Deletion of *nudB* Causes Increased Susceptibility to Antifolates in *Escherichia coli* and *Salmonella enterica*

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ABSTRACT Co-trimoxazole, a fixed-dose combination of sulfamethoxazole (SMX) and trimethoprim (TMP), has been used for the treatment of bacterial infections since the 1960s. Since it has long been assumed that the synergistic effects between SMX and TMP are the consequence of targeting 2 different enzymes of bacterial folate biosynthesis, 2 genes (*pabB* and *nudB*) involved in the folate biosynthesis of *Escherichia coli* were deleted, and their effects on the susceptibility to antifolates were tested. The results showed that the deletion of *nudB* resulted in a lag of growth in minimal medium and increased susceptibility to both SMX and TMP. Moreover, deletion of *nudB* also greatly enhanced the bactericidal effect of TMP. To elucidate the mechanism of how the deletion of *nudB* affects the bacterial growth and susceptibility to antifolates, 7,8-dihydroneopterin and 7,8-dihydropteroate were supplemented into the growth medium. Although those metabolites could restore bacterial growth, they had no effect on susceptibilities to the antifolates. Reverse mutants of the *nudB* deletion strain were isolated to further study the mechanism of how the deletion of *nudB* affects susceptibility to antifolates. Targeted sequencing and subsequent genetic studies revealed that the disruption of the tetrahydromonapterin biosynthesis pathway could reverse the phenotype caused by the *nudB* deletion. Meanwhile, overexpression of *folM* could also lead to increased susceptibility to both SMX and TMP. These data suggested that the deletion of *nudB* resulted in the excess production of tetrahydromonapterin, which then caused the increased susceptibility to antifolates. In addition, we found that the deletion of *nudB* also resulted in increased susceptibility to both SMX and TMP in *Salmonella enterica*. Since dihydroneopterin triphosphate hydrolase is an important component of bacterial folate biosynthesis and the tetrahydromonapterin biosynthesis pathway also exists in a variety of bacteria, it will be interesting to design new compounds targeting dihydroneopterin triphosphate hydrolase, which may inhibit bacterial growth and simultaneously potentiate the antimicrobial activities of antifolates targeting other components of folate biosynthesis.

KEYWORDS *nudB*, sulfamethoxazole, susceptibility, tetrahydromonapterin, trimethoprim

Co-trimoxazole, a fixed-dose combination of sulfamethoxazole (SMX) and trimethoprim (TMP), has been used as a broad-spectrum antibacterial drug since the 1960s. Initially, co-trimoxazole was used to treat urinary tract infections and lower respiratory tract infections (1, 2), but later on, it was found to have good prophylactic activity against *Pneumocystis jirovecii* pneumonia (3). The use of co-trimoxazole greatly increased with the emergence of the HIV epidemic in the 1980s, since it provided good protection against various opportunistic infections during

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the pre-antiretroviral therapy (ART) era (4–9). In recent years, researchers have found that co-trimoxazole is able to prevent the development of tuberculosis in HIV-positive patients, especially in those who have not received prior ART (10). At present, co-trimoxazole is still one of the first-line drugs for the treatment of urinary tract infections, skin and soft tissue infections, and community-acquired methicillin-resistant *Staphylococcus aureus* infections (11); however, the widespread use of co-trimoxazole has resulted in increasing resistance, just like with many other antimicrobial agents (12). Thus, it has become a challenge to improve the antibacterial activity of co-trimoxazole and reduce the emergence of co-trimoxazole-resistant bacteria at the same time.

Both SMX and TMP are antifolates, which inhibit the growth of bacterial cells by blocking bacterial folate biosynthesis. SMX and TMP target 2 different enzymes involved in bacterial folate biosynthesis: SMX targets dihydropteroate synthetase, while TMP targets dihydrofolate reductase (13, 14). This is the long-assumed reason why these 2 compounds have synergistic effects (15). If this assumption is true, it may be possible to design new compounds that target other critical enzymes involved in bacterial folate biosynthesis, which could potentiate the antibacterial effect of either SMX or TMP. To test this hypothesis, we deleted the *nudB* gene (which is involved in the *de novo* biosynthesis of folate) in both *Escherichia coli* and *Salmonella enterica* and then tested its impact on the susceptibilities to SMX and TMP. The results are reported herein.

RESULTS

Effects of *nudB* and *pabB* deletion on bacterial susceptibility to SMX and TMP in *E. coli*. It has long been assumed that the reason why SMX and TMP have a good synergistic effect is because these 2 drugs target 2 different enzymes in the bacterial folate biosynthesis pathway. If this is the case, the knockout of other genes involved in folate biosynthesis could potentiate the antibacterial effect of either SMX or TMP, or both. The *pabB* and *nudB* genes were knocked out in *E. coli*, and we tested the susceptibilities of these 2 mutants to SMX, TMP, rifampin (RIF), and ampicillin. The results showed that the deletion of *pabB* did not affect the susceptibility of *E. coli* to any of the drugs tested; however, the deletion of *nudB* made *E. coli* hypersensitive to both SMX and TMP, as determined by the MIC tests (Table 1). In addition, the MICs of SMX and TMP for the *nudB* deletion mutant were determined in the presence of exogenous dihydropteroate and 7,8-dihydroneopterin, and the results showed that these additions had no effect on SMX and TMP susceptibilities of the mutant (Table 2). The susceptibility of the *nudB* deletion mutant to other antibacterial drugs (rifampin and ampicillin) was also tested, and the results show that the deletion of *nudB* does not affect the susceptibilities to these 2 drugs (MIC for rifampin, 8 μ g/ml; MIC for ampicillin, 4 μ g/ml).

Effect of the *nudB* deletion on the growth of *E. coli*. The *nudB* gene encodes dihydroneopterin triphosphate pyrophosphohydrolase, which is also an important enzyme involved in bacterial folate biosynthesis. Previously, Gabelli et al. found that even though the deletion of *nudB* resulted in a remarkable decrease in folate concentration, the *nudB* knockout mutant did not have any growth defects in *E. coli* (16).

Growth curves of the wild-type and the *nudB* deletion strains were compared using E minimal medium with or without exogenous folate intermediate metabolites (dihydropteroate and dihydroneopterin) to determine the effect of *nudB* deletion on bacterial growth. As shown in Fig. 1A, when W3110 Δ *nudB* was grown in E minimal medium, it showed a prolonged lag phase of growth, which could be completely reversed by adding exogenous dihydroneopterin (Fig. 1C) and partially reversed by dihydropteroate (Fig. 1B).

Effect of the *nudB* deletion on the bactericidal effect of TMP against *E. coli*. TMP exposure experiments were performed to further test the effect of the *nudB* deletion on the bactericidal effect of TMP against *E. coli*. The results show that the cell numbers of

TABLE 1 Susceptibilities of different strains to TMP and SMX

Strain	MIC ($\mu\text{g/ml}$) for:	
	TMP	SMX
<i>E. coli</i> W3110	0.32	128
<i>E. coli</i> W3110/pCA24N	0.32	128
<i>E. coli</i> W3110/pCA24N- <i>nudB</i>	0.64	256
<i>E. coli</i> W3110/pCA24N- <i>folX</i>	0.32	128
<i>E. coli</i> W3110/pUC19	0.32	128
<i>E. coli</i> W3110/pUC19- <i>folM</i>	0.16	64
<i>E. coli</i> W3110 $\Delta nudB$	0.02	4
<i>E. coli</i> W3110 $\Delta nudB$ /pCA24N	0.02	4
<i>E. coli</i> W3110 $\Delta nudB$ /pCA24N- <i>nudB</i>	0.32	128
<i>E. coli</i> W3110 $\Delta nudB$ /pUC19	0.02	4
<i>E. coli</i> W3110 $\Delta nudB$ /pUC19- <i>folM</i>	0.01	1
<i>E. coli</i> W3110 $\Delta folX$	0.32	128
<i>E. coli</i> W3110 $\Delta folX$ /pCA24N	0.32	128
<i>E. coli</i> W3110 $\Delta folX$ /pCA24N- <i>folX</i>	0.32	128
<i>E. coli</i> W3110 $\Delta folM$	0.32	128
<i>E. coli</i> W3110 $\Delta folM$ /pCA24N	0.32	128
<i>E. coli</i> W3110 $\Delta folM$ /pCA24N- <i>folM</i>	0.32	128
<i>E. coli</i> W3110 $\Delta nudB \Delta folX$	0.32	128
<i>E. coli</i> W3110 $\Delta nudB \Delta folX$ /pCA24N	0.32	128
<i>E. coli</i> W3110 $\Delta nudB \Delta folX$ /pCA24N- <i>folX</i>	0.02	8
<i>E. coli</i> W3110 $\Delta nudB \Delta folM$	0.32	128
<i>E. coli</i> W3110 $\Delta nudB \Delta folM$ /pUC19	0.32	128
<i>E. coli</i> W3110 $\Delta nudB \Delta folM$ /pUC19- <i>folM</i>	0.02	8
<i>S. enterica</i> serovar Typhimurium LT2	0.32	1,024
<i>S. enterica</i> serovar Typhimurium LT2/pUC19	0.32	1,024
<i>S. enterica</i> serovar Typhimurium LT2/pUC19- <i>folM</i>	0.16	512
<i>S. enterica</i> serovar Typhimurium LT2 $\Delta nudB$	0.08	256
<i>S. enterica</i> serovar Typhimurium LT2 $\Delta nudB$ /pUC19	0.08	256
<i>S. enterica</i> serovar Typhimurium LT2 $\Delta nudB$ /pUC19- <i>folM</i>	0.04	128

the wild-type strain decreased from 7 log₁₀ CFU/ml to a little bit less than 6 log₁₀ CFU/ml after 12 h of TMP treatment; however, cell numbers of the *nudB* deletion strain decreased from 7 log₁₀ CFU/ml to less than 4.5 log₁₀ CFU/ml (Fig. 2). After 24 h of treatment, there was no further decrease in cell numbers for the wild-type strain, but for the *nudB* deletion strain, the cell numbers kept decreasing to less than 3 log₁₀ CFU/ml. From 24 h on, the cell numbers of the wild-type strain started to increase gradually, but the cell numbers of the *nudB* deletion strain remained static until 60 h of treatment. During the course of the TMP treatment, the complemented strain behaved just like the wild-type strain, while the *nudB* overexpression strain was slightly more resistant to the TMP treatment.

Identification of reverse mutations in the *nudB* deletion mutant. Thirty reverse mutants were isolated to further understand the reason why the deletion of the *nudB* gene led to the increased sensitivity to both SMX and TMP. Target sequencing was performed for 2 genes involved in tetrahydromonapterin biosynthesis (*folX* and *folM*). From Table 3, it can be seen that the sequence variations were identified in 18 of these reverse mutants: 7 of the reverse mutants have insertions in either of the 2 genes, 5 of the strains have substitutions, and 6 of them have deletions. Eleven of the 30 mutants have no mutations in the 2 genes. For those reverse mutants that have mutations in

TABLE 2 Effects of 7,8-dihydropteroate (DHP) and 7,8-dihydroneopterin (DHNP) on susceptibility of *E. coli* W3110 and *E. coli* W3110 $\Delta nudB$ to TMP

Strain	MIC ($\mu\text{g/ml}$) for:		
	TMP, SMX in absence of DHP and DHNP	TMP, SMX in presence of 20 $\mu\text{g/ml}$ DHP	TMP, SMX in presence of 20 $\mu\text{g/ml}$ DHNP
<i>E. coli</i> W3110	0.32, 128	0.32, 128	0.32, 128
<i>E. coli</i> W3110 $\Delta nudB$	0.02, 4	0.02, 4	0.02, 4

FIG 1 Growth curves of different *E. coli* strains on E minimal medium: in the absence of 7,8-dihydropteroate and 7,8-dihydroneopterin (A), in the presence of 20 μ g/ml DHP (B), and in the presence of 20 μ g/ml DHNP (C). (D and E) Growth curves of W3110 $\Delta nudB$ $\Delta folX$ strain (D) and W3110 $\Delta nudB$ $\Delta folM$ strain (E). Growth of the bacteria at 37°C was measured by taking OD₆₀₀ every 8 or 12 h using the Synergy H1 Hybrid reader (BioTek, USA). Experiments were performed in triplicate.

FIG 2 Time-kill curves of different *E. coli* strains after exposure to 2 µg/ml TMP. Data represent mean $\pm$ standard deviation (SD) of the results from 3 independent experiments. The dashed lines represent the strains treated without drugs, while the solid lines represent strains treated with 2 µg/ml TMP.

either *folX* or *folM*, their MICs for TMP were determined to be identical to that of *E. coli* W3110. In addition, six of the reverse mutants without any mutation in *folX* and *folM* were randomly selected, and their MICs for TMP were all determined to be around 0.32 µg/ml (see Table S3 in the supplemental material). For the 8 reverse mutants that have

TABLE 3 Mutations identified in reverse mutants of *E. coli* $\Delta nudB$

Strain	<i>folX</i> (nt) ^a	<i>folM</i> (nt)
KL-1	WT	WT
KL-2	Insertion at 333 ^b	WT
KL-3	WT	4-base deletion at 654 (TACT)
KL-4	Substitution 59G→A	WT
KL-5	WT	WT
KL-6	WT	WT
KL-7	WT	WT
KL-8	WT	WT
KL-9	WT	WT
KL-10	WT	WT
KL-11	WT	WT
KL-12	Insertion at 207 ^b	WT
KL-13	WT	4-base deletion at 654 (TACT)
KL-14	WT	Substitution 557T→G
KL-15	Insertion at 143 ^b	WT
KL-16	WT	WT
KL-17	Insertion at 140 ^b	WT
KL-18	Insertion at 160 ^b	WT
KL-19	Insertion at 90 ^b	WT
KL-20	WT	4-base deletion at 654 (TACT)
KL-21	Substitutions 2T→G, 5C→T Insertion at 4 (GGCCAGTC)	WT
KL-22	WT	4-base deletion at 654 (TACT)
KL-23	WT	WT
KL-24	WT	WT
KL-25	WT	4-base deletion at 654 (TACT)
KL-26	WT	Substitution 92A→C
KL-27	Insertion at 111 ^b	WT
KL-28	WT	4-base deletion at 654 (TACT)
KL-29	Substitutions 2T→G, 5C→T Insertion at 4 (GGCCAGTC)	WT
KL-30	WT	WT

^ant, nucleotides; WT, wild type.

^bInsertion of large fragment.

insertions in either *folX* or *folM*, DNA sequencing data showed that all of those insertion sequences are from a transposase: GTAATGACTCCAACCTATTGATAGTGTTCAGATAATGCCCCGATGACTTTGTCATGCAGCTCCACCGATTTTGAGAACGACAGCGACTTCCGTCCAGCCGTGCCAGGTGCTGCCTCAGATTACAGTTATGCCGCTCAATTCGCTGCGTATATCGCTTGCTGATTACGTGCAGCTTCCCTTCAGGCGGATTACATACAGCGCCAGCCATCCGTCATCCATATCACCACGTCAAAGGGTGACAGCAGGCTCATAAGACGCCCCAGCGTCGCCATAGTGCGTTACACGAATACGTGCGCAACAACCGTCTCCGGAGACTGTCATACGCGTAAAACAGCCAGCGCTGGCGCGATTTAGCCCCGACATAGCCCCACTGTTTCGTCATTTCCGCGCAGACGATGACGTCCTGCCCCGGCTGTATGCGCGAGGTTACCGACTGCGGCCTGAGTTTTTAAGTGACGTAATCATGTGTTGAGGCCAACGCCCATAATGCGGGCTGTTGCCCGCATCCAACGCCATTTCATGGCCATATCAATGATTTTCTGGTGCGTACCGGGTTGAGAAGCGGTGTAAGTGAAGTGCAGTTGCCATGTTTTACGGCAGTGAGAGCAGAGATAGCGCTGATGTCCGGCGGTGCTTTTGCCGTTACGCACCACCCGTCAGTAGCTGAACAGGAGGGACAGCTGATAGAAACAGAAGCCACTGGAGCACTCAAAAACACCATCATACACTAAATCAGTAAGTTGGCAGCATCACCAGAACCTTCG.

Effect of the disruption of the tetrahydromonapterin biosynthesis pathway on SMX and TMP susceptibility. Two double-knockout mutants (*E. coli* W3110 $\Delta nudB \Delta folX$ and *E. coli* W3110 $\Delta nudB \Delta folM$) were constructed to confirm that the disruption of the tetrahydromonapterin biosynthesis pathway could reverse the phenotype caused by the *nudB* deletion. From Table 1, it can be seen that the susceptibilities to SMX and TMP of both of the double mutants are the same as the wild-type strain, and when the intact *folX* or *folM* gene was transformed into the corresponding double mutant, it became hypersensitive to both SMX and TMP again. Meanwhile, 2 more single-knockout mutants (*E. coli* W3110 $\Delta folX$ and *E. coli* W3110 $\Delta folM$) were constructed, and the susceptibilities of these 2 mutants to both SMX and TMP were also determined. The results showed that the knockout of either *folX* or *folM* had no effect on the susceptibilities to SMX and TMP. The growth curves of the above-mentioned 4 strains were also determined parallel to the wild-type strain and the *nudB* deletion strain. As shown in Fig. 1D and E, *E. coli* W3110 $\Delta folX$, *E. coli* W3110 $\Delta nudB \Delta folX$, *E. coli* W3110 $\Delta folM$, and *E. coli* W3110 $\Delta nudB \Delta folM$ grew as normally as the wild-type strain in the E minimal medium.

Effect of *folM* overexpression on bacterial susceptibility to TMP and SMX. *FolM* catalyzes the formation of tetrahydromonapterin, which is the end product of the tetrahydromonapterin biosynthesis pathway (Fig. 3). To explore the impact of the excess production of tetrahydromonapterin on the susceptibility to TMP and SMX, the *folM* gene from *E. coli* was overexpressed in *E. coli* W3110, *E. coli* W3110 $\Delta nudB$, *S. enterica* serovar Typhimurium LT2, and *S. enterica* serovar Typhimurium LT2 $\Delta nudB$, respectively. The results showed that the overexpression of *folM* caused increased susceptibility to both SMX and TMP in all of the strains tested (Table 1).

Effect of the *nudB* deletion on bacterial susceptibility to SMX and TMP in *S. enterica*. The *nudB* gene was deleted in *S. enterica*, and susceptibility of the *nudB* deletion mutant to SMX and TMP was determined. As shown in Table 1, the deletion of *nudB* also caused increased susceptibility to both of the drugs; however, the *nudB* deletion mutant grew as normally as its parental strain in the E minimal medium (data not shown).

DISCUSSION

In this study, we found that the deletion of *nudB*, which encodes an important component of the folate *de novo* biosynthesis pathway in bacteria, led to increased susceptibility to 2 widely used antifolate drugs (SMX and TMP) in both *E. coli* and *S. Typhimurium*. Thus, it is possible to design a new antimicrobial recipe consisting of compounds targeting different components of the bacterial folate biosynthesis pathway.

The idea that the deletion of other important components of folate biosynthesis might potentiate the antibacterial activity of preexisting antifolates comes from the old story between SMX and TMP, i.e., their synergistic effect. We tried to delete several genes (*pabB*, *pabC*, *nudB*, *folB*, *folK*, *folP*, and *folC*) involved in the *de novo* biosynthesis

FIG 3 Schematic diagram of folate metabolic pathway.

of folate in *E. coli*, but only 2 mutants were obtained (*E. coli* W3110 $\Delta nudB$ and *E. coli* W3110 $\Delta pabB$). The remaining 5 mutants were still in the process of construction. The effects of the deletion of those 2 genes on bacterial growth were analyzed, and the data showed that the deletion of *pabB* did not affect bacterial growth; however, the deletion of *nudB* led to an obvious lag of growth in the E minimal medium. From the beginning, NudB was biochemically characterized to be a nucleoside triphosphate pyrophosphohydrolase, likely a member of the MutT family of proteins. However, the *nudB* gene did not complement the MutT[−] mutator phenotype; on the contrary, the deletion of *nudB* resulted in decreased frequency of spontaneous nalidixic acid-resistant mutants (17). Later on, NudB was found to be a dihydroneopterin triphosphate pyrophosphatase, a Nudix enzyme involved in folate biosynthesis, and the deletion of *nudB* led to a marked reduction in folate synthesis, although no growth defects were observed (16). The difference between our study and the previous report may result from different strain backgrounds. Our results, in combination with the previous report, confirmed that although NudB plays an important role in *de novo* folate biosynthesis, it is not the only copy of dihydroneopterin triphosphate pyrophosphatase in *E. coli*.

The effects of the deletion of those 2 genes on the bacterial susceptibility to antifolates were also tested, and the results showed that the deletion of *nudB* led to increased sensitivity to both SMX and TMP, but the deletion of *pabB* did not have any effect. PabB is responsible for the synthesis of *para*-aminobenzoic acid, one of the precursors of folate *de novo* biosynthesis. These data suggest that the synergistic effect between SMX and TMP may not result simply from targeting 2 different key enzymes of the same pathway. Thus, it was not surprising to find out that the reason why the deletion of *nudB* led to increased sensitivity to antifolates is unrelated to folate biosynthesis. Although exogenous intermediate folate metabolites could complement the growth lag caused by *nudB* deletion, they had no effect on the susceptibilities to SMX and TMP.

If we go deeper into the bacterial pterin biosynthesis, we can see that after the hydrolysis of GTP, dihydroneopterin triphosphate can be utilized by at least 2 proteins: one of those is NudB, and the other is FolX (dihydroneopterin triphosphate epimerase) (18), a key component of tetrahydromapterin biosynthesis (19). Thus, we wondered if the deletion of *nudB* would make dihydroneopterin triphosphate flux more into the tetrahydromapterin biosynthesis pathway and then lead to the increased sensitivity to both SMX and TMP. If this was the case, reverse mutations could be identified in *folX*. To test this hypothesis, reverse mutants of the *nudB* deletion mutant were isolated, and targeted sequencing was performed for *folX* and *folM*, with *folM* encoding dihydrobiopterin reductase in *E. coli* (19). As expected, of the reverse mutants isolated, more than half of them had mutations (or insertions or deletions) in either the *folX* or *folM* gene. To further verify that the mutation of *folX* or *folM* could reverse the phenotype caused by the *nudB* deletion, 2 double-knockout mutants (*E. coli* W3110 $\Delta nudB \Delta folX$ and *E. coli* W3110 $\Delta nudB \Delta folM$) were constructed, and the results showed that the susceptibilities of these 2 mutants to SMX and TMP were the same as the wild-type strain. Meanwhile, further deletion of *folX* and *folM* could also complement the lag of growth caused by the *nudB* deletion. In addition, we found that the overexpression of FolM, which was thought to be an alternative to FolA, led to increased susceptibility to both TMP and SMX. These data suggest that in *E. coli*, although other dihydroneopterin triphosphate pyrophosphatases may exist, FolX is definitely the predominant consumer of dihydroneopterin triphosphate in the absence of NudB, which is consistent with the previous observation that tetrahydromapterin production far exceeds folate production (19). As a result, the deletion of *nudB* leads to the excess production of tetrahydromapterin, which causes increased susceptibility to SMX and TMP. Since intermediate metabolites of tetrahydromapterin are very unstable, it is very difficult to measure the fluctuation of those metabolites *in vivo*. Although we now know that the deletion of *nudB* can make dihydroneopterin triphosphate flux more into the tetrahydromapterin biosynthesis pathway, it is still unclear why this makes *E. coli* hypersensitive to antifolates. Through protein BLAST, we found that a variety of

bacteria (including *E. coli*) contain components of the tetrahydromonapterin biosynthesis pathway (Table S2), but the biological functions of tetrahydromonapterin still remain unknown. The only clue we could find is that in *Pseudomonas aeruginosa* tetrahydromonapterin is the cofactor for phenylalanine hydroxylase (PhhA), which is involved in tyrosine biosynthesis (19). So far, however, no homologue of PhhA has been identified in *E. coli*. We also tried to add exogenous tyrosine (50 $\mu\text{g/ml}$) to the growth medium to see if it would affect the susceptibility to SMX and TMP, and the results showed that the addition made *E. coli* slightly more resistant to TMP (2 times the increase of the MIC). Further studies are required to reveal how the deletion of *nudB* results in hypersensitivity to both SMX and TMP.

The effect of the *nudB* deletion on the bactericidal activity of TMP was also investigated, and the results showed that the deletion of *nudB* greatly enhanced the bactericidal activity of TMP. Since a previous study showed that the deletion of *nudB* resulted in the decreased frequency of spontaneous nalidixic acid-resistant mutants (17), we wondered whether the deletion of *nudB* also led to the decreased rate of spontaneous resistance to TMP. A mutagenesis assay was performed (see the supplemental material). From the data we obtained, we could see that for the *nudB* deletion strain, no spontaneous resistant mutant could be obtained in the presence of 1 $\mu\text{g/ml}$ TMP (Table S4). To better understand why no TMP spontaneous resistant mutant could be obtained for the *nudB* deletion strain in the presence of a 1- $\mu\text{g/ml}$ concentration of the drug, 40 colonies of TMP spontaneous mutants derived from the wild-type strain and the complemented strain (20 colonies each) were selected, and their MICs for TMP were determined subsequently. The results showed that the MICs for those TMP spontaneous mutants were all determined to be 4 $\mu\text{g/ml}$, about 15 times those of their parental strain (*E. coli* W3110). Since 1 $\mu\text{g/ml}$ TMP (50 times the MIC for TMP of *E. coli* W3110 ΔnudB) was used for the mutagenesis assay and 2 $\mu\text{g/ml}$ TMP (100 times the MIC for TMP of *E. coli* W3110 ΔnudB) was used for the killing curve assay, such high concentrations of the drug might prevent the growth of spontaneous resistant mutants of *E. coli* W3110 ΔnudB . Taken together, all of the above-mentioned data suggest that the enhanced bactericidal effect of TMP caused by the *nudB* deletion is related to the increased susceptibility to TMP.

Except for *E. coli*, components of the tetrahydromonapterin biosynthesis pathway also exist in a variety of other bacteria, including many pathogenic bacteria, such as *S. enterica* and *Yersinia pestis*. This indicates that the deletion of *nudB* may cause similar phenotypes in other bacteria, such as *S. enterica*. Therefore, the *nudB* gene was also deleted in *S. enterica*. Unlike *E. coli*, the deletion of *nudB* did not affect the growth of *S. enterica*, which also proved that NudB is not the only copy of dihydroneopterin triphosphate hydrolase in *S. enterica*. As expected, the deletion of *nudB* also led to increased susceptibility to both SMX and TMP in *S. enterica*, although to a lesser extent than that of *E. coli*. This difference may be explained by the following: in *E. coli*, NudB is the major producer of dihydroneopterin monophosphate since the deletion of *nudB* causes an obvious lag of bacterial growth, but it is not the same in *S. enterica*. In addition, with the overexpression of *E. coli*, FolM also resulted in increased susceptibility to both SMX and TMP, suggesting that the excess production of tetrahydromonapterin may also be the cause of the phenotype.

Taken together, we found that the deletion of *nudB* led to a lag in bacterial growth and increased sensitivity to both SMX and TMP in *E. coli*. In addition, the deletion of *nudB* also led to increased sensitivity to both SMX and TMP in *S. enterica*. Further genetic studies showed that the deletion of *nudB* could make dihydroneopterin triphosphate flux more into the tetrahydromonapterin biosynthesis pathway, yielding an excess production of tetrahydromonapterin, which causes increased sensitivity to both SMX and TMP. Although NudB is not essential for bacterial growth, dihydroneopterin triphosphate pyrophosphate hydrolase is required for bacterial growth, since dihydroneopterin monophosphate is required for bacterial *de novo* folate biosynthesis, which needs to be catalyzed by dihydroneopterin triphosphate pyrophosphate hydrolase. On the other hand, from the data of protein BLAST and previous reports, the tetrahydromonapterin

biosynthesis pathway also exists in a variety of bacteria. Thus, it is possible to design new compounds targeting dihydroneopterin triphosphate pyrophosphate hydrolase, which may inhibit the growth of bacteria and simultaneously potentiate the antimicrobial activities of other antifolates (such as SMX and TMP).

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 W3110 and *Salmonella enterica* serovar Typhimurium LT2 were obtained from laboratory stock. They were both grown in LB liquid medium or E minimal medium (we dissolved 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g of citric H_2O , 13.09 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and 3.5 g of $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ in 1 liter of deionized water) supplemented with 0.5% D-(+)-glucose on LB agar plates (1.5% agar). The gene knockout mutants were constructed with the λ Red recombination system, as previously described (20). The mutants were verified by junction PCR and subsequent sequencing using primers that anneal to the genomic region outside the recombination locus. The double-knockout mutants were generated using the same procedures, except that the kanamycin resistance gene of the single-gene knockout mutant was first eliminated using the helper plasmid pCP20. Complemented strains of all of the mutants were constructed as follows: the *nudB* gene was amplified using the primers nudB-F and nudB-R, the *folX* gene was amplified using the primers folX-F and folX-R, and the *folM* gene was amplified using the primers folM-F and folM-R. Each of the intact wild-type genes was digested with endonuclease BglII and XbaI, cloned into pCA24N or pUC19, and then transformed into the corresponding mutant. All plasmids, primers, and strains that we used are listed in Table S1.

Drug and reagent preparation. The following antibiotics were used: sulfamethoxazole (SMX), rifampin, trimethoprim (TMP), and ampicillin. All of the antibiotics were purchased from Sigma-Aldrich. Drugs were solubilized according to the manufacturer's recommendations, and stock solutions were filter sterilized and stored in aliquots at -20°C . 7,8-Dihydropteroate powder was purchased from Schircks Laboratories (Jona, Switzerland), dissolved in 0.01 M NaOH, filter sterilized, and stored at -80°C .

Drug susceptibility tests. Bacterial cells were grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.5 to 1.0) and diluted to 10^5 CFU/ml in fresh LB. Then, 10-fold serial dilutions were plated onto the LB agar solid plates containing various concentrations of SMX, TMP, rifampin, and ampicillin: 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 $\mu\text{g/ml}$ SMX; 0, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, and 2.56 $\mu\text{g/ml}$ TMP; 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 $\mu\text{g/ml}$ RIF; and 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 $\mu\text{g/ml}$ ampicillin. Cultures were incubated overnight at 37°C . The MIC was defined as the lowest concentration of the compound required to inhibit 99% of the bacterial CFU.

Drug exposure experiments. Bacterial cells were grown to mid-log phase (OD_{600} 0.5 to 1.0) and diluted to an OD_{600} of ~ 0.1 in fresh LB medium. For TMP treatment, 2 $\mu\text{g/ml}$ TMP was used. Cultures were incubated at 37°C without shaking, aliquots of the samples were taken at regular intervals, and serial dilutions were performed before plating.

Identification of reverse mutations in *E. coli* W3110 $\Delta nudB$. *E. coli* W3110 $\Delta nudB$ was grown in LB medium to an OD_{600} of 0.8, collected by centrifugation ($4,000 \times g$, 15 min, at room temperature), and resuspended in fresh medium at a concentration of 10^8 CFU/ml. Subsequently, 100 μl of the cells was spread onto LB agar plates containing 0.1 $\mu\text{g/ml}$ TMP and incubated at 37°C overnight to select the reverse mutants. Single colonies were purified, and then liquid cultures were grown to determine the susceptibilities to both SMX and TMP. Subsequently, genomic DNA was extracted from all of the reverse mutants, and targeted sequencing of PCR amplicons of 2 genes (*folX* and *folM*) was performed. The primers folXd and folMd used in the targeted sequencing are described in Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02378-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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