin reductase (Trx-TrxR) systems represent the major defense systems against disulfide bond formation. Both glutathione reductase and thioredoxin reductase belong to the flavoproteins, which require the redox cofactor FAD or FMN for function (8). Although the Gsh-GshR system is able to protect *L. lactis* from oxidative stress, its inability to synthesize glutathione renders this system inactive in the absence of exogenous glutathione (9).

Several Trx-TrxR-related genes have also been identified in *L. lactis*, where *trxA*, *trxD*, and *nrdH* encode thioredoxin, whereas *trxB1* encodes a thioredoxin reductase (7, 10). Proliferation of a Δ *trxB1* *L. lactis* mutant was found to be severely hampered by oxygen, but the addition of reductants such as dithiothreitol (DTT) to the growth medium restored growth (7). *L. lactis* also harbors a *trxB2* gene, encoding a thioredoxin reductase-like protein (TrxB2). TrxB2 homologues are found in many bacteria, and

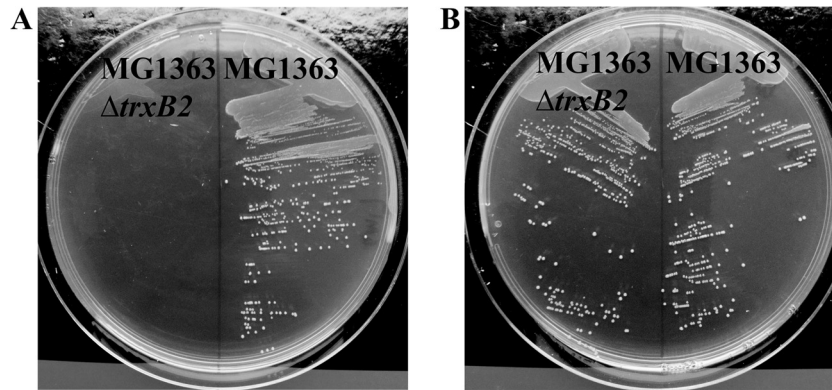


FIG 1 Inhibitory effect of oxygen on growth of a $\Delta trxB2$ mutant. The $\Delta trxB2$ mutant and its parent (MG1363) were streaked on M17 agar containing 0.2% glucose and incubated aerobically (A) and anaerobically (B). Evaluation of growth was carried out after an overnight (16-h) incubation at 30°C.

although the amino acid sequence of TrxB2 is generally very similar to that of thioredoxin reductases, these proteins lack the active-site residues CXXC, characteristic of thioredoxin reductases (7, 11, 12).

In a previous study, we found that FAD biosynthesis in *L. lactis* was impeded at high temperatures, leading to a reduced activity of flavoproteins involved in redox balance homeostasis (NADH oxidase) and anabolism (pyruvate dehydrogenase) and an oxygen-sensitive phenotype (2). Adding DTT to the growth medium did not improve growth, as was observed for the $\Delta trxB1$ mutant (our unpublished data), which could indicate that other flavoproteins with other functions might be important for growth of *L. lactis* in the presence of oxygen (2).

Although the aforementioned *trxB2* gene has been neglected in previous studies, several transcriptomics analyses have shown a remarkable up-regulation of *trxB2* under aerobic conditions (5, 13), which prompted us to further study its function and role in the aerobic growth of *L. lactis*.

In this study, we found that inactivation of the *trxB2* gene in *L. lactis* results in an oxygen-sensitive phenotype that can be reversed by heterologous expression of a ferredoxin (flavodoxin) reductase (YumC) from *Bacillus subtilis* (14). We carried out adaptive laboratory evolution (ALE) to further explain the molecular basis of the $\Delta trxB2$ mutant and discovered that nucleotide metabolism is influenced.

RESULTS

Effect of inactivating *trxB2* on aerobic growth. A simple way to explore the role of a gene is to knock it out and observe whether this changes the phenotype under various conditions. Since we suspected that the *trxB2* gene could be involved in the oxidative stress response, we decided to inactivate it under anaerobic conditions, and this resulted in strain JC085. The $\Delta trxB2$ mutant was first examined on GM17 agar plates under aerobic conditions and anaerobic conditions using the wild-type strain as a benchmark. The proliferation of the $\Delta trxB2$ mutant was severely hampered by oxygen, and no colonies formed after an overnight incubation (16 h) at 30°C, while MG1363 grew normally (Fig. 1A). Under anaerobic conditions, the $\Delta trxB2$ mutant grew just as well as the wild-type MG1363 (Fig. 1B).

Complementation of a *trxB2* allele in the $\Delta trxB2$ derivative.

On the chromosome, the *trxB2* gene is adjacent to the *ccpA* gene

(approximately 100 bp between the coding sequences [CDSs]), which encodes the important catabolite control protein A (15). It is possible, although unlikely, that deletion of *trxB2* can affect transcription of *ccpA* and in this way somehow alter the gene expression profile under aerobic conditions, which could affect growth. To rule out the possibility that such secondary effects were responsible for the oxygen-sensitive growth phenotype observed, a wild-type *trxB2* allele was introduced into the $\Delta trxB2$ mutant. Two plasmid constructs were made, where the *trxB2* gene was transcribed from either the native promoter or a synthetic promoter. These plasmids as well as plasmids without an insert were introduced into the $\Delta trxB2$ strain, and the growth profiles of the resulting strains were then determined in liquid M17 medium containing 0.2% glucose as described in Materials and Methods.

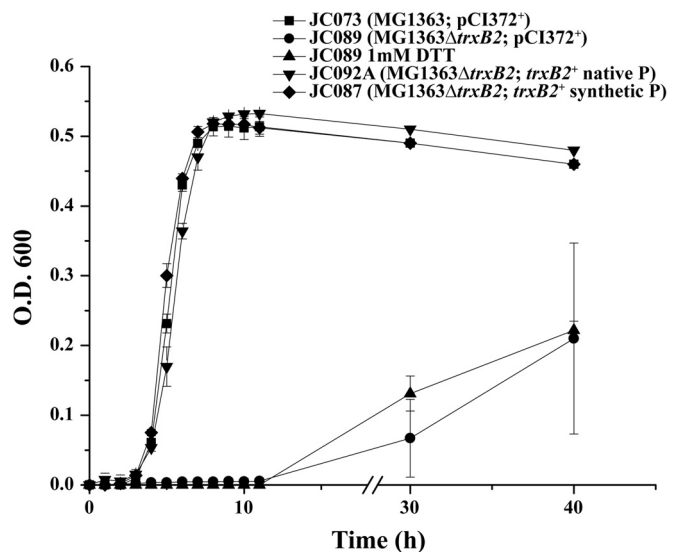


FIG 2 Complementation of the $\Delta trxB2$ mutant. The $\Delta trxB2$ mutant (JC085) containing plasmids expressing *trxB2*, either from its native promoter (JC087) or from a synthetic promoter (JC092A), were characterized in liquid M17 medium using a Bioscreen. As controls, the wild-type strain MG1363 containing the empty vector (JC073) and the $\Delta trxB2$ mutant with the empty vector (JC089) were included. In this experiment, the effect of DTT was also tested. For further information regarding growth conditions, see Materials and Methods.

TABLE 1 Comparison of the amino acid sequences of *L. lactis* TrxB2, *B. subtilis* ferredoxin (flavodoxin) reductase, and the thioredoxin reductases from *L. lactis* and *E. coli*

Sequence	% similarity	% identity	% gap	Protein length	Expected value
<i>L. lactis</i> _TrxB2	100	100	0	321	0
<i>B. subtilis</i> _FdR	62	42	3	332	4.0E-88
<i>L. lactis</i> _TrxR	41	25	5	308	4.0E-21
<i>E. coli</i> _TrxR	45	27	8	321	4.0E-29

MG1363 was included as a benchmark. Compared to the wild-type strain, the $\Delta trxB2$ mutant (JC089) exhibited an extremely long lag phase, and the final cell density was only half of that achieved by the wild-type strain after 40 h of growth (Fig. 2). Complementation with the wild-type *trxB2* allele, expressed from either the native or a synthetic promoter (yielding JC087 and JC092A), resulted in a complete recovery of growth. Addition of DTT has previously been shown to rescue aerobic growth of an *L. lactis* strain with inactivated thioredoxin reductase (*trxB1*) (7). However, adding DTT to the medium did not significantly improve growth of the $\Delta trxB2$ mutant (Fig. 2).

Heterologous expression of *yumC* from *Bacillus subtilis* in the $\Delta trxB2$ mutant. To further explore the biological function of *trxB2*, a BLAST search of the deduced amino acid sequence was performed, and the results suggested that TrxB2 was a YumC-like ferredoxin (flavodoxin) reductase (FdR) (YumC is a ferredoxin [flavodoxin] reductase found in *B. subtilis*). Next, an additional comparison of the protein similarity was carried out in which the amino acid sequence of TrxB2 was aligned with sequences for thioredoxin reductases from *L. lactis* (TrxR_Ll) and *Escherichia coli* (TrxR_Ec) and with the ferredoxin reductase (FdR) from *Bacillus subtilis* (FdR_Bc). This comparison revealed an overall high similarity (at least 40%) of TrxB2 to both the FdR and TrxRs (Table 1). Several conserved amino acid residues, which are pre-

sented in the same color in Fig. 3, were also found within the sequences. The similarity between TrxB2 and FdR_Bc was found to be 62%, which is approximately 20% higher than its similarity with TrxR_Ll and TrxR_Ec (Table 1). Furthermore, both TrxB2 and FdR_Bc were found to lack the conserved cysteine residues Cys146 and Cys149 (in yellow) in the sequences compared to the thioredoxin reductases (Fig. 3).

Since the ferredoxin (flavodoxin) reductase feature of FdR_Bc (YumC) was confirmed previously (14), heterologous expression of FdR_Bc was carried out to see if this protein could complement the $\Delta trxB2$ mutant, which would support the conclusion that TrxB2 functions as a ferredoxin (flavodoxin) reductase. Figure 4 shows the growth profile of the $\Delta trxB2$ mutant expressing FdR_Bc. FdR_Bc indeed was able to complement the *trxB2* mutation, and the strain grew just as well as the wild-type strain (Fig. 4).

Adaptive evolution of the $\Delta trxB2$ mutant and genome resequencing. Although the sequence comparison and the complementation experiment strongly indicated that TrxB2 is a ferredoxin (flavodoxin) reductase, its involvement in aerobic growth remains unclear. Suppressor mutations alleviating the effect of the $\Delta trxB2$ mutant could provide important leads to the role of this protein, and for this reason an adaptive laboratory evolution experiment was carried out. After 2 weeks of evolution, corresponding to around 80 generations of growth, two fast-growing mutants

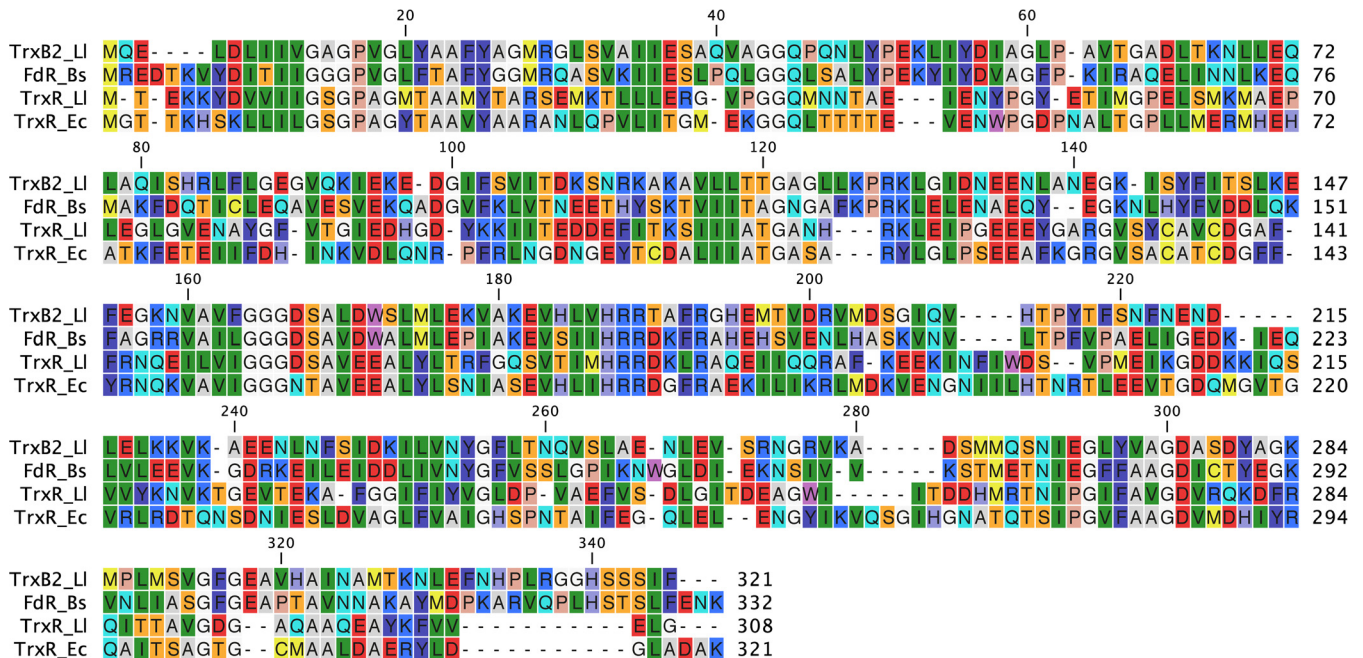


FIG 3 Alignment of amino acid sequences for TrxB2, ferredoxin (flavodoxin) reductase, and thioredoxin reductase from different organisms. Ll, *L. lactis*; Bc, *B. subtilis*; Ec, *E. coli*. In the text, when referring to a particular amino acid position, the numbers shown above the sequences are used.

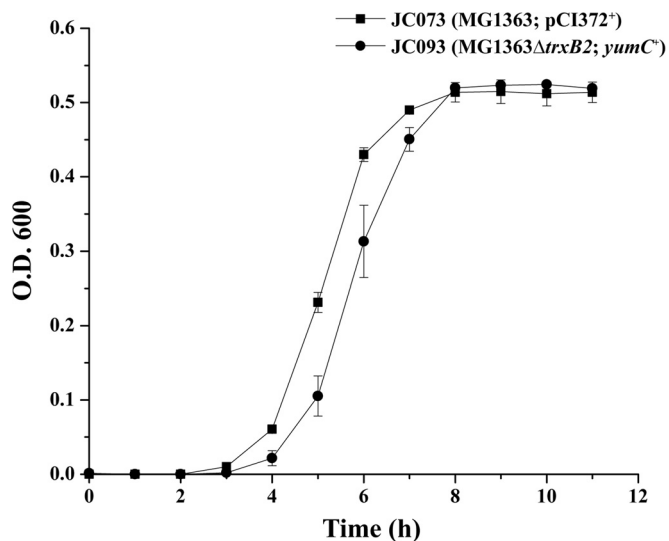


FIG 4 Effect of heterologous expression of *yumC* on the growth of the Δ *trxB2* mutant. The Δ *trxB2* mutant (JC085) containing a plasmid expressing *B. subtilis yumC* from a synthetic promoter (JC093) and the wild-type strain with an empty plasmid (JC073) were characterized in liquid M17 medium using a Bioscreen. For further information regarding growth conditions, see Materials and Methods.

(JC091B and JC091C) were isolated from two independent evolutionary lines. Subsequent growth experiments showed that these two mutants had become oxygen tolerant and could grow just as well as the wild-type strain (Fig. 5).

Genome resequencing revealed one mutation in JC091B and three in JC091C, and these mutations were at different locations (Table 2). Intriguingly, in both isolates, a single mutation was found in the *nrdI* gene, which encodes a ribonucleotide reductase NrdI (flavodoxin). In JC091B, a single nucleotide substitution (T to C) resulted in an amino acid change of tyrosine 45 to histidine, and in JC091C, a substitution of T to G resulted in a change of phenylalanine 15 to leucine in NrdI. When NrdI of *L. lactis* was aligned with NrdI of *B. subtilis* (16), the Tyr45-to-His substitution in JC091B was found to be located next to the FMN binding residue (YTT), and the Phe15-to-Leu substitution was on the flanking region of the FMN binding residue (SKTGNV) within 2 amino acids (see Fig. S1 in the supplemental material). One additional single nucleotide polymorphism (SNP) (reference position 446912) was found in JC091C, which was located in the intergenic region preceding the 5' untranslated region of the gene *treR* and *llmg_0453*. These two genes encode a trehalose operon transcriptional repressor and sucrose-specific PTS enzyme IIABC, respectively. A large gene deletion (~12 kb) was also noticed in JC091C. The lost fragment contains 12 genes (*llmg_1347* to *llmg_1358*), and most of them encode tellurium resistance-related proteins.

Effect of adding deoxynucleosides on the aerobic growth of the Δ *trxB2* mutant. The occurrence of different mutations in *nrdI* in two independently adapted mutants suggested that TrxB2 might be involved in ribonucleotide reduction. To test this hypothesis, aerobic growth in the presence of deoxyribonucleosides was assessed. Addition of deoxyribonucleosides had a dramatically positive effect on the Δ *trxB2* mutant, where the lag phase was reduced to 4 h, after which the growth profile was very similar to that of MG1363 (Fig. 6). In contrast, no increase in optical density

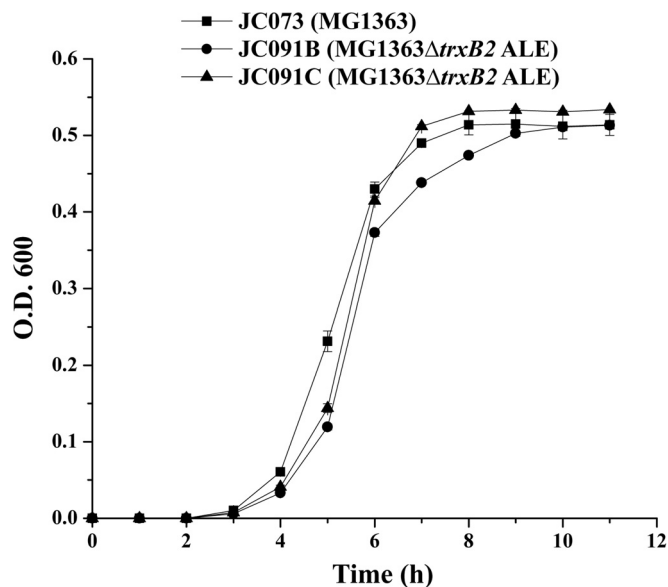


FIG 5 Growth profiles of the strains obtained from the aerobic adaptive evolution of the Δ *trxB2* mutant. The adapted strains JC091B and JC091C as well as the wild-type strain containing the empty plasmid (JC073) were characterized in liquid M17 medium using a Bioscreen. For further information regarding growth conditions, see Materials and Methods.

(OD) was observed for the Δ *trxB2* mutant growing without exogenous deoxyribonucleosides within 24 h.

Inactivation of the *yumC* gene in *B. subtilis* and complementation by *trxB2*. The *yumC* gene is one of the essential genes for *B. subtilis*, and a direct inactivation was not achievable (17). Therefore, the *yumC* gene was conditionally inactivated using the integration vector pMUTIN2 (18). After integration, the original promoter had been replaced by P_{spac} , the expression of which can be tightly controlled by isopropyl- β -D-thiogalactopyranoside (IPTG) (18). The *yumC*-inactivated mutant JC0109 was not able to grow on LB agar plates without addition of IPTG (data not shown). When the *trxB2* gene from *L. lactis* was expressed in JC0109, normal colonies formed on LB agar plates in the absence of IPTG (see Fig. S2B in the supplemental material), whereas only a few colonies formed for JC0109 carrying the empty vector (see Fig. S2A). In contrast to their beneficial effect on aerobic growth of the *L. lactis* Δ *trxB2* mutant, deoxynucleosides failed to compensate for the lethal phenotype of *yumC* knockout strain (data not shown).

DISCUSSION

In this study, we found that the *trxB2* gene has an essential role in *L. lactis* when the bacterium grows in the presence of oxygen, whereas growth is unaffected in the absence of oxygen (Fig. 1). Deletion of the catabolic regulator gene *ccpA*, which is adjacent to the *trxB2* gene on the chromosome, has been shown to have an effect on not only carbon catabolism but also on the behavior and the tolerance to oxidative stresses under aerobic conditions in *L. lactis* (19). To rule out such secondary effects, we carried out a complementation experiment where *trxB2* was expressed from a plasmid in the mutant, and this demonstrated that the phenotype observed indeed was due to the deletion of *trxB2*.

The *trxB2* gene is annotated as encoding a thioredoxin reduc-

TABLE 2 Variations identified on the chromosomes of the aerobically adapted mutants

Strain	Reference position	Variation type	Nucleotide change	Gene	Protein	Amino acid change
JC091B	1513984	SNP	T → C	<i>nrdI</i>	Ribonucleotide reductase NrdI	Tyr45 → His
JC091C	1513896	SNP	T → G	<i>nrdI</i>	Ribonucleotide reductase NrdI	Phe15 → Leu
	446912	SNP	C → T		Intergenic	
	1321264–1333763	Deletion		<i>llmg_1347-llmg_1358</i>		

tase due to its high similarity with these proteins (15), but growth of the $\Delta trxB2$ mutant could not be restored by adding DTT, as has been observed to work for thioredoxin reductase mutants (7). This observation in conjunction with the fact that the active-site residues CXXC (Cys146 and Cys149 in Fig. 3) are lacking, indicate that the *trxB2* gene would have another function for aerobic growth.

YumC represents a novel type of FdR which exists in many bacteria and which was recently characterized (14). It functions as an FdR, as it is able to reduce cytochrome *c* at a high rate using NADPH as an electron donor in the presence of ferredoxin (14). Two arguments support the conclusion that TrxB2 functions as a YumC-like FdR in *L. lactis*. First, the amino acid sequence of TrxB2 is very similar to that of the YumC-like FdR from *B. subtilis* (Table 1; Fig. 3), and second, YumC is able to suppress the oxygen-sensitive phenotype of the $\Delta trxB2$ mutant (Fig. 4).

FdRs have been found to be involved in many different biological processes, such as photosynthesis, nitrogen fixation, and reduction of cytochrome P450 in both prokaryotes and eukaryotes (20). Four types of FdR have been identified so far: (i) a plant type, including cyanobacterial FdR; (ii) a bacterial type, such as the *E. coli* FdR; (iii) a mitochondrial type or adrenodoxin reductase type; and (iv) a novel bacterial type represented by YumC in *B. subtilis* (14). The amino acid sequences of YumC-like FdRs are

more similar to that of TrxR than those of other types of FdR, but their biological role has remained unclear (14).

To the best of our knowledge, no studies have reported an important role for YumC-like ferredoxin (flavodoxin) reductases for aerobic growth of bacteria. After a simple ALE experiment for the $\Delta trxB2$ mutant under aerobic conditions, independent SNPs were identified in *nrdI*. *nrdI* encodes a flavodoxin involved in the maintenance of the diferric-tyrosyl radical cofactor in the class Ib ribonucleotide reductase (RnR) (see Fig. S3 in the supplemental material) (21). The fact that two independent suppressor mutations appear in *nrdI* suggests that the *trxB2* gene is involved in ribonucleotide reduction. This was further substantiated by the addition of deoxynucleosides to the growth medium, which could restore growth of the $\Delta trxB2$ mutant when oxygen was present (Fig. 6).

In *L. lactis*, formation of deoxynucleotides is conducted by the class Ib RnR (*nrdEF*) and class III RnR (*nrdDG*), which function under aerobic and anaerobic conditions, respectively (22), where the class Ib RnR rely on a diferric-tyrosyl radical cofactor which requires oxygen for generation (see Fig. S3 in the supplemental material), and the class III RnRs utilize an oxygen-sensitive glycol radical (23). Therefore, when the class Ib system is disabled in *L. lactis*, normal growth is possible only when oxygen is depleted or exogenous deoxynucleosides are provided (22). In the current model of the class Ib *nrdEF* RnR, NrdI functions as an electron reductant of the tyrosyl radical metalloprotein NrdF (met-NrdF) in the maintenance pathway (reduction of the diferric-tyrosyl radical complex) (see Fig. S3 in the supplemental material) as its analogue YfaE in the class Ia RnR in other bacteria (21). Besides this, flavodoxin together with the second-type FdR are also involved in activation of the class III RnR enzyme (the anaerobic type) in bacteria such as *E. coli* (24, 25). In the current model, NrdI serves as a one-electron reductant, while two electrons should be transferred simultaneously for the reduction of met-NrdF to diferrous NrdF (see Fig. S3). Therefore, a ferredoxin (flavodoxin) reductase is most likely needed to facilitate efficient electron transfer (21). However, the existence of this partner FdR has not been experimentally confirmed yet, and currently it is the only missing component in the model (21). It was suspected that in *E. coli*, a Fre-like ferredoxin (flavodoxin) reductase could be involved, but inactivation of the *fre* gene failed to influence aerobic growth of *E. coli*, which indicates that other undiscovered participants are involved in the process (26, 27). The positive effect of deoxynucleosides and the mutations in *nrdI* on the aerobic growth of the $\Delta trxB2$ mutant are the two arguments for suggesting that TrxB2 participates in the formation of deoxynucleotides by the class Ib *nrdEF* in *L. lactis*. The chemical reductant DTT did not have any significant effects on the proliferation of the $\Delta trxB2$ mutant when oxygen was present (Fig. 2), although DTT at a high pH (pH 8.8) has been shown to be able to lead to reduction of the tyrosyl radical

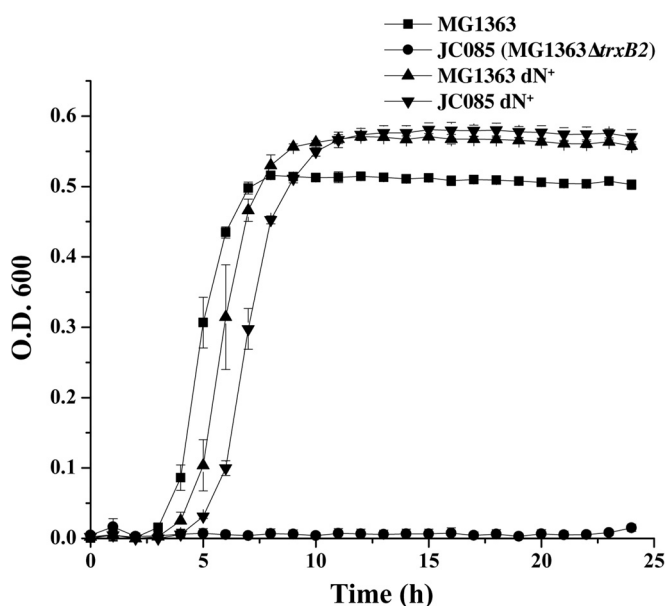


FIG 6 Positive effect on growth of the $\Delta trxB2$ mutant of deoxynucleosides. The wild-type MG1363 and its *trxB2* deletion derivative (JC085) were characterized in liquid M17 medium using a Bioscreen. Cultures to which deoxynucleosides were added are indicated with “dN+.” For further information regarding growth conditions, see Materials and Methods.

metalloprotein (28). The reason for the lack of a stimulatory effect is probably a too-slow reaction rate, especially at the lower pH (below 7) optimal for *L. lactis* growth (28).

In *B. subtilis*, *yumC* has been found to be indispensable for proliferation, which was demonstrated by replacing the native promoter of *yumC* with one that is IPTG inducible (17). We also replaced the native promoter with an IPTG-inducible promoter and observed the same behavior. When *trxB2* was heterologously expressed in this strain, the proliferation continued in the absence of IPTG, thus allowing us to demonstrate that TrxB2 can also be substituted for YumC (see Fig. 2SB in the supplemental material). Few colonies were observed for the control strain bearing the empty vector (pHT254) (see Fig. 2SA), but these are most likely mutants that had been affected in *lacI* and/or *P_{spac}* in pMUTIN2 on the chromosome and therefore express *yumC*. This conclusion is also strengthened by the observation that colonies can be found only in the streak where the cell density is the highest (see Fig. 2SA). Deoxynucleosides did not improve the viability of the *yumC* mutant, which indicates that YumC could have other/additional important roles in the life cycle of *B. subtilis*, and this result might not be surprising, because the role of essential genes differs among organisms due to differences in their natural environment (29). The *yumC* gene was indispensable for *B. subtilis* (17), while *L. lactis* was still able to survive when *trxB2* was deleted (Fig. 2).

Another intriguing finding of this study is that mutations in *nrdI* were able to compensate for the loss of TrxB2 and allow normal growth under aerobic conditions. Flavodoxin depends on a noncovalently bound FMN as the redox-active component, and the bound FMN can be in two different redox states (semiquinone or hydroquinone) during its involvement in various oxidation-reduction reactions (30). Amino acid substitutions near the FMN binding residues have been shown to perturb the redox potential of the different forms of bound FMN in NrdI to various degrees (30, 31). We found that growth was not completely halted without TrxB2 under aerobic conditions, and this observation, together with the finding that it is possible to select the *nrdI* suppressor mutants, indicates that there is a redundancy in the capacity for reducing this tyrosyl radical metalloprotein (met-NrdF) in *L. lactis*. Whether the two amino acid substitutions, which are located close to the FMN binding sites, result in a perturbation of the redox potential and how this potentially could assist growth in the absence of TrxB2 require further investigation.

The results of this study shed light on the biological role of the newly identified YumC-type FdR and provide new information that could help to refine the current model describing the class Ib *nrdEF* RnR systems that are involved in aerobic ribonucleotide reduction in both prokaryotes and eukaryotes, where the existence of a partner FdR for NrdI has been suggested. Further investigations should be conducted to fully understand the interactions between FdR (TrxB2), flavodoxin (NrdI), and the tyrosyl radical metalloprotein (NrdF) in *L. lactis* revealed in this study.

MATERIALS AND METHODS

Strains and growth conditions. *L. lactis* MG1363 (32), *B. subtilis* 168 (ATCC 23857), and the derivatives thereof used in this study are listed in Table S1 in the supplemental material. *E. coli* TOP10 (Invitrogen) was used for cloning purposes in this study. M17 broth (33) supplemented with 0.2% glucose was utilized as the growth medium for *L. lactis*. *B. subtilis* and *E. coli* were aerobically cultivated in lysogeny broth (LB) (34) at

37°C. When necessary, a mixture of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine (Sigma) was supplied at a concentration of 1 g/liter. Both chloramphenicol and erythromycin were supplied at a concentration of 5 µg/ml for *L. lactis* and *B. subtilis*, and ampicillin was supplied at a concentration of 100 µg/ml for *E. coli*.

Growth assessment for *L. lactis* was carried out in the following manner: overnight cultures in test tubes were diluted 2,000 times in fresh medium, and 200 µl culture was transferred into a honeycomb 2 plate (Oy Growth Curves Ab Ltd.). Growth experiments were carried out using a Bioscreen-C automated growth curve analysis system (Oy Growth Curves Ab Ltd.) at 30°C under an air atmosphere, where cell density was monitored by measuring optical density (OD) at 600 nm. To keep cells in suspension, shaking was performed prior to each measurement.

For the strict anaerobic conditions, cells were incubated in a 2.5-liter anaerobic jar, where the anaerobic environment was generated using the AnaeroGen system (Thermo Scientific).

DNA techniques All manipulations were performed as described by Sambrook et al. (34). A description of the PCR primers used can be seen in Table S2 in the supplemental material. PfuX7 polymerase (35) was used for PCR applications. Chromosomal DNA from *L. lactis* was isolated by using the method described for *E. coli* with the modification that cells were treated with 20 µg of lysozyme per ml for 2 h instead of 30 min. Cells of *L. lactis* were made electrocompetent by growth in GM17 medium containing 1% glycine and transformed by electroporation as previously described by Holo and Nes (36). The plasmid vector pCS1966 (37) was used for deleting genes in *L. lactis*. Generally, when chromosomal genes were being deleted, 800-bp regions upstream and downstream of the deleted region were PCR amplified and inserted into pCS1966. The resulting plasmids were used as described previously (37).

Deleting the *trxB2* gene. The derivative of pCS1966 for deleting the *trxB2* gene was constructed as described above using primers JC0140 and JC0141 and primers JC0142 and JC0143. Deletion was verified using the primers JC0182 and JC0183. The strain containing the *trxB2* deletion was designated JC085.

Construction of the Δ *trxB2* mutant complemented with the *trxB2* allele on a plasmid. A *trxB2* allele was introduced into the Δ *trxB2* mutant on the vector pCI372 (38) expressed from either a native promoter or a synthetic promoter. For the native promoter, the CDS including the promoter region of *trxB2* was amplified using the primers JC0178 and JC0179. The *groESL* terminator region from *L. lactis* MG1363 was also amplified using the primers JC0009 and JC0010. After digestion using EcoRI/XbaI and XbaI/PstI (Invitrogen), respectively, these two fragments were ligated into the multiple cloning sites of the vector pCI372 and introduced into JC085. Successful transformants were selected on agar plates under the strict anaerobic conditions described above, where growth of the Δ *trxB2* mutant is unaffected.

For expression from synthetic promoters, the CDS of *trxB2* was fused to a library of synthetic promoters using a previously published method (39) using the primers JC0206 and JC0207. The resulting PCR product was digested with EcoRI and PstI and ligated into pCS4518, which is a pCI372 derivative containing a *gusA* gene as the reporter, and subsequently introduced into JC085. The following procedure was the same as the one used for the native promoter.

Heterologous expression of *yumC* in the Δ *trxB2* mutant. The procedure used for heterologous expression of *yumC* was identical to the one used for expressing *trxB2* in the Δ *trxB2* mutant using the synthetic promoter as mentioned above. The nucleotide sequence of *yumC* in *B. subtilis* subsp. *subtilis* strain 168 was obtained from NCBI (GenBank no. CP010052.1). The template nucleotide sequence was codon optimized for *L. lactis* (see Fig. S4 in the supplemental material) and synthesized by GenScript. The PCR amplification was performed using the primers JC0212 and JC0213.

Sequence analysis. All the nucleotide and deduced amino acid sequences of *nrd* were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>).

Alignment and comparison of amino acid sequences were conducted using CLC, Main Workbench (Qiagen).

Procedure for adaptive evolution. The evolution was started from the $\Delta trxB2$ derivative of *L. lactis* MG1363 (JC089) and conducted using a serial-transfer regime for two independent lines in M17 containing 0.2% glucose and 5 μ g/ml chloramphenicol. For this purpose, 5 ml culture in a 20-ml test tube was incubated in a temperature-controlled shaker (200 rpm) at 30°C. When the culture entered the stationary phase, 0.5 ml culture was transferred into a new test tube with 4.5 ml fresh medium, which corresponds to 3.32 generations of growth in each tube. Each week, a culture sample was saved in 25% glycerol at -80°C in order to track the evolution. In total, cells were adapted under aerobic conditions for 77 generations. Culture from the final tubes of the two independent evolutionary lines was streaked on plates and incubated aerobically. In this way, two single colonies that grew similarly to MG1363 were isolated from each of the evolutionary lines, and the clones were designated JC091B and JC091C.

Genome resequencing and mutation discovery. Genomic DNA of the mutant was purified using a DNeasy blood and tissue kit (Qiagen) and the quality was checked by DNA electrophoresis and NanoDrop 1000 (Thermo Scientific) analysis. Genome sequencing was performed by the Beijing Genomics Institute (BGI). The procedure, described briefly, was as follows. A 2- μ g portion of genomic DNA was randomly sheared using a nebulizer (Illumina), and the ends were repaired using polynucleotide kinase and Klenow enzyme. The 5' ends of the DNA fragments were phosphorylated, and a single adenine base was added to the 3' ends using Klenow exo^{+} (Illumina). Following ligation of a pair of Illumina adaptors to the repaired ends, the DNA was amplified in 10 cycles, using adaptor primers (Illumina), and fragments of around 150 bp were isolated using agarose gel electrophoresis. Sequencing libraries were quantified with a 2100 BioAnalyzer DNA 1000 chip (Agilent) as well as the Picogreen fluorescence assay (Invitrogen). Cluster generations were performed on an Illumina cluster station using 11 pmol of sequencing libraries. A total of 38 cycles of sequencing were carried out using the Illumina Ix genome analyzer system according to the manufacturer's specifications. CLC Genomics Workbench (Qiagen) was used for mapping the reads, SNP and DIP (deletion-insertion polymorphism) detection, and identification of genomic rearrangement using the published genome sequence of *L. lactis* MG1363 (15) as the reference.

Inactivation of *yumC* and heterologous expression of *trxB2* in *B. subtilis*. For inactivation of *yumC*, a 500-bp fragment which contained the leader sequence and 5' region of *yumC* was amplified using primers JC0347 and JC0348. The PCR product was digested with BamHI and EcoRI and cloned into the MCS of pMUTIN2. The plasmid was transformed into *B. subtilis* using the standard protocol developed by Jarmer et al. (40), except that erythromycin and IPTG were supplied for preparation of competent cells. Integrants were screened on LB agar plates containing 5 μ g/ml erythromycin and 0.3 mM IPTG (Sigma). Verification of successful integrants was performed by PCR using primers JC0189/JC0342 followed by Sanger sequencing. The successful integrant, where the expression of *yumC* was controlled by addition of IPTG, was designated JC0109.

For the heterologous expression of *trxB2* from *L. lactis* in JC0109, the *trxB2* allele, which was led by the synthetic promoter, was PCR amplified using primers JC0179 and JC0352 from the chromosome DNA of JC092A. The PCR fragment was digested with BamHI and XbaI and ligated with pHT254, which was amplified using primers JC0354 and JC0355 and treated with the same restriction enzymes. The resulting plasmid and pHT254 were transformed into JC0109 according to the abovementioned protocol, resulting in JC0111 and JC0112, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01132-15/-/DCSupplemental>.

Figure S1, TIF file, 1.1 MB.

Figure S2, TIF file, 1.4 MB.

Figure S3, TIF file, 0.2 MB.

Figure S4, TIF file, 0.5 MB.

Table S1, PDF file, 0.1 MB.

Table S2, PDF file, 0.04 MB.

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