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Glucose Uptake Pathway-Specific Regulation of Synthesis of Neotrehalosadiamine, a Novel Autoinducer Produced in *Bacillus subtilis*▽

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Neotrehalosadiamine (3,3′-diamino-3,3′-dideoxy-α,β-trehalose; NTD) is an amino-sugar antibiotic produced by several *Bacillus* species that functions as an autoinducer by activating its own biosynthetic operon, *ntdABC*. We previously reported that the introduction of a certain *rpoB* mutation (*rpoB5*) into *Bacillus subtilis* enables the cells to overproduce NTD. *B. subtilis* mini-Tn*10* transposant libraries have been screened for genes that affect NTD production. Inactivation of *ccpA*, which encodes a major transcriptional regulator of carbon catabolite regulation, markedly reduced NTD production. By contrast, inactivation of *glcP*, which is situated just downstream of *ntdABC* and encodes a glucose/mannose:H^+ symport permease, stimulated NTD production. Overexpression of *glcP* led to the repression of *ntdABC* expression (and thus NTD production) in response to GlcP-mediated glucose uptake. These results suggest that CcpA-mediated catabolite activation of *ntdABC* expression occurs in response to the increase of the in vivo concentration of fructose-1,6-bisphosphate via glucose-6-phosphate and that GlcP-mediated glucose repression of *ntdABC* expression occurs in association with the increase of the in vivo concentration of unphosphorylated glucose. In addition, Northern analysis showed that *glcP* is transcribed from the *ntdABC* promoter through transcription readthrough at the *ntdABC* transcription terminator site, which enables NTD to function as a modulator of glucose uptake through the stimulation of *ntdABC-glcP* transcription, even in wild-type (*rpoB*$^+$) cells. A trace amount (0.5 to 3 μg/ml) of NTD was sufficient to ensure expression of *glcP*, thus demonstrating the physiological role of "antibiotic" in the producing bacteria by functioning as an autoinducer for glucose uptake modulation.

Carbon catabolite regulation (involving repression and activation) is a ubiquitous phenomenon whereby bacteria alter the expression of a large number of genes in response to the availability of carbon sources in their surroundings and is dependent upon the organism's ability to take up sugar. *Bacillus subtilis* has at least three different pathways for glucose uptake. The first is the glucose-specific phosphoenolpyruvate-dependent phosphotransferase system (glucose-PTS), which is widespread among bacteria and plays an essential role in both the transport and the phosphorylation of glucose (31, 32). Encoded by the *ptsGHI* operon, glucose-PTS is the predominant glucose uptake pathway of *B. subtilis* and promotes carbon catabolite regulation mediated by a master transcriptional regulator, CcpA (4). The remaining two pathways are the non-PTS-type transporters GlcP (30) and GlcU (7). GlcP is a proton-dependent glucose/mannose symport permease and a member of the major facilitator superfamily (29). Paulsen et al. (30) showed that cells lacking functional GlcP import 30% less glucose than wild-type cells and that GlcP contributes partially to glucose-promoted catabolite repression. The gene encoding GlcU and a downstream *gdh* gene encoding glucose dehydrogenase form an operon that is transcribed in forespores after the onset of sporulation (26). Thus, GlcU probably functions in forespores during sporulation or in germinating spores rather than in vegetative cells. In contrast to PTS-mediated sugar uptake, which has been extensively studied, little is known about the regulation of GlcP and GlcU.

glcP, the gene encoding GlcP, is located just downstream of an operon consisting of three genes, *ntdA*, *ntdB*, and *ntdC* (formerly *yhjL*, *yhjK*, and *yhjJ*, respectively). We previously used heterologous expression in *Escherichia coli* to establish that *ntdABC* encodes all the enzymes required for the biosynthesis of an antibiotic, neotrehalosadiamine (NTD; 3,3′-diamino-3,3′-dideoxy-α,β-trehalose) (Fig. 1A) (18). Although *B. subtilis* normally produces lower-than-detectable levels of NTD, we were able to induce overproduction of NTD in *B. subtilis* by introducing a mutation into RNA polymerase (RNAP) and selecting for the rifampin (Rif)-resistant phenotype (18). Interestingly, transcriptional analysis using the *ntdABC* promoter (P_{ntdABC}) fused to *lacZ* revealed that the inactivation of the NTD biosynthesis operon shuts off its own promoter. Conversely, P_{ntdABC} was dose-dependently activated by the addition of purified NTD. A transcriptional regulator, NtdR, whose gene is situated upstream of the *ntdABC* operon in the opposite orientation, mediates the NTD-dependent activation of P_{ntdABC}. Thus, NTD appears to function as an autoinducer, at least for its own biosynthetic operon. Still, the mechanism by which *ntdABC* expression is regulated is not fully understood.

Autoinduction has been extensively studied in several bacteria in relation to quorum-sensing systems, which are important for various physiological processes, including acquisition of competence, sporulation, antibiotic production, motility, biofilm formation, bioluminescence, and virulence (3, 6, 9, 10, 19, 20, 22, 33). Whereas in gram-positive bacteria, most of

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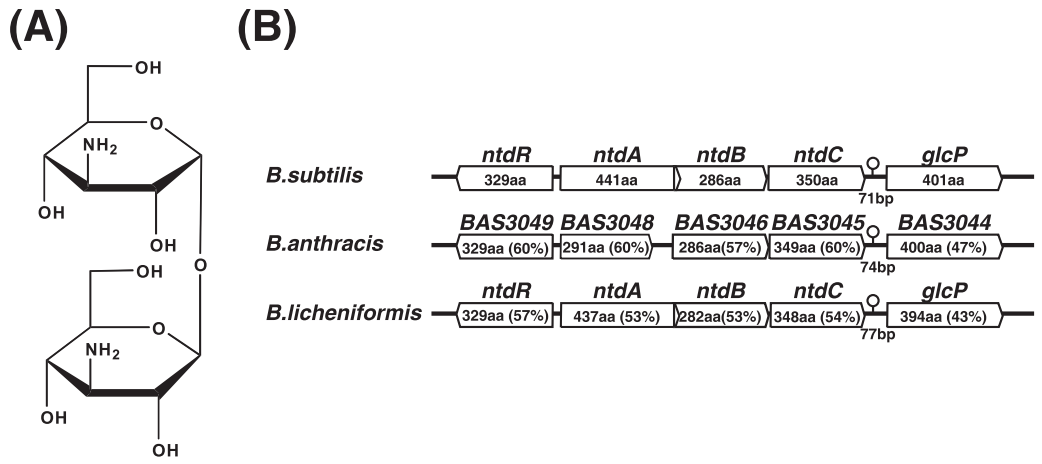


FIG. 1. Structure of NTD and organization of the NTD biosynthesis operon in *Bacillus* species. (A) Chemical structure of NTD. (B) The *ntdABC* operon region of *B. subtilis* is compared with those of *B. anthracis* and *B. licheniformis*. Amino acid lengths or amino acid identities (indicated as percentages in parentheses) with the corresponding *B. subtilis* proteins (for *B. anthracis* and *B. licheniformis*) are shown. The BAS3048 gene of *B. anthracis* has a frameshift mutation (possibly by 1 base deletion), which resulted in a protein product smaller than the *B. subtilis* NtdA.

these signaling molecules are peptides or modified peptides (6, 19, 20), NTD is unique in that it is an amino-sugar. NTD was originally identified as an antibiotic produced by *Bacillus pumilus* (36) and *Bacillus circulans* (27) that inhibits growth of *Staphylococcus aureus* and *Klebsiella pneumoniae*. In addition, genome sequencing revealed that *Bacillus anthracis* and *Bacillus licheniformis* contain orthologues for a complete set of NTD biosynthetic enzymes and its transcriptional regulator, NtdR (Fig. 1B). This suggests that NTD is used as a common "language" between *B. subtilis* and its close relatives. Here we present evidence that glucose can exert alternative effects on *ntdABC* gene expression in *B. subtilis*, depending upon the organism's uptake pathway. The significance of NTD as a novel signaling molecule that modulates glucose metabolism is discussed.

MATERIALS AND METHODS

Bacterial strains and their construction. *B. subtilis* strains used in this study are listed in Table 1. All strains except JCM1465 and 168 were derived from strain 61884 (*trpC2 aspB66*) (28). Strain GM273 (Δ*ptsGHI*::*erm*) was provided by J. Deutscher, and QB7103 (*crh*::*aphA3 ptsH1*) was kindly provided by I. Martin-Verstraete via J. Deutscher.

Strain TI138 (Δ*ntdABC*::*cat*) was constructed as follows. The 5′ fragment of *ntdA* (501 bp) and the 3′ fragment of *ntdC* (554 bp) were amplified by PCR with the primers HindIII-ntdAF (5′-ccaagcttattggaggtactgttcATGCA-3′) and Sse8387I-ntdAR (5′-cctgcaggTAATAGCATCGGTTCCACTG-3′) for *ntdA*, and Sse8387I-ntdCF (5′-cctgcagGTAAGAAGACGAGTGGATCAT-3′) and EcoRI-ntdCR (5′-ggaattcCTAGTTGACTGCTGAAACAGACAT-3′) for *ntdC*. Capital letters indicate the coding region of each gene, while the underlined sequences are the restriction sites for HindIII, Sse8387I, or EcoRI, used for cloning the resulting PCR product; lowercase letters indicate the noncoding region. The amplified DNAs were cloned into pCR2.1 (Invitrogen), generating pCR2.1-ΔntdA and pCR2.1-ΔntdC. The HindIII-EcoRI fragment derived from pCR2.1-ΔntdA was subcloned into the corresponding region of pUC18, resulting in pUC18-Δ*ntdA*. The Sse8387I-EcoRI fragment derived from pCR2.1-ΔntdC was inserted into the corresponding region of pUC18-ΔntdA, generating pUC18-ΔntdABC. An Sse8387I fragment of the *cat* gene from pCR2.1-*cat* (15) was inserted at the Sse8387I site of pUC18-ΔntdABC. The resulting plasmid, pUC18-ΔntdABC::cat, was linearized with EcoRI and used in the transformation of *B. subtilis* 61884.

Strains TI167 (*ntdC*-pMutinT3-T_{ntdABC}), TI168 (T_{ntdABC}-pMutinT3-*glcP*) and TI168Δ4 (T_{ntdABC}-pMutinT3-Δ*glcP*) were constructed by integrating plasmid pMutinT3 (25) upstream or downstream of the *ntdABC* transcriptional terminator (T_{ntdABC}). A DNA fragment containing the C-terminal coding region of *ntdC* or the N-terminal coding region of *glcP* was amplified by PCR with the following specific primer pairs: HindIII-ntdCF (5′-ctctaagcttCTGTGGTTTTAGCATTG AGG-3′) and BamHI-ntdCR (5′-cgggatcctaGTTGACTGCTGAAACAGAC-3′) for *ntdC*, and HindIII-glcPF (5′-ctctaagcttatagggtgtaatgaATGTTAA-3′) and BamHI-glcPR (5′-cgggatccAACAAATGAACCAACTGTCG-3′) for *glcP*. For construction of strain TI168Δ4, HindIII-ΔglcPF (5′-ctctaagcttatagggtgtaatgaA TGTAA-3′) was used instead of HindIII-glcPF for PCR. The capital letters indicate the coding region of each gene, while the underlined sequences are the restriction sites for HindIII or BamHI used for cloning the resulting PCR product. The amplified DNAs were cloned into pCR2.1, generating pCR2.1-ntdC, pCR2.1-glcP, and pCR2.1-ΔglcP, respectively, after which the cloned fragments were fully sequenced to confirm their correctness. The HindIII-BamHI fragments were inserted into the corresponding region of pMutinT3, generating pMutinT3-ntdC, pMutinT3-glcP, and pMutinT3-ΔglcP, respectively, which were used to transform *B. subtilis* 61884, after which strains TI167, TI168, and TI168Δ4 were selected on L agar plates based on erythromycin (Erm) resistance. To generate strains TI171 and TI172, strain TI138 (Δ*ntdABC*::*cat*) was transformed with the plasmids pMutinT3-ntdC and pMutinT3-glcP, respectively.

Growth conditions. *B. subtilis* strains were grown overnight on L agar plates (17) at 30°C, after which they were inoculated into S7N medium (17) and incubated at 37°C. Both media were supplemented as required with tryptophan (Trp; 50 μg/ml) and aspartate (Asp; 2 mM for L medium or 20 mM for S7N medium). S7N medium contained 1% glucose, if otherwise not stated. Purified NTD (prepared in our laboratory) was used at appropriate concentrations for the induction of *ntdABC*. Isopropyl-β-D-thiogalactopyranoside (IPTG; 2 mM) was used for the activation of the *spac* promoter (P_{spac}). Rif (1 μg/ml), Erm (0.5 μg/ml), spectinomycin (Spc; 100 μg/ml), kanamycin (5 μg/ml), and chloramphenicol (Cm; 5 μg/ml) were used for the selection of *B. subtilis* transformants. Ampicillin (100 μg/ml) was used for the selection of *E. coli* transformants.

NTD assay. Cells were grown in S7N medium for 24 h, as described above, after which the cultures were centrifuged and the resultant supernatants were appropriately diluted, and a 50-μl sample was applied to a paper disk (8-mm diameter; Advantec). The paper disk was then placed on an NTD assay plate (S7N soft agar [0.7%] supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-Gal], Spc, Cm, Trp, and Asp) inoculated with the *B. subtilis* indicator strain TI130R5 (*rpoB5 ntdA*::Tn*10 amyE*::P_{ntdABC}-*lacZ*), which lacks the ability to synthesize NTD because of the presence of a transposon inserted within *ntdA*. After incubation at 37°C for 24 h, the diameter of the blue zone (representing the NTD activity) was measured. Purified NTD (>95% purity) was used as the standard, where linearity of the standard curve was found in the concentration range of 5 to 100 μg/ml NTD.

Transposon mutagenesis. For transposon mutagenesis, the temperature-sensitive mini-Tn*10*-containing plasmid pIC333 was used as described by Steinmetz and Richter (35). Plasmid pIC333 was introduced into *B. subtilis* 84R5 at 28°C, after which transformants were selected for Erm resistance. The transformant colonies were then used to inoculate 20 independent cultures (1 ml each) of L medium containing Spc. During the exponential phase of growth (optical density

TABLE 1. Strains used in this study

Strain	Genotype or description	Construction, reference, or source[a]
JCM1465	Prototroph (type species for *Bacillus subtilis*)	JCM[b]
168	*trpC2* (Marburg strain)	Laboratory stock
QB7103	*trpC2 crh*::*aphA3 ptsH1 amyE*::(PΔB *levD′-lacZ cat*)	11
GM273	*trpC2*Δ*ptsGHI*::*erm*	5
61884	*trpC2 aspB66*	28
84R5	*trpC2 aspB66 rpoB5*	18
84R2	*trpC2 aspB66 rpoB2*	18
84R6	*trpC2 aspB66 rpoB6*	18
84R32	*trpC2 aspB66 rpoB32*	18
84R5-UP1	*trpC2 aspB66 rpoB5 glcP*::Tn*10*	This study
84R5-DN1	*trpC2 aspB66 rpoB5 ymfI*::Tn*10*	This study
84R5-DN2	*trpC2 aspB66 rpoB5 ccpA*::Tn*10*	This study
TI122	*trpC2 aspB66 amyE*::(P_{ntdABC}-*lacZ cat*)	18
TI122-UP1	*trpC2 aspB66 glcP*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	84R5-UP1→TI122
TI122R5	*trpC2 aspB66 rpoB5 amyE*::(P_{ntdABC}-*lacZ cat*)	18
TI122R5-UP1	*trpC2 aspB66 rpoB5 glcP*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	84R5-UP1→TI122R5
TI122R5-DN1	*trpC2 aspB66 rpoB5 ymfI*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	84R5-DN1→TI122R5
TI122R5-DN2	*trpC2 aspB66 rpoB5 ccpA*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	84R5-DN2→TI122R5
TI127R5	*trpC2 aspB66 rpoB5 ntdR*::*neo amyE*::(P_{ntdABC}-*lacZ cat*)	18
TI130	*trpC2 aspB66 ntdA*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	TI130R5→TI122
TI130R5	*trpC2 aspB66 rpoB5 ntdA*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	18
TI138	*trpC2 aspB66* Δ*ntdABC*::*cat*	pUC18-ΔntdABC::cat →61884
TI167	*trpC2 aspB66 ntdC*-pMutinT3-T_{ntdABC}	pMutinT3-ntdC→61884
TI167R5	*trpC2 aspB66 rpoB5 ntdC*-pMutinT3-T_{ntdABC}	TI167→84R5
TI168	*trpC2 aspB66* T_{ntdABC}-pMutinT3-*glcP*	pMutinT3-glcP→61884
TI168R5	*trpC2 aspB66 rpoB5* T_{ntdABC}-pMutinT3-*glcP*	TI168→84R5
TI168Δ4	*trpC2 aspB66* T_{ntdABC}-pMutinT3-Δ*glcP*	pMutinT3-ΔglcP→61884
TI168R5Δ4	*trpC2 aspB66 rpoB5* T_{ntdABC}-pMutinT3-Δ*glcP*	TI168Δ4→84R5
TI171	*trpC2 aspB66* Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}	pMutinT3-ntdC→TI138
TI171R5	*trpC2 aspB66 rpoB5* Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}	84R5→TI171
TI171R2	*trpC2 aspB66 rpoB2* Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}	84R2→TI171
TI171R6	*trpC2 aspB66 rpoB6* Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}	84R6→TI171
TI171R32	*trpC2 aspB66 rpoB32* Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}	84R32→TI171
TI172	*trpC2 aspB66* Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*	pMutinT3-glcP→TI138
TI172R5	*trpC2 aspB66 rpoB5* Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*	84R5→TI172
TI172R2	*trpC2 aspB66 rpoB2* Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*	84R2→TI172
TI172R6	*trpC2 aspB66 rpoB6* Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*	84R6→TI172
TI172R32	*trpC2 aspB66 rpoB32* Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*	84R32→TI172
TI179	*trpC2 aspB66* Δ*ptsGHI*::*erm amyE*::(P_{ntdABC}-*lacZ cat*)	GM273→TI122
TI179R5	*trpC2 aspB66 rpoB5* Δ*ptsGHI*::*erm amyE*::(P_{ntdABC}-*lacZ cat*)	TI179→TI122R5
TI184R5	*trpC2 aspB66 rpoB5 crh*::*aphA3 amyE*::(P_{ntdABC}-*lacZ cat*)	QB7103→TI122R5
TI185R5	*trpC2 aspB66 rpoB5 crh*::*aphA3 ptsH1 amyE*::(P_{ntdABC}-*lacZ cat*)	QB7103→TI179R5
TI191R5	*trpC2 aspB66 rpoB5 ntdR*::*neo glcP*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)	TI127R5→TI122R5-UP1
TI196R5	*trpC2 aspB66 rpoB5 ptsH1 amyE*::(P_{ntdABC}-*lacZ cat*)	TI185R5→TI179

[a] Arrows indicate construction by transformation (with congression in some cases).
[b] JCM, Japan Collection of Microorganisms.

at 650 nm [OD_{650}], 0.5), the temperature was shifted from 28°C to 42°C, and the incubation was continued for an additional 4 h. Appropriate dilutions of these cultures were then spread onto L agar plates containing Spc. We selected colonies that are resistant to Spc but sensitive to Erm. The mutants with altered NTD production were identified using the NTD assay plate (see above) after the isolates were cultivated for 24 h in S7N medium containing 1% glucose. The chromosomal region targeted by the transposon was rescued as follows. Chromosomal DNA from each transposant was prepared and digested with EcoRI or HindIII. The mini-Tn*10* transposon-inserted gene, along with its flanking region, was cloned into *E. coli* JM109 by self-religation and sequenced using primers Tn10-F (5′-GCCGCGTTGGCCGATTC-3′) and Tn10-R (5′-GATATTCACGGTTTAC-3′).

Northern blot analysis. Cells grown on L agar plate were inoculated into S7N medium, cultured at 37°C with vigorous shaking, and harvested at the indicated times, and total cellular RNA was prepared using Isogen reagent (Nippon Gene) according to the protocols provided by the manufacturer. The RNAs were subjected to electrophoresis in the presence of formamide, transferred onto a membrane (Hybond-N^+; Amersham), and hybridized with a digoxigenin-labeled RNA probe. For RNA dot blotting, RNAs were spotted directly onto a membrane. Transcription of the RNA probes was driven by the T7 promoter in the pCR2.1 vector. To prepare the template for the *ntdB* probe, the DNA fragment containing the full-length *ntdB* coding region was amplified by PCR using primers ntdBF (5′-ATGTTATTAAGC AAGAAATCGGAG-3′) and ntdBR (5′-TTATTTCCTCCTCATGAATCCA-3′). The amplified DNA was then cloned into pCR2.1 to generate pCR2.1-ntdB. Plasmid pCR2.1-ntdB together with pCR2.1-ntdC and pCR2.1-glcP (see above) served as a template for in vitro transcription. A digoxigenin RNA labeling kit (SP6/T7) was purchased from Roche Diagnostics and used for RNA labeling.

Assay of β-Gal activity. Strains were grown aerobically in S7N medium, after which β-galactosidase (β-Gal) activity was measured as described previously (16). One unit is equivalent to $1{,}000 \times A_{420}/OD_{650}$/min, where A_{420} is the absorbance at 420 nm.

RESULTS

NTD biosynthesis is regulated by CcpA-mediated catabolite activation. We previously showed that a single Ser487→Leu substitution (*rpoB5* mutation) within the β subunit of RNAP

TABLE 2. Characterization of genes that affect NTD production

Strain	Tn*10* inserted gene	Function or similarity	NTD titer (μg/ml)[a]
61884	NA[b]	NA	<5
84R5	NA	NA	200
84R5-UP1	*glcP*	Glucose/mannose:H^+ symporter	700
84R5-DN1	*ymfI*	Similar to 3-oxoacyl-acyl carrier protein reductase	<5
84R5-DN2	*ccpA*	Catabolite control protein A	5

[a] *B. subtilis* strains were grown overnight on L agar plates at 30°C, after which they were inoculated into S7N medium containing excess (1%) glucose and incubated for 24 h at 37°C. NTD titers were determined by using paper disk agar diffusion assays with *B. subtilis* TI130R5 (*rpoB5 ntdA*::Tn*10 amyE*::P_{ntdABC}-*lacZ*) as the indicator strain (see Materials and Methods). NTD titers for strains 84R5 and 84R5-UP1 were determined after appropriate dilution of their cultured broth. Purified NTD (>95% purity) was used as the standard. At least two independent experiments were performed.

[b] NA, not applicable.

TABLE 3. Expression of *ntdABC-lacZ* in various *B. subtilis* mutants

Strain	Relevant genotype	P_{ntdABC}-*lacZ* (U/OD_{650})[a]	
		−NTD	+NTD
TI122R5		330 ± 47	410 ± 74
TI122R5-UP1	*glcP*::Tn*10*	440 ± 98	490 ± 130
TI122R5-DN1	*ymfI*::Tn*10*	23 ± 7.2	300 ± 8.5
TI122R5-DN2	*ccpA*::Tn*10*	48 ± 33	200 ± 92
TI179R5	Δ*ptsGHI*	150 ± 79	220 ± 18
TI184R5	*crh*::*aphA3*	270 ± 62	470 ± 14
TI196R5	*ptsH1*	170 ± 18	430 ± 33
TI185R5	*crh*::*aphA3 ptsH1*	26 ± 12	180 ± 82

[a] Cells were grown for 15 h at 37°C in S7N medium containing excess (1%) glucose with or without 500 μg/ml NTD. β-Gal activity is expressed in units/cell optical density at 650 nm. The values are the means ± standard deviations of three or more experiments conducted independently.

causes a dramatic activation of NTD production in *B. subtilis* (18). We then carried out transposon mutagenesis of the *B. subtilis rpoB5* mutant (strain 84R5) using the mini-Tn*10* delivery plasmid pIC333 and identified the NTD biosynthesis operon. During the course of that transposon mutagenesis, three other insertion mutants were also found to be affected on NTD production. One mutant represented by strain 84R5-UP1 produced 3.5-fold more NTD than the 84R5 strain, while the other two mutants represented by strains 84R5-DN1 and 84R5-DN2, respectively, displayed dramatically reduced NTD production (Table 2). A backcross transformation revealed that the insertion mutation was directly responsible for the observed phenotype (data not shown), and the genes inactivated by the transposon insertion in each strain were identified through target rescue and sequencing (Table 2). The 84R5-UP1 mutant carried a mini-Tn*10* insertion within the N-terminal coding region of *glcP*. The 84R5-DN1 mutant carried the insertion within *ymfI*, which encodes an uncharacterized protein with similarity to 3-oxoacyl-acyl carrier protein reductase, and the 84R5-DN2 mutant carried the insertion within *ccpA*, which encodes a major transcription factor mediating carbon catabolite regulation (12).

To further analyze the effects of these mutations, each insertion was introduced into reporter strain TI122R5 carrying the *ntdABC* promoter (P_{ntdABC}) fused to *lacZ* at the *amyE* locus, after which the β-Gal activities in resultant strains were measured. In this way, we were able to study the possible effect of the mutations on the promoter activity for *ntdABC* expression. As shown in Table 3, the β-Gal activity in each mutant was in good agreement with the mutants' abilities to produce NTD. The CcpA-mediated catabolite regulation is widely believed to depend on either of two Ser46-phosphorylated proteins: the histidine-containing protein HPr, which is a component of PTS (1, 5, 8), or its paralogue, Crh (11). These Ser46-phosphorylated proteins (HPr and Crh) stimulate CcpA to bind catabolite-responsive element (*cre*). We therefore tested the effect of these mutations (*ptsH* and *crh*, which encode HPr and Crh proteins, respectively) on the expression of P_{ntdABC}-*lacZ*. Similar to the results obtained with a *ccpA* mutation, the double mutation of *crh* and *ptsH* (but not *crh* or *ptsH* mutation alone) diminished the expression of P_{ntdABC}-*lacZ* (Table 3), supporting the intrinsic role of CcpA in NTD production. Unlike the *glcP* mutant, a mutant lacking glucose-PTS (Δ*ptsGHI*) also exhibited a lower expression level. The reduced expression of P_{ntdABC}-*lacZ* observed in all of these mutants was restored extensively by adding purified NTD (Table 3), which can be taken as an indication that *ccpA* and *ymfI* play a role, directly or indirectly, in the supply of substrate(s) for NTD biosynthesis, the synthesis of which is dependent on a glucose activation mechanism. In fact, as shown in Fig. 2, the expression of P_{ntdABC}-*lacZ* was induced at the stationary phase in a glucose-dependent manner; almost no induction was observed in wild-type strain TI122 under the glucose-limited (0.1%) condition. Further evidence supporting the above notion came from the fact that the addition of NTD (200 μg/ml) markedly stimulated *ntdABC* expression (reaching 103 U/OD_{650} in the $rpoB^+$ strain), even in cells cultured with 0.1% glucose, demonstrating a bypass of the requirement of glucose for *ntdABC* expression in the presence of exogenous NTD (data not shown). On the other hand, the enhanced *ntdABC* expression observed in the *glcP* mutant appears to contradict the positive effect of glucose on NTD production, as mentioned above. We therefore further investigated the regulatory function of GlcP on *ntdABC* expression.

***glcP* is cotranscribed with the NTD biosynthesis operon via transcriptional readthrough.** To analyze the role of GlcP during NTD production, we first compared the growth and expression of P_{ntdABC}-*lacZ* in the wild type with those in the *glcP* mutant strains using S7N medium which contained an excess amount (1%) of glucose (Fig. 3). We found that the *glcP* mutant grew somewhat slower than the parent strain, presumably due to the impaired glucose uptake. The *glcP* mutation resulted in a dramatic elevation of β-Gal activity in both the $rpoB^+$ wild-type and the *rpoB5* mutant strains. It is noteworthy that the elevation of β-Gal activity was detected even at the exponential growth phase, indicating that GlcP protein exists and functions throughout the entire growth phase. In addition, because NtdR is a transcriptional activator of *ntdABC* (18), we then measured the *ntdR* promoter (P_{ntdR})-*lacZ* expression in the *glcP* mutant. We found that whereas the *glcP* mutation induced expression of P_{ntdABC}-*lacZ*, the expression of P_{ntdR}-*lacZ* in the *glcP* mutant was rather less than that in the $glcP^+$ parental strain, especially during the late growth phase (data not shown). However, in a

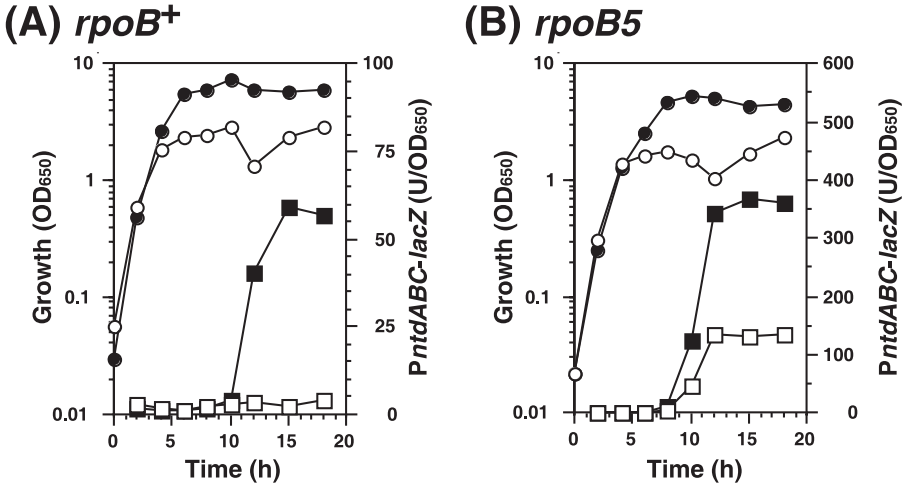


FIG. 2. Growth and transcription of P_{ntdABC}-*lacZ* in the *B. subtilis* wild type and *rpoB5* mutant strains. Strains TI122 (wild type) (A) and TI122R5 (*rpoB5* mutant) (B) were grown in the presence of limited (0.1%, open symbols) or excess (1%, closed symbols) glucose. Culture samples were withdrawn at the indicated times, and culture densities (OD_{650}, circles) and β-Gal activities (squares) were measured. At least two independent experiments were performed, and one of them is shown.

mutant (strain TI191R5) lacking functional NtdR, the effect of the *glcP* mutation on P_{ntdABC}-*lacZ* expression was no longer observed (data not shown). From these results, it is concluded that, although NtdR is an essential element for *ntdABC* expression even in the *rpoB5* mutant or *glcP* mutant strain, a basal level of *ntdR* expression is sufficient to fully activate P_{ntdABC}.

Since no obvious promoters exist downstream of the *ntdABC* transcriptional terminator (T_{ntdABC}), we hypothesized that *glcP* may be cotranscribed along with *ntdABC* via transcription readthrough. To test this idea, we subjected the transcripts to Northern blotting using probes for *ntdC* and *glcP* (Fig. 4A). As expected, in *rpoB5* mutant cells grown to mid-exponential phase (OD_{650}, 0.5), the *ntdABC* transcript (3.2 kb) and a transcription readthrough product (4.5 kb) were both clearly detected using an *ntdC* probe (Fig. 4B, left panel). By contrast, when the *glcP* probe was used, only the readthrough product was detected (Fig. 4B, right panel). Likewise, these transcripts (*ntdABC* and *ntdABC-glcP*) were observed in transition (OD_{650}, 1.5) and stationary growth phase (OD_{650}, 8.0) (data not shown). These results indicate that P_{ntdABC} is the sole promoter of *glcP* expression. Although it was difficult to detect the *ntdABC-glcP* readthrough transcript in *rpoB*$^+$ wild-type cells, the *ntdABC* transcript (though slight) was readily detectable, even during the exponential growth phase (Fig. 4B), a time when only basal levels of P_{ntdABC} activity were seen (see Fig. 3). These results suggest that a basal level of *ntdABC* expression (as seen during the exponential growth phase [see Fig. 2]) is sufficient for cells to express *glcP*, at least under the experimental conditions used here.

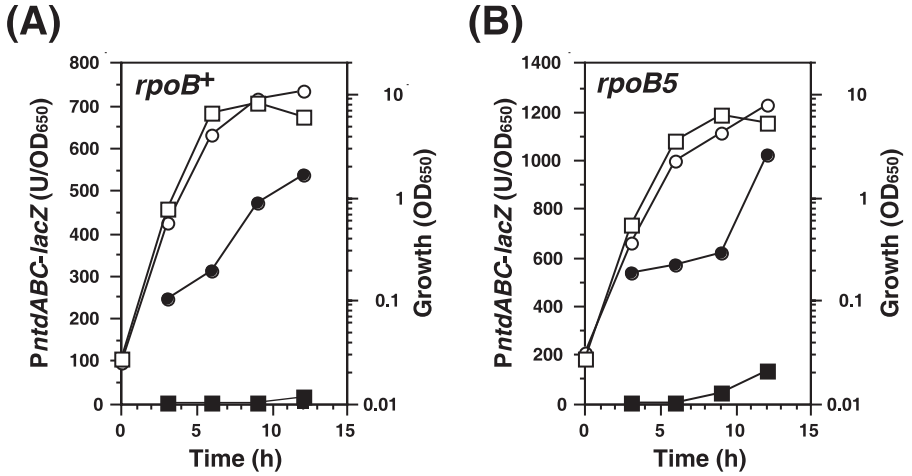


FIG. 3. Effect of *glcP* mutation on the expression of P_{ntdABC}-*lacZ* in *B. subtilis* wild-type and *rpoB5* mutant strains. The expression of P_{ntdABC}-*lacZ* in *B. subtilis rpoB*$^+$ wild-type (A) and *rpoB5* mutant (B) strains is shown. Strains TI122 (*rpoB*$^+$, squares), TI122-UP1 (*rpoB*$^+$ *glcP*::Tn*10*, circles), TI122R5 (*rpoB5*, squares), and TI122R5-UP1 (*rpoB5 glcP*::Tn*10*, circles) were grown in S7N medium containing excess (1%) glucose. Culture samples were withdrawn at the indicated times, and culture densities (OD_{650}, open symbols) and β-Gal activities (closed symbols) were measured. At least three independent experiments were performed, and one of them is shown.

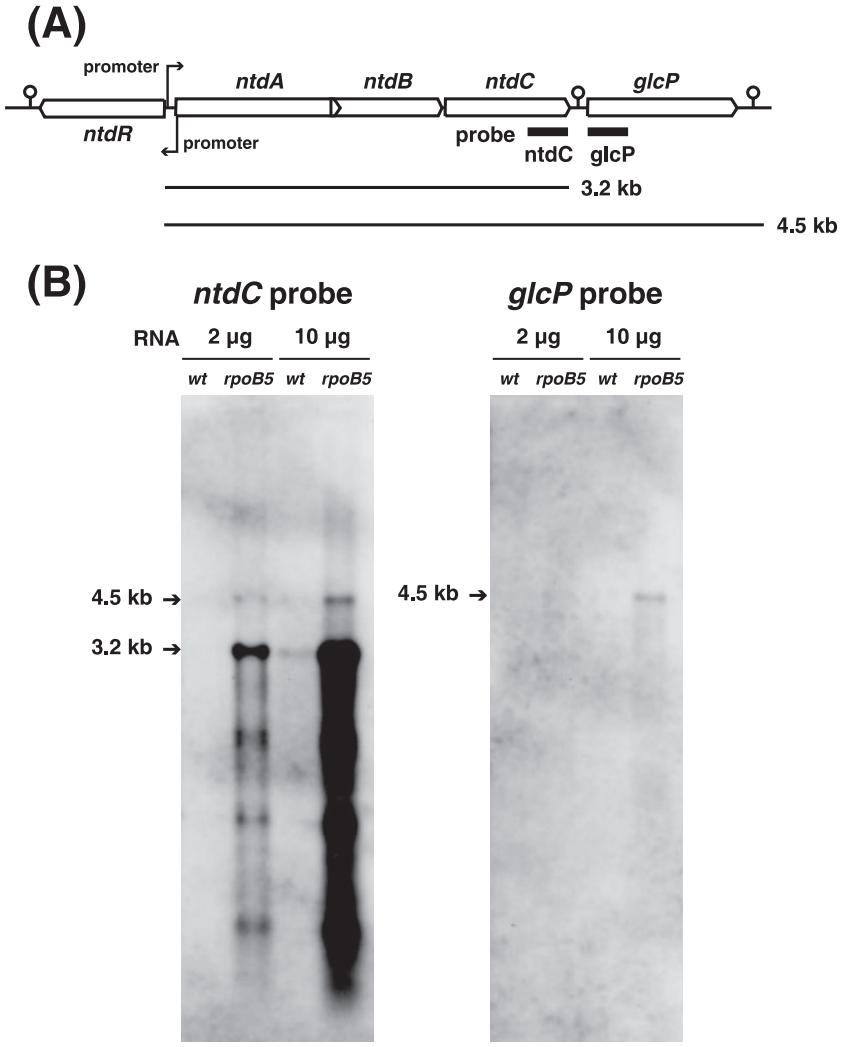


FIG. 4. Northern analysis of *ntdABC* transcription. (A) Structure of the *ntdABC* operon. Promoters and transcriptional terminators are indicated. The two transcripts of the *ntdABC* operon and the probes used for Northern analysis are shown. (B) Northern analysis of the *ntdABC* transcript using probes for *ntdC* and *glcP*. *B. subtilis* strains 61884 (wild-type) and 84R5 (*rpoB5* mutant) were grown in S7N medium containing excess (1%) glucose until OD_{650} reached 0.5, after which total cellular RNA was prepared from each strain using Isogen reagent. RNA samples (2 or 10 μg) were subjected to electrophoresis, transferred to a membrane, and then hybridized with the RNA probe for *ntdC* (left panel) or *glcP* (right panel) shown in panel A. Lane wt, wild type.

Expression of the NTD biosynthesis operon is repressed by GlcP-mediated glucose uptake. To further analyze the role of GlcP in NTD biosynthesis, we next inserted the pMutinT3 plasmid immediately upstream or downstream of the T_{ntdABC} of the *rpoB*$^+$ wild-type and *rpoB5* mutant strains, yielding *glcP*-interrupted strains carrying *ntdC*-pMutinT3-T_{ntdABC} and T_{ntdABC}-pMutinT3-*glcP*, respectively (Fig. 5A). These strains enabled us to control the expression of *glcP* via the IPTG-dependent promoter P_{spac}. In addition, a *glcP*-disrupted strain carrying T_{ntdABC}-pMutinT3-Δ*glcP* was also constructed. In this strain, T nucleotide in the second codon of *glcP* was deleted, resulting in a −1 frameshift and a nonsense mutation at Leu2 (TTA)→ochre (TAA). Initially, we directly detected the *ntdABC* transcript in the presence or absence of IPTG using RNA dot blotting with an *ntdB* probe (Fig. 5B). With the *rpoB*$^+$ wild-type genetic background, induction of *glcP* (in the presence of IPTG) apparently repressed *ntdABC* expression in both the TI167 and TI168 strains (Fig. 5B). With the *rpoB5* mutant genetic background, however, this repressive effect was no longer observed in strain TI167R5 (but not in TI168R5), possibly due to the blockade of *glcP* transcription by T_{ntdABC} in TI167R5. An alternative explanation for the absence of the repressive effect is that *glcP* is expressed in TI167 and TI167R5 less than in TI168 and TI168R5, and the lower concentration of GlcP is sufficient to repress transcription in wild-type *rpoB*$^+$ cells but not in *rpoB5* mutant cells. Given that the addition of IPTG had no effect on *ntdABC* expression in the Δ*glcP* strains (TI168Δ4 and TI168R5Δ4) as a control experiment, we concluded that expression of *ntdABC* is repressed by a GlcP protein-mediated mechanism.

Using *glcP*-interrupted strain TI168, we tested whether or not GlcP-dependent glucose uptake is responsible for the down-regulation of *ntdABC* transcription (Fig. 5C). When *glcP* was expressed in the absence of glucose (Fig. 5C), the expres-

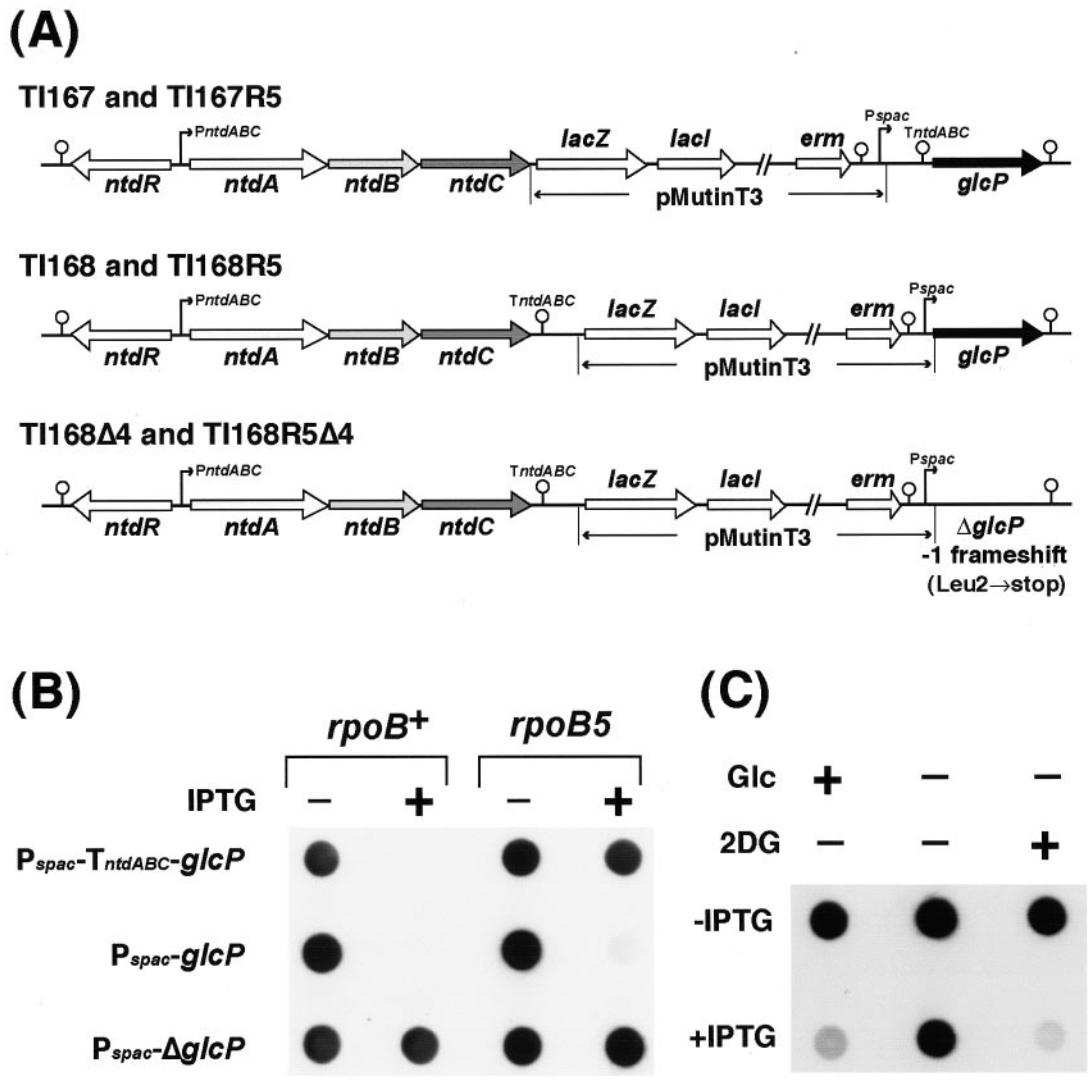
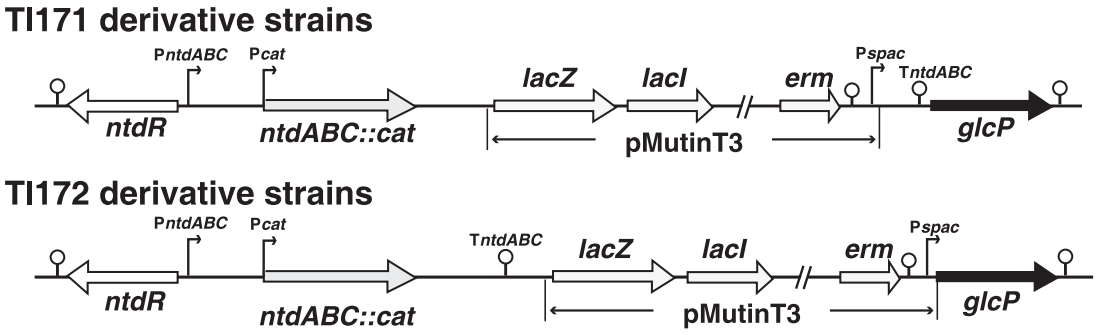


FIG. 5. Repression of *ntdABC* transcription by GlcP-mediated glucose transport. (A) Construction of *glcP*-interrupted strains. *B. subtilis* *glcP*-interrupted strains carrying *ntdC*::pMutinT3 or *glcP*::pMutinT3 were constructed by integrating a plasmid, pMutinT3, immediately upstream or downstream of T_{ntdABC}, respectively. These strains enabled us to control the expression of the *glcP* gene via the IPTG-dependent P_{spac}. A *glcP*-disrupted strain carrying Δ*glcP*::pMutinT3 also was constructed. In this strain, T nucleotide in the second codon of *glcP* was deleted, resulting in a −1 frameshift and a nonsense mutation at Leu-2 (TTA) (→ochre [TAA]). (B) Repression of *ntdABC* by induction of *glcP*. Total cellular RNAs were prepared from cells of strains TI167 (*rpoB*$^+$ *ntdC*::pMutinT3), TI168 (*rpoB*$^+$ *glcP*::pMutinT3), TI168Δ4 (*rpoB*$^+$ Δ*glcP*::pMutinT3), TI167R5 (*rpoB5 ntdC*::pMutinT3), TI168R5 (*rpoB5 glcP*::pMutinT3), and TI168R5Δ4 (*rpoB5* Δ*glcP*::pMutinT3), which were grown for 10 h in S7N medium containing excess (1%) glucose with or without 2 mM IPTG. RNA samples (10 μg each) were spotted directly onto a membrane and hybridized with the RNA probe for *ntdB*. (C) Repression of *ntdABC* by GlcP-mediated sugar transport. *B. subtilis* strain TI168 (*rpoB*$^+$ *glcP*::pMutinT3) was grown in S7N medium containing excess (1%) glucose (Glc) until OD_{650} reached 1.0, after which the cells were harvested by centrifugation and transferred into the same S7N medium (left lane), S7N medium without glucose (center lane), or S7N medium supplemented with 1% 2-deoxy-glucose (2DG) instead of glucose (right lane). Where indicated, IPTG was added to final a concentration of 2 mM just after the cells were transferred. After incubation for an additional 4 h, cells were harvested and RNA samples (4 μg each) were spotted directly onto a membrane and hybridized with the RNA probe for *ntdC*.

sion of *ntdABC* was derepressible. However, the addition of 2-deoxy-glucose (a nonmetabolizable carbon source) instead of glucose entirely repressed *ntdABC* expression in cells expressing *glcP*, indicating that GlcP protein alone does not function as a repressor for *ntdABC*. The repression of *ntdABC* by glucose was also observed in a *ccpA* mutant genetic background (data not shown). Thus, GlcP-mediated glucose uptake appears to negatively regulate expression of the NTD biosynthesis operon in a CcpA-independent manner, which led us to conclude that glucose is able to act both as an inducer and as a repressor of *ntdABC* expression in *B. subtilis*, depending upon its uptake pathway, although we cannot completely eliminate the possibility that GlcP together with glucose regulates expression of the operon.

Mutant RNAP efficiently recognizes the *ntdABC* transcription termination site. The results shown in Fig. 5B imply that the readthrough frequency at T_{ntdABC} in the *rpoB5* mutant strain is less than that in the *rpoB*$^+$ wild-type strain. Accord-



rpoB allele	β-Gal activity (U/OD_{650})		Readthrough (%)
	P-*lacZ*	P-T_{ntdABC}-*lacZ*	
rpoB$^+$	62.7 ± 10.9	11.2 ± 1.5	17.8 ± 0.7
rpoB5(S487L)	187.0 ± 18.4	20.7 ± 3.2	11.0 ± 0.6
rpoB2(H482R)	54.0 ± 7.2	17.1 ± 1.1	31.8 ± 2.2
rpoB6(H482Y)	64.3 ± 11.2	21.3 ± 4.9	32.9 ± 1.9
rpoB32(H482P)	68.4 ± 7.9	24.3 ± 1.7	35.6 ± 1.6

FIG. 6. Readthrough frequency at the *ntdABC* terminator site for various *rpoB* mutants. (Upper panel) Construction of strains for the T_{ntdABC} transcription readthrough assay. To avoid the effect of endogenously synthesized NTD, the *ntdABC* operons in strains TI167 and TI168 were replaced with *cat* genes. The *cat* gene used here contains a weak promoter. In a TI171 derivative strain carrying Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}, β-Gal activity represents the total promoter activity, whereas in a TI172 derivative strain carrying Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*, β-Gal activity represents the activity resulting only from transcription readthrough at T_{ntdABC}. Therefore, the transcription readthrough level can be expressed as the β-Gal activity resulting from transcription readthrough divided by the activity representing the total promoter activity. (Lower panel) TI171 and TI172 derivative strains containing each *rpoB* allele were cultivated for 15 h in S7N medium (containing 1% glucose) supplemented with purified NTD (500 μg/ml) and then harvested, after which measurements of β-Gal activities were made. Readthrough frequencies are expressed as β-Gal activity in TI172 derivative strains divided by the activity in a TI171 derivative strain. β-Gal activities are shown as the means ± standard deviations of three or more experiments.

ingly, we compared the readthrough frequency of the *rpoB*$^+$ wild-type strain with those of various *rpoB* mutant strains at T_{ntdABC}. To avoid the effect of endogenously synthesized NTD, *ntdABC* in the strains TI167 and TI168 was deleted and replaced with the *cat* gene, generating strains TI171 and TI172, respectively (Fig. 6). The *cat* gene used here contains a weak promoter (P_{cat}). In the TI171 derivative strains carrying Δ*ntdABC*::*cat*-pMutinT3-T_{ntdABC}, β-Gal activity represents the total promoter activity from P_{ntdABC} and P_{cat}, whereas in the TI172 derivative strains carrying Δ*ntdABC*::*cat*-T_{ntdABC}-pMutinT3-*glcP*, β-Gal activity represents only the activity resulting from transcription readthrough at T_{ntdABC}. Therefore, the transcription readthrough level can be expressed as the β-Gal activity resulting from transcription readthrough divided by the activity representing the total promoter activity. In this way, the readthrough frequency at T_{ntdABC} was measured using various *rpoB* mutants. Expectedly, the *rpoB5* mutant revealed a decreased readthrough frequency compared to that in the *rpoB*$^+$ wild-type strain (Fig. 6). In contrast, the readthrough frequencies for other *rpoB* mutants harboring a His482→Arg (*rpoB2*), a His482→Tyr (*rpoB6*), or a His482→Pro (*rpoB32*) substitution within the RNAP β subunit were even higher than that in the *rpoB*$^+$ wild-type strain. The *rpoB5* RNAP mutant, which carries a Ser487→Leu substitution within its β subunit, thus appears to have a greater ability to recognize T_{ntdABC} than the wild-type RNAP or the His482 mutants described above.

NTD functions in *rpoB*$^+$ wild-type cells at concentrations below the detectable level. Although NTD activity normally could not be detected (with a bioassay) in culture broth conditioned by the *B. subtilis* wild-type strain (*rpoB*$^+$) (Table 2), an insertional mutation of *glcP* led to increased *ntdABC* expression (Fig. 3A) and, in turn, detectable NTD production (data not shown). In addition, we previously reported that the insertional mutation of *ntdA* causes a loss of promoter activity (18). Taking these facts together, it is possible that NTD can function for *ntdABC* expression at a concentration level below that which is detectable (5 μg/ml). We therefore determined the minimum concentration of NTD required for autoinduction using the reporter strain TI130, which contains a transposon inserted within *ntdA*. As shown in Fig. 7, *B. subtilis* cells sensed the exogenously added NTD at concentrations higher than 0.5 μg/ml. The amount of NTD produced by the *B. subtilis rpoB*$^+$

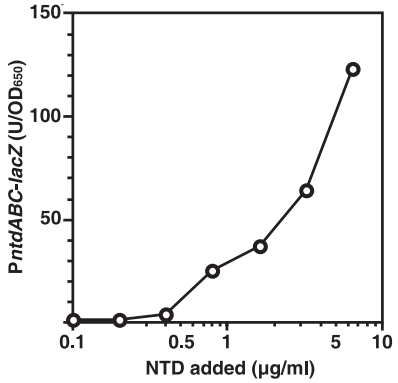


FIG. 7. Minimum concentration of NTD needed for *ntdABC* transcriptional activation. *B. subtilis* strain TI130 [*ntdA*::Tn*10 amyE*::(P_{ntdABC}-*lacZ cat*)] was grown for 10 h in S7N medium (containing 1% glucose) supplemented with various concentrations of NTD, after which culture samples were withdrawn and their β-Gal activities were measured. At least four independent experiments were performed, and one of them is shown.

61884 strain was 1 to 3 μg/ml, as calculated based on the expression level of P_{ntdABC}-*lacZ* in the *rpoB*$^+$ strain. Two other *B. subtilis* standard strains (JCM1465 and 168) and a number of *B. subtilis* strains isolated from soil also produced levels of NTD that were low but detectable with a bioassay (about 10 μg/ml). It is therefore concluded that the NTD produced in a wide variety of *B. subtilis* spp., though in small amounts, is able to function as a glucose uptake modulation factor in this bacterial group.

DISCUSSION

Bacteria are able to monitor their surroundings by releasing and detecting signaling molecules called autoinducers. This process enables bacteria to control gene expression so they can adapt to changes within their environment. Among the different classes of autoinducers, the best studied is LuxI, an acylhomoserine lactone autoinducer produced by *Vibrio fischeri* (reviewed by Schauder and Bassler [33]). Moreover, *B. subtilis* is known to express two oligopeptide autoinducers, ComX and CSF (competence and sporulation factors), but many gram-positive autoinducer systems are currently under study (reviewed by Dunny and Leonard [6]). We previously reported that NTD is a novel autoinducer needed for transcription of its own biosynthesis operon in *B. subtilis* (18). This notion is confirmed in the present work, making NTD the third known autoinducer produced by *B. subtilis*. Our work also demonstrates that the downstream *glcP* gene is coexpressed with the *ntdABC* operon through transcription readthrough (Fig. 4). In other words, GlcP-mediated glucose uptake is under the control of NTD. Although the *B. subtilis rpoB*$^+$ wild-type strain produces less-than-detectable amounts of NTD, our results indicate that a trace amount (0.5 to 3 μg/ml) of NTD is sufficient to ensure expression of *glcP*. Thus, by stimulating the transcription of *ntdABC-glcP*, NTD functions as a glucose uptake modulator in *B. subtilis* and probably in other NTD-producing bacteria, with a gene arrangement identical to that in *B. subtilis*. This conclusion is important because glucose metabolism takes a central place in a wide variety of metabolic systems. Another significant aspect of our finding is based on the fact that in addition to its function as an autoinducer, NTD has an antimicrobial activity against certain bacteria, possibly by inhibiting the cell wall synthesis, as demonstrated by the decline of antimicrobial activity in the presence of 1 mM D-glucosamine (T. Inaoka and K. Ochi, unpublished data). This is particularly intriguing, since the intrinsic biological role of antibiotics (if any) in the producing bacteria has not been verified (or has been largely overlooked) so far. Many bacteria, including *B. subtilis* (34), often produce antibiotics. NTD therefore may offer a feasible system for the study of possible physiological roles of "antibiotics" in the producing bacteria.

The effect of glucose on NTD production is enigmatic. The expression of *ntdABC* is apparently regulated, perhaps indirectly, by a mechanism related to CcpA-mediated carbon catabolite activation. Consistent with this idea are the observations that expression of P_{ntdABC}-*lacZ* was induced during the stationary phase in a glucose-dependent manner (Fig. 2) and that NTD production was severely impaired in a *ccpA* mutant (Tables 2 and 3). That the addition of purified NTD to the growth medium stimulated P_{ntdABC}-*lacZ* expression in *ccpA* and *ymfI* mutants suggests that these genes are involved in de novo synthesis of NTD, perhaps playing a role for the supply of a precursor(s) for NTD biosynthesis. At present, however, we cannot exclude the possibility that CcpA directly regulates *ntdABC* expression. Although there is no *cre* CcpA-binding motif in the *ntdABC* promoter region, a number of other genes subject to catabolite activation also lack a *cre* motif (2, 23, 24). It was somewhat surprising that GlcP-mediated glucose uptake repressed *ntdABC* expression in a CcpA-independent manner (Fig. 5C). This finding appears to contradict the positive effect of glucose on NTD production, but one possible explanation is that glucose can exert alternative (positive and negative) effects on *ntdABC* transcription, depending upon the uptake pathway. As summarized in Fig. 8, glucose transported through the predominant uptake pathway (glucose-PTS) is phosphorylated to glucose-6-phosphate during the permeation process and is then rapidly metabolized in the glycolytic pathway. In response to high cellular glycolytic activity, a glycolytic intermediate fructose-1,6-bisphosphate stimulates phosphorylation of HPr and Crh at Ser46 by activating the HPr kinase/phosphorylase (HprK), eventually resulting in CcpA-dependent catabolite activation (11). However, P_{ntdABC} remained repressed until the cells entered the stationary phase.

The repression seen during the exponential growth phase appears to depend solely on the glucose transport mediated by GlcP (Fig. 5C). Although details of the mechanism remain unclear, it is likely that a presumptive transcription regulator of *ntdABC* expression linked to GlcP-dependent glucose uptake (designated X in Fig. 8) might be involved. Within the framework of this notion, NtdR per se can be one of the candidates for X. Another hypothesis is that glucose itself transported by GlcP might interfere with the synthesis of a precursor of NTD. Investigation of the molecular mechanism underlying this glucose repression mediated by GlcP is now in progress. Such a study may provide a clue for uncovering the regulation mechanism of gene expression for NTD synthesis and also may help to further our understanding of the intrinsic mechanism of catabolite regulation in *B. subtilis*. In that regard, the *glcP* mutation partially but specifically relieves glucose- and su-

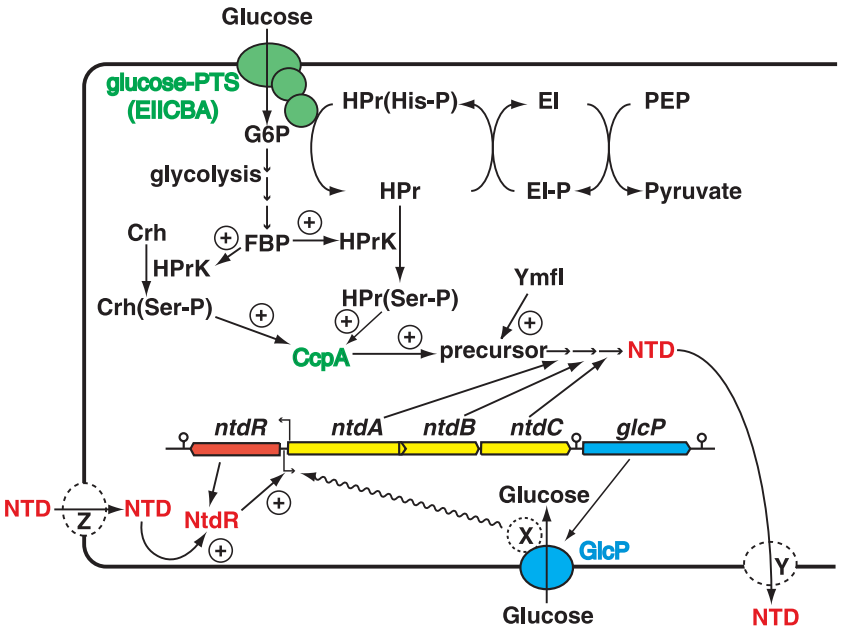


FIG. 8. Proposed model of NTD production in *B. subtilis*. Glucose transported through glucose-PTS (EIICBA) positively regulates NTD production. This activation mechanism is based on the CcpA-dependent catabolite activation mechanism. Under conditions of high glycolytic activity, fructose-1,6-bisphosphate stimulates the phosphorylation of HPr and Crh at Ser46 by activating HprK, leading to CcpA-dependent catabolite activation (11). CcpA and YmfI proteins appear to be involved, directly or indirectly, in de novo synthesis of a precursor(s) for NTD, rather than in *ntdABC* promoter regulation. By contrast, *ntdABC* is repressed by GlcP-dependent glucose uptake. Possibly, a presumptive transcriptional regulator (designated X) linking to GlcP-dependent glucose uptake participates in this regulation. The transcriptional activator NtdR binds directly to NTD and activates the *ntdABC* promoter (18). NTD transporters (Y and Z), which are required for export or import of NTD, have not yet been identified. Positive effects are represented as arrows (→), while the wavy line represents a regulation of the *ntdABC* promoter by a presumptive transcriptional regulator, X. The glucose-PTS is comprised of enzyme I (EI), enzyme II (EIICBA), and HPr. G6P, glucose-6-phosphate; FBP, fructose-1,6-bisphosphate.

crose-promoted catabolite repression of the gluconate operon (30).

Another point of interest is the mechanism underlying the gene activation induced by a certain Rif-resistant *rpoB* mutation. The *rpoB* mutations also reportedly activate antibiotic production in several *Streptomyces* spp. (13, 14, 21, 37). These earlier studies suggest that the mechanism of gene activation involves the alteration of the ternary structure of RNAP. We previously showed that a mutant RNAP (Ser487→Leu, corresponding to the *rpoB5* mutation) efficiently recognizes housekeeping sigma factor (σ^A)-dependent promoters, including P_{ntdABC} (18). In agreement with that work, a quantitative analysis using surface plasmon resonance also revealed that this mutated RNAP binds to P_{ntdABC} more efficiently than the wild-type RNAP (H. Aoki, T. Inaoka, and K. Ochi, unpublished results). In addition to those earlier works, the present work shows that the readthrough frequency at T_{ntdABC} of the *rpoB5* mutant strain was less than that of the *rpoB*$^+$ wild-type strain (Fig. 6B), indicating that the RNAP mutant acquired a superior ability to recognize not only the promoter but also the transcription terminator, thus leading to a synergistic activation of NTD production. We do not know at present whether or not the enhancement of the transcription termination caused by the mutant RNAP is specific to the *ntdABC-glcP* operon. Moreover, very marked differences in *ntdABC* expression eventuated between *rpoB*$^+$ and *rpoB5* mutant cells (Fig. 5B), despite rather small differences (at most twofold) in readthrough frequencies (Fig. 6). Although further work is needed for more-convincing evidence of the mutant RNAP effect on transcription readthrough, these novel findings would be useful not only for basic microbiology but also for constructing new systems for screening secondary metabolites and enhancing the ability of strains to produce useful compounds.

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