Impact of Activation of the Neotrehalosdiamine/Kanosamine Biosynthetic Pathway on the Metabolism of *Bacillus subtilis*

Natsumi Saito,^a Huong Minh Nguyen,^{b,c} Takashi Inaoka^c

^aNational Institute of Technology, Tsuruoka College, Tsuruoka, Yamagata, Japan

^bInstitute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Viet Nam

^cFood Research Institute, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, Japan

ABSTRACT The pentose phosphate (PP) pathway is one of the major sources of cellular NADPH. A *Bacillus subtilis* *zwf* mutant that lacks glucose-6-phosphate dehydrogenase (the enzyme that catalyzes the first step of the PP pathway) showed inoculum-dose-dependent growth. This growth defect was suppressed by *glcP* disruption, which causes the upregulation of the autoinducer neotrehalosdiamine (NTD)/kanosamine biosynthetic pathway. A metabolome analysis showed that the stimulation of NTD/kanosamine biosynthesis caused significant accumulation of tricarboxylic acid (TCA) cycle intermediates and NADPH. Because the major malic enzyme YtsJ concomitantly generates NADPH through malate-to-pyruvate conversion, *de novo* NTD/kanosamine biosynthesis can result in an increase in the intracellular NADPH pool via the accumulation of malate. In fact, a *zwf* mutant grew in malate-supplemented medium. Artificial induction of *glcP* in the *zwf* mutant caused a reduction in the intracellular NADPH pool. Moreover, the correlation between the expression level of the NTD/kanosamine biosynthesis operon *ntdABC* and the intracellular NADPH pool was confirmed. Our results suggest that NTD/kanosamine has the potential to modulate carbon energy metabolism through an autoinduction mechanism.

IMPORTANCE Autoinducers enable bacteria to sense cell density and to coordinate collective behavior. NTD/kanosamine is an autoinducer produced by *B. subtilis* and several close relatives, although its physiological function remains unknown. The most important finding of this study was the significance of *de novo* NTD/kanosamine biosynthesis in the modulation of the central carbon metabolism in *B. subtilis*. We showed that NTD/kanosamine biosynthesis caused an increase in the NADPH pool via the accumulation of TCA cycle intermediates. These results suggest a possible role for NTD/kanosamine in carbon energy metabolism. As *Bacillus* species are widely used for the industrial production of various useful enzymes and compounds, the NTD/kanosamine biosynthetic pathway might be utilized to control metabolic pathways in these industrial strains.

KEYWORDS *Bacillus subtilis*, autoinducer, kanosamine, metabolome, neotrehalosdiamine

Autoinduction is a gene regulation mechanism that relies on self-produced signaling molecules called autoinducers. By monitoring the accumulation of autoinducers in the surrounding environment, bacteria can sense cell population density and adapt accordingly by altering gene expression (quorum sensing) (1–4). This mechanism is widespread in the bacterial world and is known to control various important processes, including competence, sporulation, motility, biofilm formation, and virulence. In general, Gram-negative bacteria communicate using acyl-homoserine lactones (5), while Gram-positive bacteria use peptides (6). Moreover, a furanosyl borate diester, autoinducer 2 (AI-2), acts as a universal signal for interspecies communication (7).

Neotrehalosdiamine (NTD; 3,3'-diamino-3,3'-dideoxy- α,β -trehalose) is a structurally unique autoinducer consisting of two kanosamine (3-amino-3-deoxy-D-glucose) residues

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Address correspondence to Takashi Inaoka, tina2672@affrc.go.jp.

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FIG 1 Chemical structures of NTD and kanosamine.

with a 1,1'- α,β linkage (Fig. 1). Several *Bacillus* species, including *Bacillus subtilis*, possess the ability to produce NTD, although its physiological role remains unknown (8–10). NTD induces at least the expression of its biosynthetic operon *ntdABC* by binding to the transcriptional activator NtdR (10). Biochemical analyses performed by another research group showed that the *ntdABC* operon exclusively contains enzymes to produce the kanosamine moiety from glucose-6-phosphate (G6P) (11, 12). However, the enzyme that catalyzes the last step of NTD biosynthesis (i.e., the formation of the 1,1'- α,β linkage of kanosamine moieties) has not been identified.

The expression of *ntdABC* is repressed during the exponential growth phase, even in the presence of excess NTD. This repression is exerted by the function of GlcP, which was annotated as a glucose/mannose:H⁺ symport permease (13). The gene encoding GlcP, *glcP*, is located immediately downstream of *ntdABC* and is cotranscribed with *ntdABC* via transcriptional readthrough at the *ntdABC* transcriptional terminator site (14). Our previous transcriptional analysis revealed that *ntdABC* expression is apparently repressed through GlcP-mediated glucose transport (14). Moreover, disruption of the *glcP* gene caused derepression of *ntdABC*, thereby leading to NTD/kanosamine overproduction. Considering that *glcP* mutant cells can incorporate glucose as well as wild-type cells do (14), it seems that GlcP acts as a glucose sensor for controlling *ntdABC* expression, rather than for glucose uptake. Although the glucose incorporated through GlcP is unphosphorylated, the glucose incorporated through the predominant glucose uptake pathway, i.e., the glucose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (glucose-PTS), is phosphorylated to G6P during the permeation process (15). As G6P can serve as a substrate for the first step of the NTD/kanosamine biosynthetic pathway, glucose has a positive effect on NTD/kanosamine biosynthesis through the glucose-PTS. Furthermore, NTD production is severely reduced by disruption of *ccpA* (14), which encodes a master regulator for carbon catabolite regulation (16). This indicates that NTD production is also regulated by the CcpA-dependent catabolite activation mechanism, either directly or indirectly. Thus, glucose has both positive and negative effects on NTD production, depending on the uptake pathway. Therefore, we hypothesized that NTD is an extracellular signal for sensing both glucose availability and cell density.

FIG 2 Autoinduction effect of kanosamine on the *ntdABC* promoter in *B. subtilis*. (A) The *B. subtilis* reporter strains TI352 (wild type), TI481 (*ntdR*), and TI482 (*ntdA*) were inoculated into S7N medium without (white bars) or with (gray bars) kanosamine. Kanosamine was added to the growth medium at a concentration of 200 μ g/ml (0.93 mM). Culture samples were collected at 16 h, and β -galactosidase activities were measured. The asterisks denote significant differences compared with the values obtained in the absence of kanosamine ($P < 0.05$). (B) The *B. subtilis* reporter strain TI482 (*ntdA*) was inoculated into S7N medium containing kanosamine (open circles) or NTD (closed circles) at the indicated concentration. Culture samples were collected at 16 h, and β -galactosidase activities were measured. The line indicates the basal activity of the *ntdABC* promoter in cells grown in nonsupplemented medium. The average values from three independent experiments are shown. The error bars represent the standard deviations.

In this work, we began to investigate the physiological role of NTD in glucose metabolism. The pentose phosphate (PP) pathway is a major source of the reducing power of NADPH and metabolic intermediates for biosynthetic processes. Because both the NTD/kanosamine biosynthetic pathway and PP pathway branch from G6P, we examined the correlation between these two pathways. During the course of this study, we found that upregulation of the NTD/kanosamine biosynthetic pathway can suppress the growth defect of a *zwf* mutant lacking glucose-6-phosphate dehydrogenase, which is the enzyme that catalyzes the first step of the PP pathway. A metabolome analysis revealed that the activation of the NTD/kanosamine biosynthetic pathway resulted in an increase in the intracellular NADPH level, leading to recovery of the growth of the *zwf* mutant. The correlation between NTD/kanosamine biosynthesis and the intracellular NADPH level is discussed.

RESULTS

Kanosamine also acts as an autoinducer of the *ntdABC* operon. We first examined whether kanosamine also acts as an autoinducer of *ntdABC*. Using reporter strains carrying an *ntdABC* promoter (P_{ntdABC})-lacZ fusion at the *amyE* locus, the effect of kanosamine on P_{ntdABC} activity was monitored (Fig. 2A). Addition of excess kanosamine to the growth medium apparently activated P_{ntdABC} in an NTD/kanosamine nonproducer strain, TI482 (*ntdA::Tn10*), indicating that extracellular kanosamine can act as an autoinducer of P_{ntdABC} . This effect was not observed in the *ntdR* mutant, indicating that kanosamine-driven autoinduction is mediated by NtdR in addition to NTD. As shown in Fig. 2B, the responsiveness of the promoter to kanosamine was comparable to that observed for NTD (Fig. 2B). Thus, *B. subtilis* can respond to both NTD and kanosamine at extracellular concentrations of 0.3 μ M or higher. In the wild-type strain, the amount of NTD/kanosamine produced under our culture condition was estimated to be approximately 5 to 10 μ M, as predicted from the activity of P_{ntdABC} (Fig. 2).

Activation of the NTD/kanosamine biosynthetic pathway can suppress the growth defect caused by *zwf* disruption. The NTD/kanosamine biosynthetic pathway and the PP pathway both branch from G6P. In the first step of the PP pathway, G6P dehydrogenase, which is encoded by the *zwf* gene, converts G6P to 6-phospho-gluconolactone. To examine whether NTD/kanosamine production is affected by the knockout of the *zwf* gene, several *zwf* disruption mutants [i.e., TI443 (*zwf*), TI444 (*zwf glcP*), and TI445 (*zwf ntdABC glcP*)] were constructed. The *zwf* mutant grew as well as the wild-type 168 strain in liquid LB medium with 1% (vol/vol) of the inoculum. However, when the LB overnight culture was inoculated into semisynthetic S7N medium containing excess glucose and glutamate, this mutant

FIG 3 Growth characteristics of *B. subtilis* *zwf* mutants. The *B. subtilis* 168 (wild type) (open circles) and TI443 (*zwf*) (closed circles) strains were grown in LB medium for 16 h at 37°C. An appropriate volume of the overnight culture was inoculated into S7N medium, and growth was monitored by measuring the OD₆₅₀. The inoculum was 2% (vol/vol) (A), 1% (vol/vol) (B), or 0.5% (vol/vol) (C). (D) The growth of the *B. subtilis* 168, TI443, NTD/kanosamine overproducer TI444 (*zwf glcP*), and NTD/kanosamine nonproducer TI445 (*zwf ntdABC glcP*) strains with the 1% inoculum is shown. The average values from three independent experiments are shown. The error bars represent the standard deviations.

showed an inoculation-volume-dependent growth. Although the *zwf* mutant grew with 2% (vol/vol) of the inoculum (Fig. 3A), a lower-dose inoculum (1 or 0.5%) caused growth arrest or cell lysis (Fig. 3B and C). Inoculation-volume-dependent growth was also observed in an experiment using water-washed cells (data not shown). Supplementation with LB (2%), casamino acid (0.1%), purines (0.2 mM each adenosine and guanosine), or pyrimidines (0.2 mM each thymidine and cytidine) had no significant effect on the growth of the *zwf* mutant (data not shown). Hence, this growth phenomenon was unlikely to be caused by any carry-over effect. Interestingly, the NTD/kanosamine-overproducing *zwf glcP* double mutant grew with 1% (vol/vol) of the inoculum (Fig. 3D) but not 0.5% (data not shown). Further disruption of the NTD/kanosamine biosynthesis operon *ntdABC* completely negated the effect of *glcP* disruption on cell growth (Fig. 3D). No significant restoration of the growth of the *zwf* mutant was observed in S7N medium supplemented with NTD or kanosamine at the concentration of 1 mM, because NTD/kanosamine-driven autoinduction was prevented during the exponential growth phase, even in the presence of excess NTD/kanosamine (data not shown). These results suggest that the activation of the *de novo* NTD/kanosamine biosynthetic pathway can cause alterations in metabolism, thereby suppressing the growth defect of the *zwf* mutant.

The effect of upregulation of the NTD/kanosamine biosynthetic pathway on metabolism. To clarify the metabolic changes, we performed a metabolome analysis (Fig. 4). The disruption of *zwf* caused a remarkable decrease in the intracellular levels of PP pathway intermediates, resulting in decreased levels of NADPH and phosphoribosyl pyrophosphate (PRPP) (Fig. 4). The signal corresponding to kanosamine was observed only in the *zwf*

FIG 4 Effect of upregulation of NTD/kanosamine biosynthetic pathway on central carbon metabolism. The *B. subtilis* 168 (wild type), TI443 (*zwf*), TI444 (*zwf glcP*), and TI445 (*zwf ntdABC glcP*) strains were grown in LB medium for 16 h at 37°C. One milliliter of the overnight culture (Continued on next page)

glcP double mutant, indicating that the NTD/kanosamine biosynthetic pathway was actually activated only in the *zwf glcP* mutant. Although no significant increases in the intracellular levels of PP pathway intermediates were observed even in the *zwf glcP* mutant, the intracellular NADPH level in this mutant was increased as much as that observed in the wild-type strain. Conversely, no significant increase in the intracellular NADPH level was observed in the *zwf ntdABC glcP* triple mutant. A similar result was obtained in an NADPH assay that was performed using the crude extract of each strain (see Fig. S1 in the supplemental material), which suggests that upregulation of the NTD/kanosamine biosynthetic pathway can stimulate NADPH production without a contribution from the oxidative PP pathway. The metabolome profile revealed that the activation of the NTD/kanosamine biosynthetic pathway had a significant effect on glycolysis/gluconeogenesis and the tricarboxylic acid (TCA) cycle, rather than on the PP pathway. The intermediates of these metabolic pathways accumulated only in the *zwf glcP* mutant (Fig. 4). As the conversion of isocitrate to 2-oxoglutarate (2OG) by isocitrate dehydrogenase (IDH) in the TCA cycle is one of the major sources of NADPH generation, we assessed the effect of NTD/kanosamine on IDH activity. However, *glcP* disruption had no significant effect on IDH activity (data not shown). Therefore, we considered that the NTD/kanosamine biosynthesis stimulated another route for NADPH generation. In the second step of the NTD/kanosamine biosynthetic pathway, NtdA catalyzes the conversion of 3-oxoglucose 6-phosphate to kanosamine 6-phosphate through a pyridoxal phosphate (PLP)-dependent amination reaction (12). Because PLP reacts with a glutamate to form pyridoxamine phosphate (PMP) and 2OG (11), the activation of the NTD/kanosamine biosynthetic pathway can cause the accumulation of 2OG. In turn, the accumulated 2OG can enter the TCA cycle, thus increasing the other TCA cycle intermediates, including malate (Fig. 4). *B. subtilis* possesses four malic enzymes (MaeA, MalS, MleA, and YtsJ), which catalyze the decarboxylation of malate to pyruvate with concomitant reduction of NAD⁺ or NADP⁺. YtsJ, which is the major malic enzyme, utilizes NADP⁺, whereas the remaining enzymes utilize NAD⁺ (17). Thus, the accumulation of malate can lead to an increase in the intracellular NADPH level through malate-to-pyruvate conversion by YtsJ. Accordingly, we confirmed the effect of several TCA cycle intermediates (malate, succinate, and fumarate) on the growth of the *zwf* mutant. Strikingly, the addition of malate to S7N medium restored the growth of the *zwf* mutant, although the maximum cell mass reached only half of that of the wild-type strain (Fig. 5A). Moreover, the intracellular NADPH level in cells that were grown in malate-supplemented medium was increased as much as 2-fold compared with that detected in cells grown in nonsupplemented medium (Fig. 5B). Neither succinate nor fumarate had a recovery effect on the growth of the *zwf* mutant (data not shown). In *B. subtilis*, malate is a preferred carbon source that is cointilized with glucose (18). It has been reported that the expression of *maeN*, which encodes the malate-specific uptake transporter, is induced by malate (19). In contrast, the expression of the C₄-dicarboxylic transporter for succinate and fumarate, which is encoded by *dctP*, is subjected to catabolite repression in the presence of glucose or malate (20, 21). Therefore, it is not surprising that malate alone restored the growth of the *zwf* mutant. We sought to construct a *zwf ytsJ* double mutant. However, this attempt was unsuccessful, suggesting that simultaneous disruption of *zwf* and *ytsJ* causes synthetic lethality (data not shown).

Correlation between *ntdABC* expression and the intracellular NADPH level. The expression of *ntdABC* is repressed through GlcP-mediated glucose transport (14). To confirm the correlation between *ntdABC* expression and the intracellular NADPH level,

FIG 4 Legend (Continued)

was inoculated in 50 ml of S7N medium and cultivated until the OD₆₅₀ reached 0.2, and the cells were collected by filtration. The metabolites in each cell were analyzed using CE-TOF MS. The y axis indicates the amount of metabolites (picomoles per OD₆₅₀ unit per milliliter of culture). 3PG/2PG and kanosamine/mannosamine/glucosamine were not differentiated by the CE-TOF MS method used. The average values from three independent experiments are shown. The error bars represent the standard deviations. Abbreviations: 1,3-BPG, 1,3-bis-phosphoglycerate; 2-OG, 2-oxoglutarate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; AcCoA, acetyl coenzyme A; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F1,6P, fructose 1,6-bis-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; Glu, glutamate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; PRPP, phosphoribosyl pyrophosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; SucCoA, succinyl coenzyme A; X5P, xylulose 5-phosphate.

FIG 5 Effect of malate in the *B. subtilis* *zwf* mutant. (A) The *B. subtilis* 168 (wild type) and TI443 (*zwf*) strains were grown in LB medium for 16 h at 37°C. The overnight culture was inoculated at 1:100 into S7N medium without or with 0.2% malate. Growth was monitored by measuring the OD₆₅₀. (B) At an OD₆₅₀ of 0.2 to 0.3, cells were collected by centrifugation and used for the NADPH assay. The average values from three independent experiments are shown. The error bars represent the standard deviations. The asterisks denote significant differences compared with the value obtained in the absence of malate ($P < 0.05$).

we constructed two engineered strains in which the expression of *glcP* is controlled by the isopropyl- β -D-thiogalactopyranoside (IPTG)-dependent *spac* promoter (P_{spac}) (Fig. 6A). For this purpose, the pMutinT3 plasmid was integrated immediately downstream (TI451) or upstream (TI480) of the *ntdABC* terminator (T_{ntdABC}). The TI451 (*zwf ntdABC-T_{ntdABC}-pMutinT3-glcP*) strain showed growth arrest even in the absence of IPTG (without artificial induction of *glcP*) because of the leaky expression of *glcP* from P_{spac} (see Fig. S2 in the supplemental material). This suggests that a very low level of *glcP* expression was sufficient to repress the expression of *ntdABC*. In contrast, the TI480 (*zwf ntdABC-pMutinT3-T_{ntdABC}-glcP*) strain grew normally in the absence of IPTG (Fig. 6B). The growth of the TI480 strain was inhibited in a dose-dependent manner by the addition of IPTG to the growth medium (Fig. 6B). The addition of IPTG at a concentration of 30 μ M caused growth arrest. A transcriptional analysis revealed that an induction of *ntdA* of at least 50-fold compared with that in the wild-type strain 168 was required for the growth of the *zwf* mutant (Fig. 6C). Furthermore, the intracellular level of NADPH was apparently reduced in an IPTG-dependent manner (Fig. 6D). Thus, the increased expression of *ntdA* resulted in an increase in the intracellular NADPH pool, thus restoring the growth of the *zwf* mutant. Our results suggest that NTD/kanosamine functions as a metabolic modulator through autoinduction.

DISCUSSION

This study found a significant correlation between NTD/kanosamine biosynthesis and the intracellular NADPH pool. Our results suggest a possible role for NTD/kanosamine in the metabolism of *B. subtilis* and probably other NTD/kanosamine-producing close relatives.

The major routes for NADPH generation are considered to be the oxidative PP pathway and IDH in the TCA cycle. Under our culture condition, the intracellular NADPH level in a *zwf* mutant was only approximately 20% of that detected in the wild-type strain, indicating that NADPH generation largely depends on the oxidative PP pathway (Fig. 4; see also Fig. S1 in the supplemental material). We found that the *zwf* mutant exhibited inoculum-dose-dependent growth in S7N medium (Fig. 3A to C). Even in LB medium, with a much-lower-dose inoculum (below 0.01%), this mutant showed an extremely extended growth lag (unpublished result). These observations suggest that the PP pathway plays a more critical role in growth with a lower-dose inoculum than in growth with a higher-dose inoculum. The PP pathway is also important for supplying

(A)

(B)

(C)

(D)

FIG 6 Effect of *glcP* expression on the intracellular NADPH pool. (A) For artificial control of *glcP* expression, the *B. subtilis* strain TI480 was constructed by integrating the pMutinT3 plasmid immediately upstream of *T_{ntdABC}*. In this strain, *glcP* is transcribed from the IPTG-dependent *P_{spac}* promoter via transcriptional readthrough at *T_{ntdABC}*. (B) Overnight culture was inoculated at 1:100 into S7N medium containing IPTG at the indicated concentrations. The growth was monitored by measuring the OD₆₅₀. When the culture OD₆₅₀ reached 0.2 to 0.3, cells were collected by centrifugation and used for RT-qPCR analysis (C) or NADPH assay (D). The relative expression of *ntdA* is expressed as the ratio compared with that detected in wild-type strain 168. The average values from at least four independent experiments are shown. The error bars represent the standard deviations. The asterisks denote significant differences compared with the value obtained in the absence of IPTG ($P < 0.05$).

precursors of nucleotide synthesis. Although a marked reduction of PRPP was observed in the *zwf* mutant (Fig. 4), no significant reduction in nucleotide pools, with the exception of IMP, was observed (data not shown). Moreover, the addition of purine or pyrimidine nucleosides to the S7N medium did not restore the growth of the *zwf* mutant (data not shown). Therefore, we considered that the major cause of the growth defect observed in this mutant was the limited availability of the intracellular NADPH pool. In support of this hypothesis, a significant correlation between the NADPH level and growth was observed (Fig. 6B and D). Cell growth starting from a lower cell density might require much more NADPH for anabolism. We have already isolated several *zwf* suppressor mutants that can grow with a lower-dose inoculum (unpublished results). Further analysis of these suppressor mutations will help elucidate the role of the PP pathway in growth with a lower-dose inoculum.

The intracellular NADPH pool was recovered in the *zwf glcP* double mutant but not in the *zwf ntdABC glcP* triple mutant, indicating that activation of NTD/kanosamine biosynthetic pathway can stimulate NADPH production without the contribution of the PP pathway (Fig. 4; Fig. S1). IDH in the TCA cycle is an alternative route for NADPH generation. In exponentially growing cells, the genes that encode the first three TCA cycle enzymes (i.e., citrate synthase, aconitase, and IDH) are synergistically repressed by a readily metabolized carbon-nitrogen source, such as glucose and glutamate/glutamine (22, 23). Moreover, the disruption of *glcP* had no significant effect on IDH activity (data not shown). It appears that IDH had a limited contribution to NADPH generation in the *zwf glcP* mutant, suggesting the stimulation of another route for NADPH generation. One possible route is the malic enzyme YtsJ, which catalyzes the decarboxylation of malate with concomitant generation of NADPH. The activity of the NADP-dependent malic enzyme in *zwf* mutant was approximately 2-fold higher than that in the wild-type strain (see Fig. S3 in the supplemental material). However, neither the *glcP* disruption nor kanosamine *per se* had any detectable effect on the activity. Because the first

steps of the TCA cycle are repressed under nutrient-rich conditions, it is possible that YtsJ-mediated NADPH production is restricted at the intracellular malate level. Considering that upregulation of the NTD/kanosamine biosynthetic pathway caused the accumulation of several TCA cycle intermediates, including malate (Fig. 4), the NTD/kanosamine biosynthetic pathway can contribute to YtsJ-mediated NADPH generation. In fact, addition of malate to S7N medium caused an increase in the intracellular NADPH level (Fig. 5B). It should be noted that the intracellular NADPH level in the *zwf* mutant growing in malate-supplemented medium reached only approximately half of that detected in the wild-type strain (Fig. 5B). We cannot exclude the possibility of the effect of another factor or factors on NADPH generation or consumption in the *zwf glcP* mutant.

One of the most important findings of this study was that NTD/kanosamine had a significant impact on the central carbon metabolism of *B. subtilis*. The TCA cycle intermediate 2OG is a key metabolite that lies at the junction between the carbon and nitrogen metabolic pathways. This metabolite coordinates carbon and nitrogen metabolism in response to nutrient availability (24). Under nutrient-rich conditions, such as in our culture medium, which contained excess glucose and glutamate, only a small portion of pyruvate enters the TCA cycle (22, 23). Hence, the accumulated pyruvate feeds into overflow pathways that produce acetate and acetoin, both of which are excreted into the environment. Our metabolome data revealed that upregulation of the NTD/kanosamine biosynthetic pathway caused a significant increase in the 2OG pool (Fig. 4). The accumulation of 2OG was also observed in our preliminary metabolome analysis using the *zwf⁺ glcP* mutant (data not shown). It appears that NTD/kanosamine synthesis stimulated the later reactions of the TCA cycle (conversions from 2OG to malate) via the accumulation of 2OG. Considering that the expression of the acetoin biosynthesis operon *alsSD* was markedly reduced in the *zwf⁺ glcP* mutant (25), NTD/kanosamine biosynthesis might support a metabolic shift from glycolysis to gluconeogenesis by increasing the intracellular 2OG pool in stationary-phase cells. Although further analysis is needed to clarify the role of NTD/kanosamine in metabolism, NTD/kanosamine has the potential to play a role as a metabolic modulator in response to nutrient availability.

MATERIALS AND METHODS

Bacterial strains and chemicals. All *B. subtilis* strains used in this study were derived from strain 168 and are listed in Table 1. Strain 1012 *zwf gntZ* (*zwf::neo gntZ::spc*) was kindly provided by Uwe Sauer (26). For the selection of *B. subtilis* transformants, neomycin (3 μ g/ml), spectinomycin (100 μ g/ml), chloramphenicol (4 μ g/ml), and erythromycin (0.5 μ g/ml) were used. NTD was previously prepared in our laboratory (10). Kanosamine hydrochloride was purchased from Santa Cruz Biotechnology, Inc. (USA). L-Malate monosodium salt was added to the growth medium at a final concentration of 0.2%. IPTG was used for the artificial expression of *glcP*.

Growth conditions. *B. subtilis* strains were maintained on 2 $\times$ LB agar medium (20 g of tryptone, 10 g of yeast extract, 10 g of NaCl, and 15 g of agar per liter). Because the *zwf* mutants exhibited a dilution-sensitive growth, cells were never isolated as a single colony, with the exception of the selection of *zwf* transformants. To monitor growth in S7N medium (10 mM ammonium sulfate, 5 mM potassium phosphate [pH 7.0], 100 mM MOPS [morpholinepropanesulfonic acid; adjusted to pH 7.0 by KOH], 2 mM MgCl_2 , 0.7 mM CaCl_2 , 50 μ M MnCl_2 , 5 μ M FeCl_3 , 2 μ M thiamine, 20 mM glutamate, 1% glucose, 50 μ g/ml tryptophan, and 0.1% nutrient broth [Difco]), *B. subtilis* cells were inoculated in liquid LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) and cultivated for 16 h at 37°C with vigorous shaking. Subsequently, an appropriate volume of the culture was transferred into S7N medium and cultivated at 37°C with vigorous shaking.

β -Galactosidase assays. β -Galactosidase activities were measured as described previously (27). The specific activities were calculated as $(A_{420} \times 1,000)/(\text{min} \times \text{ml of culture} \times \text{OD}_{650})$, where OD_{650} is the optical density at 650 nm.

Metabolome analysis. A 16-h culture grown in LB medium was diluted 50-fold with S7N medium and cultivated at 37°C until the OD_{650} reached 0.2. Cells (10 absorbance units [$\text{ml of culture} \times \text{OD}_{650}$]) were collected by vacuum filtration using a filter with a pore size of 0.4 μ m (Millipore) and quickly washed with 10 ml of Milli-Q water twice. The filter was immersed in 2 ml of methanol containing 2.5 μ M (each) internal standards (methionine sulfone, morpholineethanesulfonic acid [MES], and D-camphor-10-sulfonic acid) for quantification. Cells were then removed from the filter in a bath sonication. A 1.6-ml portion of the cell suspension was mixed with an equal volume of chloroform and 640 μ l of Milli-Q water. After vortexing and centrifugation, the aqueous layer was recovered and filtrated using

TABLE 1 Strains used in this study

Strain	Genotype	Source or reference ^a
168	<i>trpC2</i>	Laboratory stock
1012 <i>zwf gntZ</i>	<i>leuA8 metB5 zwf::neo gntZ::spc</i>	26
TI122	<i>trpC2 aspB66 amyE::P_{ntdABC}-lacZ cat</i>	10
TI127	<i>trpC2 aspB66 ntdR::neo amyE::P_{ntdABC}-lacZ cat</i>	10
TI130	<i>trpC2 aspB66 ntdA::Tn10 amyE::P_{ntdABC}-lacZ cat</i>	14
TI138	<i>trpC2 aspB66 ntdABC::cat</i>	14
TI167	<i>trpC2 aspB66 ntdC-pMutinT3-T_{ntdABC}-glcP</i>	14
TI168	<i>trpC2 aspB66 ntdC-T_{ntdABC}-pMutinT3-glcP</i>	14
TI214	<i>trpC2 aspB66 glcP::spc</i>	25
TI352	<i>trpC2 amyE::P_{ntdABC}-lacZ cat</i>	TI122→168
TI415	<i>trpC2 glcP::spc</i>	TI214→168
TI420	<i>trpC2 ntdC-T_{ntdABC}-pMutinT3-glcP</i>	TI168→168
TI422	<i>trpC2 ntdABC::cat glcP::spc</i>	TI138→TI415
TI443	<i>trpC2 zwf::neo</i>	1012 <i>zwf gntZ</i> →168
TI444	<i>trpC2 zwf::neo glcP::spc</i>	TI443→TI415
TI445	<i>trpC2 zwf::neo ntdABC::cat glcP::spc</i>	TI443→TI422
TI451	<i>trpC2 zwf::neo ntdC-T_{ntdABC}-pMutinT3-glcP</i>	TI443→TI420
TI479	<i>trpC2 ntdC-pMutinT3-T_{ntdABC}-glcP</i>	TI167→168
TI480	<i>trpC2 zwf::neo ntdC-pMutinT3-T_{ntdABC}-glcP</i>	TI443→TI479
TI481	<i>trpC2 ntdR::neo amyE::P_{ntdABC}-lacZ cat</i>	TI127→TI352
TI482	<i>trpC2 ntdA::Tn10 amyE::P_{ntdABC}-lacZ cat</i>	TI130→TI352

^aThe arrows indicate construction by transformation of the latter strain with the genomic DNA of the former strain.

Ultrafree-MC ultrafilter devices (Millipore) for metabolome analysis. After the filtrate was dried, the residue was dissolved in 25 μ l of Milli-Q water containing 200 μ M 3-aminopyrrolidine and trimesate as the internal standards for migration time correction. A metabolome analysis was conducted by capillary electrophoresis-time of flight mass spectrometry (CE-TOF MS). CE-TOF MS was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer, an isocratic high-performance liquid chromatography (HPLC) pump, a CE-MS adapter kit, and a CE-electrospray ionization (ESI) MS sprayer kit (Agilent Technologies). The system was controlled by the Agilent Chem Station software for CE. The instrumental conditions used for analysis were as described previously (28).

NADPH assay. One milliliter of LB overnight culture was inoculated into 100 ml of 57N medium and incubated at 37°C with vigorous shaking until the OD₆₅₀ reached 0.2 to 0.3. The cells were then collected by centrifugation (6,000 rpm for 5 min at 25°C), washed with phosphate-buffered saline (PBS), and centrifuged again. After the supernatant was discarded, the cell pellet was kept at −20°C until use. The intracellular NADPH level was measured using an Amplitude colorimetric NADPH assay kit (AAT Bioquest, USA), according to the manufacturer's recommendations with slight modification. In brief, the cell pellet was resuspended in 0.5 ml of lysis buffer (component D in the kit; AAT Bioquest, USA) supplemented with 1 μ l of DNase I (5 U/ μ l; TaKaRa, Japan) and incubated at room temperature for 30 min. After the cell debris was removed, the resulting crude extract was subjected to an NADPH assay. The NADPH level was normalized by adjusting the concentration of total protein. Protein concentrations were determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce, USA).

Reverse transcription-quantitative PCR. For reverse transcription-quantitative PCR (RT-qPCR), total cellular RNA was prepared as described previously (25). First-strand cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio, Japan) and random hexamer primers, according to the manufacturer's instructions. Real-time PCR was performed using the Thunder Bird SYBR qPCR mix (Toyobo, Japan) on an ABI 7300 real-time PCR system (Applied Biosystems, USA). 16S rRNA was used for the normalization of RNA levels. The oligonucleotides used for the quantification of *ntdA* and 16S were described previously (25).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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