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CodY-Mediated Regulation of Guanosine Uptake in *Bacillus subtilis*▽

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CodY is a global transcriptional regulator known to control expression of more than 100 genes and operons in *Bacillus subtilis*. Some of the most strongly repressed targets of CodY, the *nupNOPQ* (formerly, *yufNOPQ*) genes, were found to encode a guanosine transporter. Using DNase I footprinting experiments, we identified two high-affinity CodY-binding sites in the regulatory region of the *nupN* gene. The two sites are located 50 bp upstream and 163 bp downstream of the transcription start site. The downstream site was responsible for 6- to 8-fold *nupN* repression in the absence of the upstream site. When the upstream site was intact, however, only a minor contribution of the downstream site to *nupN* regulation could be detected under the conditions tested. Both sites contained 15-bp CodY-binding motifs with two mismatches each with respect to the consensus sequence, AATTTTCWGTTTTAA. However, the experimentally determined binding sites included additional sequences flanking the 15-bp CodY-binding motifs. An additional version of the 15-bp CodY-binding motif, with 5 mismatches with respect to the consensus but essential for efficient regulation by CodY, was found within the upstream site. The presence of multiple 15-bp motifs may be a common feature of CodY-binding sites.

Bacillus subtilis cells are able to take up purine and pyrimidine nucleosides, allowing these compounds to serve as substrates for nucleotide biosynthesis via salvage pathways and as sources of carbon and nitrogen (40). Although two major transporters for pyrimidine and purine nucleosides, NupC and NupG, respectively, have been described in *B. subtilis*, mutants lacking NupC or NupG retain considerable ability to take up pyrimidine or purine nucleosides (19, 33). Thus, additional transporters for both pyrimidine and purine nucleosides remain to be identified (2, 19, 33).

The *B. subtilis nupNOPQ* (formerly, *yufNOPQ*) genes appear to encode the components of an ABC transport system of unknown specificity. The first gene of this apparent operon, *nupN*, codes for an apparent lipoprotein that could serve as the substrate-binding, specificity-determining subunit of the transporter. The putative product of *nupN* is homologous (43 to 50% identity) to several proteins that are involved in purine and pyrimidine nucleoside transport in *Treponema pallidum*, *Streptococcus mutans*, and *Lactococcus lactis* (9, 26, 41). The *nupO* and *nupPQ* genes appear to encode the ATP-binding protein and integral membrane proteins typical of ABC transporters, respectively.

The *nupNOPQ* genes were previously shown to be among the genes that are most highly repressed by CodY as detected by DNA microarray analysis and *lacZ* fusions (29). CodY is a global transcriptional regulator that controls expression of more than 100 genes and operons in *B. subtilis*.

Many of the CodY-regulated genes are involved in nitrogen or carbon metabolism (14, 29, 37–39). The main role of CodY in *B. subtilis* appears to be establishing the temporal hierarchy of utilization of various nitrogen (and carbon) compounds under conditions of nutrient excess. CodY homologs are also present in most other low-G+C, Gram-positive bacteria and in many species have been shown to coordinate expression of virulence-associated functions with expression of metabolic genes (see references 12, 21, 25, and 38 and references therein).

The activity of *B. subtilis* CodY as a DNA-binding protein (20, 23, 35) is increased by interaction with two types of effectors, branched-chain amino acids (isoleucine, leucine, and valine [ILV]) (10, 15, 22, 23, 30, 36) and GTP (17, 29, 31, 36), allowing the bacteria to change the pattern of CodY-dependent gene expression in response to the availability of nutrients in the growth medium.

We demonstrate here that the *nupNOPQ* genes encode a previously unidentified transporter for guanosine and, possibly, other nucleosides. Unexpected aspects of the mechanism by which CodY mediates repression of transcription from the *nupN* promoter are also described.

MATERIALS AND METHODS

Bacterial strains and culture media. The *B. subtilis* strains constructed in this study were all derivatives of strain SMY (44) and are described in Table 1 and in the text. *Escherichia coli* strain JM107 (43) was used for isolation of plasmids. Bacterial growth in DS nutrient broth and minimal TSS medium was as described previously (5).

DNA manipulations. Methods for common DNA manipulations, transformation, primer extension, DNA sequencing, and sequence analysis were performed as previously described (5, 6, 32). Procedures for gel shift experiments and DNase I footprinting were described in detail previously (5, 6, 32). Chromosomal DNA of *B. subtilis* strain SMY or plasmids constructed in this work were used as templates for PCR. The oligonucleotides used in this work are described in Table 2. All cloned PCR-generated fragments were verified by sequencing.

Construction of an *nupN* (*yufN*)-null mutant. The 0.35-kb PCR fragments containing the 5′ or 3′ part of the *nupN* gene and the adjacent sequences, respectively, were cloned in two steps as contiguous inserts between the XbaI and HindIII or HindIII and XhoI sites of pBB544 (4). Then, the XbaI-XhoI fragment of the resulting plasmid, pBB1625, was recloned in the integrative plasmid pBB1579. pBB1579 (Neo^r) is a derivative of pBB544 that contains the *clpB-bgaB* construct expressing thermostable *Bacillus stearothermophilus* β-galactosidase from the constitutive *clpB* promoter of *Staphylococcus aureus*. The 2.1-kb *clpP-bgaB* fragment was synthesized by PCR using pMAD (1) as a template and cloned between the KpnI and NgoMI sites of pBB544.

The resulting plasmid, pBB1654, containing an in-frame deletion within the

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source or reference[a]
CU4121	*purM*::Tn*917* (*erm*) *trpC2*	BGSC
PS251	*codY*::(*erm*::*spc*) *trpC2*	P. Serror
SMY	Prototroph	44
YXJAd	*nupG*::pMutin1 (*erm*)	NBRP
NUPNd	*nupN*::pMutin2 (*erm*)	NBRP
NUPOd	*nupO*::pMutin2 (*erm*)	NBRP
BB1888	*lacA*::*tet*	6
BB2511	Δ*amyE*::*spc lacA*::*tet*	6
BB2686	Δ*amyE*::[*erm* Φ(*nupN503p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1474
BB2809	Δ*amyE*::[*erm* Φ(*nupN276p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1520
BB2899	Δ*amyE*::[*erm* Φ(*nupN347p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1521
BB2900	Δ*amyE*::[*erm* Φ(*nupN120p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1522
BB2935	Δ*amyE*::[*erm* Φ(*nupN367p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1534
BB2936	Δ*amyE*::[*erm* Φ(*nupN140p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1535
BB2952	Δ*amyE*::[*erm* Φ(*nupN367p1-lacZ*)] *lacA*::*tet*	BB2511 × pBB1542
BB2953	Δ*amyE*::[*erm* Φ(*nupN380p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1543
BB2954	Δ*amyE*::[*erm* Φ(*nupN140p1-lacZ*)] *lacA*::*tet*	BB2511 × pBB1544
BB2955	Δ*amyE*::[*erm* Φ(*nupN153p*$^+$-*lacZ*)] *lacA*::*tet*	BB2511 × pBB1545
BB2972	Δ*amyE*::[*erm* Φ(*nupN380p21-lacZ*)] *lacA*::*tet*	BB2511 × pBB1554
BB2973	Δ*amyE*::[*erm* Φ(*nupN380p22-lacZ*)] *lacA*::*tet*	BB2511 × pBB1555
BB2974	Δ*amyE*::[*erm* Φ(*nupN153p21-lacZ*)] *lacA*::*tet*	BB2511 × pBB1556
BB2975	Δ*amyE*::[*erm* Φ(*nupN153p22-lacZ*)] *lacA*::*tet*	BB2511 × pBB1557
BB3014	Δ*amyE*::[*erm* Φ(*nupN153p2-lacZ*)] *lacA*::*tet*	BB2511 × pBB1574
BB3015	Δ*amyE*::[*erm* Φ(*nupN153p3-lacZ*)] *lacA*::*tet*	BB2511 × pBB1575
BB3322	Δ*nupN amyE*::*spc lacA*::*tet*	BB2511 × pBB1654
BB3401	Δ*amyE*::[*erm* Φ(*nupN347p11-lacZ*)] *lacA*::*tet*	BB2511 × pBB1676
BB3402	Δ*amyE*::[*erm* Φ(*nupN503p12-lacZ*)] *lacA*::*tet*	BB2511 × pBB1677
BB3431	Δ*amyE*::[*erm* Φ(*nupN380p3/11-lacZ*)] *lacA*::*tet*	BB2511 × pBB1691
BB3484	Δ*nupN amyE*::*spc lacA*::*tet purM*::Tn*917* (*erm*)	BB3322 × CU4121 DNA
BB3486	Δ*nupN nupG*::pMutin1 (*erm*) *amyE*::*spc lacA*::*tet*	BB3332 × YXJAd DNA
BB3491	Δ*amyE*::[*erm* Φ(*nupN380p3-lacZ*)] *lacA*::*tet*	BB2511 × pBB1698
BB3492	Δ*amyE*::[*erm* Φ(*nupN380p11-lacZ*)] *lacA*::*tet*	BB2511 × pBB1699
BB3493	Δ*amyE*::[*erm* Φ(*nupN347p12-lacZ*)] *lacA*::tet	BB2511 × pBB1700
BB3499	Δ*nupN nupG*::pMutin1 (*erm*) *amyE*::*spc lacA*::*tet purM*::(Tn*917*::*neo*)	BB3485 × YXJAd DNA
BB3501	*nupG*::pMutin1 (*erm*) *lacA*::*tet*	BB1888 × YXJAd DNA
BB3502	*nupG*::pMutin1 (*erm*) *lacA*::*tet purM*::(Tn*917*::*neo*)	BB3501 × BB3499 DNA
BB3546	*purM*::(Tn*917*::*neo*)	SMY × BB3485 DNA
BB3597	Δ*amyE*::[*erm* Φ(*nupN153p23-lacZ*)] *lacA*::*tet*	BB2511 × pBB1724
BB3598	Δ*amyE*::[*erm* Φ(*nupN153p24-lacZ*)] *lacA*::*tet*	BB2511 × pBB1725
BB3599	Δ*amyE*::[*erm* Φ(*nupN153p2/21-lacZ*)] *lacA*::*tet*	BB2511 × pBB1726

[a] BSCG, Bacillus Genetic Stock Center; NBRP, National BioResource Project (NIG, Japan): *B. subtilis* (http://www.shigen.nig.ac.jp/bsub/). × denotes transformation by plasmid or chromosomal DNA.

nupN gene was introduced by a single-crossover homologous recombination event into the *nupN* chromosomal locus of strain BB2511 (*amyE lacA*). White Neo^s colonies indicating excision of pBB1654 from the chromosome were searched for on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), the colored substrate of β-galactosidase. The presence of the *nupN* deletion in strain BB3322 was confirmed by analyzing the size of the chromosomal *nupN* allele by PCR.

Construction of transcriptional *lacZ* fusions. Plasmid pBB1474 containing a transcriptional *nupN503-lacZ* fusion was created by cloning in pHK23 (6) the XbaI- and HindIII-treated 0.52-kb *nupN* PCR product created with oligonucleotides oBB311 and oBB312 as the forward and reverse primers, respectively. pBB1474 contained a G-to-A substitution at position −176 with respect to the *nupN* transcription start point (see below) that was apparently introduced by PCR and was unlikely to affect *nupN* expression. (This mutation was later deleted when *nupN380-lacZ* and most other fusions were constructed.) Plasmids pBB1521 (*nupN347p*$^+$*-lacZ*), pBB1534 (*nupN367p*$^+$*-lacZ*), and pBB1543 (*nupN380p*$^+$*-lacZ*) carrying a 347-bp, 367-bp, or 380-bp version of the *nupN* regulatory region truncated by 156, 136, or 123 bp at the 5′ end, respectively, were created in a manner similar to pBB1474, but with oligonucleotide oBB365 or oBB379 as the forward PCR primer, respectively.

Plasmids pBB1520 (*nupN276p*$^+$*-lacZ*), pBB1522 (*nupN120p*$^+$*-lacZ*), and pBB1545 (*nupN153p*$^+$*-lacZ*), carrying 276-bp, 120-bp, or 153-bp versions of the *nupN* regulatory region truncated by 227 bp at the 3′ end were created in a manner similar to pBB1474, pBB1521, or pBB1543, respectively, but with oligonucleotide oBB357 as the reverse PCR primer.

Plasmid pBB1535 (*nupN140p*$^+$*-lacZ*), carrying a 140-bp version of the *nupN* regulatory region truncated by 136 bp at the 5′ end and 227 bp at the 3′ end, was created using oligonucleotides oBB367 and oBB357 as forward and reverse PCR primers, respectively.

B. subtilis strains carrying various *lacZ* fusions at the *amyE* locus (Table 1) were isolated after transforming strain BB2511 (*amyE*::*spc lacA*) with the appropriate plasmids, by selecting for resistance to erythromycin, conferred by the plasmids, and screening for loss of the spectinomycin resistance marker, which indicated a double-crossover, homologous recombination event. Strain BB2511 and all of its derivatives have very low endogenous β-galactosidase activity due to a null mutation in the *lacA* gene (8a).

Mutations in CodY-binding sites. Plasmids pBB1542 (*nupN367p1-lacZ*) and pBB1544 (*nupN140p1-lacZ*) were constructed as described above for pBB1534 (*nupN367p*$^+$*-lacZ*) and pBB1535 (*nupN140p*$^+$*-lacZ*), but using mutagenic oligonucleotide oBB378 as the forward PCR primer.

Plasmids pBB1554 (*nupN380p21-lacZ*), pBB1555 (*nupN380p22-lacZ*), pBB1556 (*nupN153p21-lacZ*), pBB1557 (*nupN153p22-lacZ*), pBB1574 (*nupN153p2-lacZ*), pBB1575 (*nupN153p3-lacZ*), pBB1698 (*nupN380p3-lacZ*), pBB1724 (*nupN153p23-lacZ*), and pBB1725 (*nupN153p24-lacZ*) were constructed as described above for pBB1543 (*nupN380p*$^+$*-lacZ*) or pBB1545 (*nupN153p*$^+$*-lacZ*), but using appropriate mutagenic oligonucleotides specified in Table 2 as forward PCR primers.

TABLE 2. Oligonucleotides used in this work

Oligonucleotide type and name	Sequence[a]	Specificity
Flanking primers		
Forward		
oBB67	5′-GCTTCTAAGTCTTATTTCC	*erm* (pHK23)
oBB311	5′-CAATATCTAGATAAGAAAACGCACTGC	*nupN503*
oBB365	5′-TTAAATCTAGATGCGAAATTCTATTTATTTC	*nupN347*, 5′ flank of *nupN*
oBB367	5′-CTAAATCTAGATAATTTTTAAAAAATTATGCG	*nupN367*
oBB378	5′-CTAAATCTAGATAATTTT**CAG**AAAATTATGCG	*nupNp1*
oBB379	5′-TTTTATCTAGATAATTCTAAAAATAGATAA	*nupN380*
oBB390	5′-TTTTATCTAGATAATTCT**G**AAAATAGATAA	*nupNp21*
oBB391	5′-TTTTATCTAGATAATT**g**TAAAAATAGATAA	*nupNp22*
oBB400	5′-TTTTATCTAGATAATTCTAAAAATAGATAATTTTTAA**c**AAATTATGC	*nupNp2*
oBB401	5′-TTTTATCTAGATAATTCTAAAAATAGATAA**a**TTTTAAAAAATTATG	*nupNp3*
oBB412	5′-AAAAAGGTACCGTCTAGTTAATGTGTAAC	*clpB* (pMAD)
oBB437	5′-CCTACAAGCTTGATCAAGACGGAG	3′ flank of *nupN*
oBB567	5′-GATCCTCTAGATAATTCTA**c**AAATAG	*nupNp23*
oBB568	5′-GATCCTCTAGATAA**a**TCTAAAAATAG	*nupNp24*
Reverse		
oBB102	5′-CACCTTTTCCCTATATAAAAGC	*lacZ* (pHK23)
oBB253	5′-GGTTTTCCCGGTCGAC	*lacZ* (pHK23)
oBB312	5′-ACAAGAAGCTTCGCCAATCCGATTTTTC	*nupN503*, 5′ flank of *nupN*
oBB357	5′-CGGAAAAGCTTCCGGGCGGAAAACC	*nupN276*
oBB408	5′-CGAGAGCCGGCTAAACCTTCCCGGCTTC	*bgaB* (pMAD)
oBB438	5′-TTTTCCTCGAGCAACGCGTGTATTTC	3′ flank of *nupN*
Internal mutagenic primers		
Forward		
oBB502	5′-CCATTTGT**A**ATTT**T**TCAGAAAATTTTATC	*nupNp11*
oBB504	5′-CCATTTGTTATTATCA**c**AAAATTTTATC	*nupNp12*
Reverse		
oBB501	5′-TTTCTGA**A**AAT**T**ACAAATGGAATGCGC	*nupNp11*
oBB503	5′-T**g**TGATAATAACAAATGGAATG	*nupNp12*

[a] The altered nucleotides are in boldface; those conferring up mutations in the CodY-binding motif are in uppercase, and those conferring down mutations are in lowercase. The restriction sites are underlined.

Plasmid pBB1726 (*nupN153p2/21-lacZ*) was constructed as described above for pBB1556 (*nupN153p21-lacZ*), but using pBB1574 (*nupN153p2-lacZ*) as the PCR template.

The p11 mutation in the *nupN347* regulatory region was introduced by two-step overlapping PCR. In the first step, a product containing the 5′ part of the *nupN* regulatory region was synthesized by using oligonucleotide oBB365 as the forward primer and mutagenic oligonucleotide oBB501 as the reverse primer. A product containing the 3′ part of the *nupN* regulatory region was synthesized by using mutagenic oligonucleotide oBB502 as the forward primer and oBB312 as the reverse primer. The PCR products were used in a second, splicing step of mutagenesis as overlapping PCR templates to generate a modified fragment containing the entire *nupN* regulatory region; oligonucleotides oBB365 and oBB312 served as the forward and reverse PCR primers, respectively. The spliced PCR product was digested with XbaI and HindIII and cloned in pHK23, as described above, to create pBB1676 (*nupN347p11-lacZ*). Plasmid pBB1691 (*nupN380p3/11-lacZ*) was created in a similar manner by using pBB1698 (*nupN380p3-lacZ*) as the PCR template and oBB401 instead of oBB365. Plasmid pBB1699 (*nupN380p11-lacZ*) was created by using pBB1691 (*nupN380p3/11-lacZ*) as the PCR template and oBB379 and oBB312 as primers.

The p12 mutation in the *nupN503* regulatory region [pBB1677 (*nupN503p12-lacZ*)] was introduced by two-step overlapping PCR, as described above, by using oligonucleotide pairs oBB67 and oBB503 or oBB504 and oBB312 in the first PCR step and oBB67 and oBB312 in the second PCR step. pBB1700 (*nupN347p12-lacZ*) was created by using pBB1677 (*nupN503p12-lacZ*) as the PCR template and oBB365 and oBB312 as primers.

Guanosine uptake. Cells were grown at 37°C in TSS medium until mid-exponential phase, collected under vacuum on 0.45-μm-pore nitrocellulose filters, washed, and resuspended at an optical density at 600 nm (OD_{600}) of ≈0.8 in the same medium without NH_4Cl. Further incubation of cells was at 25°C. [^{3}H]guanosine (Moravek Biochemicals) was added to 1 μCi/ml (0.14 μM), and 160-μl samples were taken at the indicated time, collected immediately under vacuum on 0.45-μm-pore nitrocellulose filters, washed with 5 ml of TSS without NH_4Cl but containing 10 μg/ml guanosine, dried, and counted using Ecoscint H scintillation liquid (National Diagnostics). The protein concentration was determined in sonicated cell samples using Bio-Rad protein assay reagent. A 1-ml culture at an OD_{600} of 1 contained 127.5 μg of protein.

Labeling of DNA fragments. The PCR products containing the regulatory region of the *nupN* gene were synthesized using vector-specific oligonucleotides oBB67 and oBB102 as the forward and reverse primers, respectively. oBB67 starts 96 bp upstream of the XbaI site used for cloning, and oBB102 starts 36 bp downstream of the HindIII site that serves as a junction between the promoters and the *lacZ* part of the *nupN* fusion. oBB102 (which would prime synthesis of the template strand of the PCR products) was labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP.

Purification of CodY. Wild-type CodY was purified to near homogeneity as described previously (6).

Enzyme assays. β-Galactosidase specific activity was determined as described previously (8).

RESULTS

Phenotype of a *nupN* (*yufN*)-null mutant. A large DNA segment internal to the *nupN* gene was deleted without altering the reading frame of the distal part of the gene (see Materials and Methods). The deletion removed 84% of the *nupN* coding region. When introduced into the *B. subtilis* chromosome, the *nupN* mutation had no apparent effect on growth of cells in nutrient broth (DS) or in defined medium (TSS-glu-

cose-ammonium). Moreover, the *nupN*-null mutation did not affect the ability of a *purM* strain, which is blocked in *de novo* purine biosynthesis, to use the nucleoside inosine, adenosine, guanosine, or xanthosine as a substrate for purine nucleotide biosynthesis by the salvage pathway.

B. subtilis NupG (YxjA) has been previously reported to be a major transporter of purine nucleosides inosine and guanosine. The *nupG*-null mutant apparently retained about 20% of its parent strain's ability to take up the nucleosides, indicating the existence of another transporter(s) with overlapping specificity (19). In our tests, the *purM nupG* double-null mutant (strain BB3502) was able to utilize inosine, adenosine, or guanosine to satisfy its auxotrophic requirement on solid minimal medium. (Addition of xanthosine allowed very slow growth.) However, the triple mutant strain BB3499 (*purM nupG nupN*) had a strong defect in the ability to grow with guanosine as the sole source of purines. No effect of the *nupN* mutation on the cell's ability to utilize inosine or adenosine or xanthosine as the sole purine source on solid medium was detected in the *nupG purM* genetic background (data not shown). In liquid minimal medium containing 0.2 mM guanosine, the *purM* and *purM nupN* mutants grew with a generation time of 63 to 65 min, the *purM nupG* strain had a generation time of 80 min, and the *purM nupG nupN* mutant grew extremely slowly, with a generation time of 5.5 h. All four strains grew at similar rates when provided with 0.2 mM adenosine or guanine (data not shown).

A similar phenotype of very slow growth with guanosine as the sole purine source was observed when a *nupO*-null mutation was combined with *nupG* and *purM* mutations (data not shown). Thus, *nupN* appears to encode a component, presumably the substrate-binding protein, of a guanosine uptake system that likely involves the products of the *nupOPQ* genes as well. The residual ability of the *nupN nupG purM* or *nupO nupG purM* triple-null mutants to grow with guanosine as the sole purine source, albeit very slowly, indicates that still another, minor transporter of guanosine is functional in *B. subtilis* cells.

Guanosine uptake. As shown previously (19), inactivation of the *nupG* gene led to a significant reduction in the rate of guanosine incorporation into resting cells (Fig. 1A). (Our uptake measurements do not preclude guanosine metabolism and incorporation of the guanosine derivatives into other low- and high-molecular-weight cellular compounds.) A null mutation in the *nupN* gene (strain BB3322) did not lead to any detectable defect in guanosine uptake. However, in the *nupG nupN* double-null mutant strain (BB3486), guanosine uptake was reduced to an almost undetectable level (Fig. 1A). Thus, NupN and NupG are together responsible for the bulk of guanosine uptake, though, as our growth experiments indicated, another low-activity guanosine transporter apparently exists in *B. subtilis*.

Guanosine uptake in strain BB3501, provided mostly by NupN, was nearly abolished in the presence of 50 μM inosine or adenosine (357-fold excess over guanosine), suggesting that NupN binds these purine nucleosides and may also transport them (Fig. 1B). Xanthosine and the pyrimidine nucleosides cytidine, uridine, and thymidine (at 50 μM) reduced guanosine uptake partially (Fig. 1B) (data not shown), implying that NupN binds all of these nucleosides and suggesting that NupNOPQ may be a nucleoside transporter of broad specificity but with various affinities for different substrates.

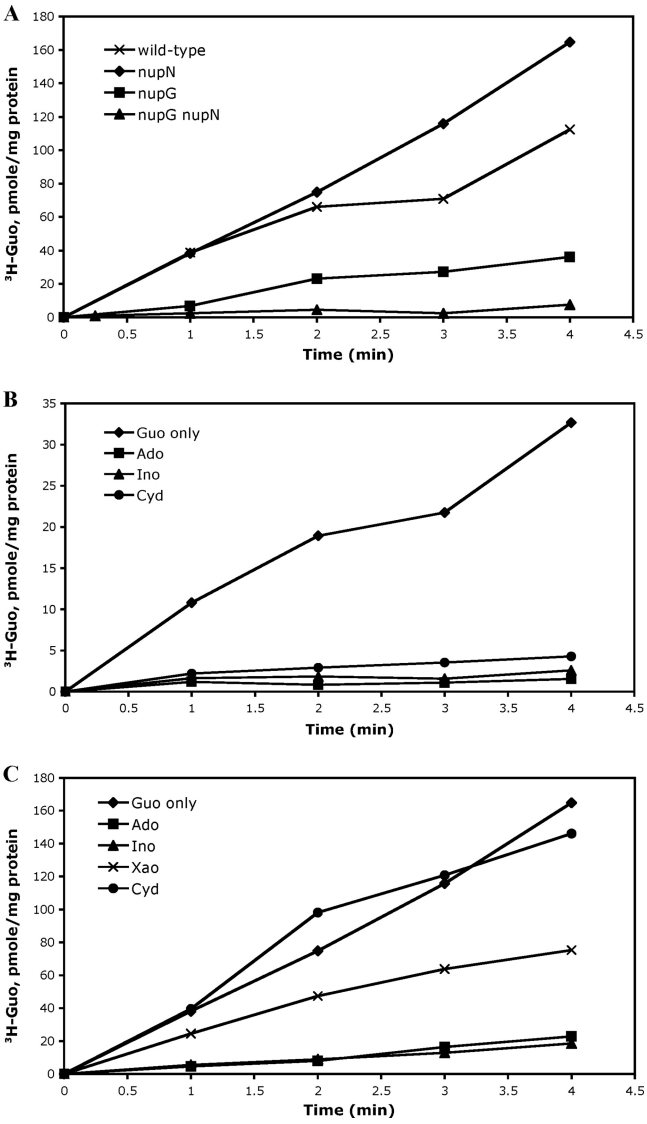

FIG. 1. Preliminary characterization of the roles of NupNOPQ and NupG in nucleoside uptake. (A) Roles of NupNOPQ and NupG in guanosine uptake. Cells of strains BB2511 (wild-type), BB3322 (*nupN*), BB3501 (*nupG*), and BB3486 (*nupG nupN*) were assayed for guanosine incorporation as described in Materials and Methods. (B) Effect of nucleosides on guanosine uptake by NupNOPQ. Cells of strain BB3501 (*nupG*) were assayed for guanosine incorporation in the absence (Guo only) or presence of other nucleosides (at 50 μM) as described in Materials and Methods. Guo, guanosine; Ado, adenosine; Ino, inosine; Cyd, cytidine. (C) Effect of nucleosides on guanosine uptake by NupG. Cells of strains BB3322 (*nupN*) were assayed for guanosine incorporation in the absence (Guo only) or presence of other nucleosides (at 50 μM) as described in Materials and Methods. Xao, xanthosine.

The uptake of guanosine in strain BB3322, mediated mostly by NupG, was efficiently reduced by addition of inosine or adenosine, slightly reduced by xanthosine, and not affected by the presence of cytidine, uridine, or thymidine, indicating that NupG is a dedicated purine nucleoside transporter (Fig. 1C) (data not shown).

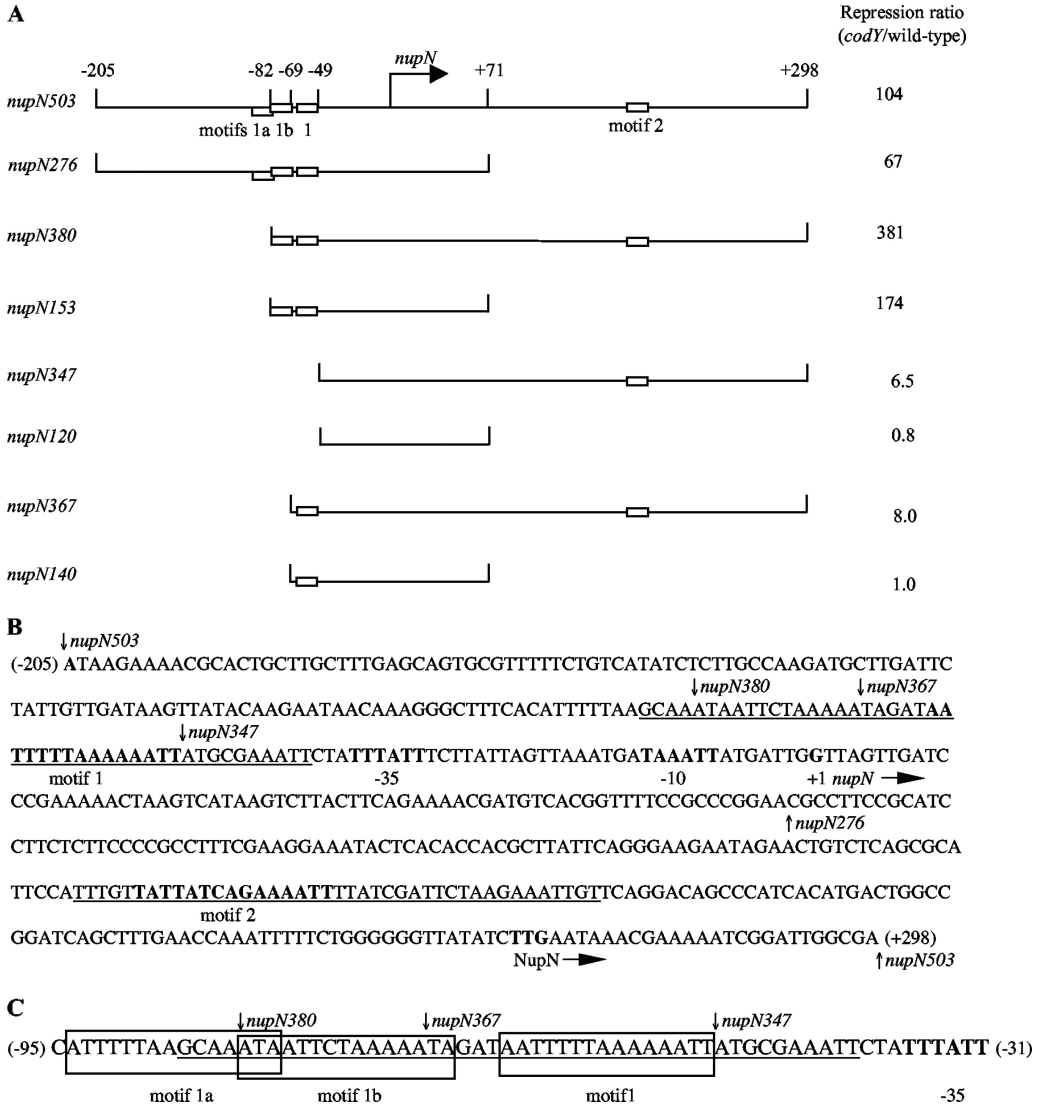

FIG. 2. Plasmid maps and the sequence of the *nupN* regulatory region. (A) Schematic maps of the *nupN* inserts used to construct *lacZ* fusions. The location of the transcription start point is indicated by the bent arrow. CodY-binding motifs are shown as rectangles. The coordinates indicate the boundaries of different fusions with respect to the transcription start point. The repression ratio is the ratio of expression values for the corresponding fusions in the *codY*-null mutant and wild-type strain in the 16-amino-acid-containing medium. (B) Sequence of the coding (nontemplate) strand of the *nupN* regulatory region. The likely initiation codon, −10 and −35 promoter regions, transcription start site, and CodY-binding motifs 1 and 2 are in boldface. The direction of transcription and translation is indicated by the arrows. The sequences protected by CodY in DNase I footprinting experiments on the template strand of DNA are underlined. The boundaries of DNA fragments used to construct various *lacZ* fusions are indicated by vertical arrows. The coordinates of the 5′ and 3′ ends of the sequence with respect to the transcription start point are shown in parentheses. Note the incorrect annotation of the *nupN* initiation codon in older databases. (C) Motifs in the *nupN* CodY-binding site I. The sequences protected by CodY in DNase I footprinting experiments on the template strand of DNA are underlined. The sequences of motifs 1a, 1b, and 1 are boxed. The −35 promoter region is in boldface. The boundaries of DNA fragments used to construct various *lacZ* fusions are indicated by vertical arrows. The coordinates of the 5′ and 3′ ends of the sequence with respect to the transcription start point are shown in parentheses.

CodY-dependent regulation of the *nupNOPQ* genes. The *nupN* gene appears to be the first gene of a four-gene (*nupNOPQ*) operon. We constructed a transcriptional fusion (*nupN503-lacZ*) containing a 503-bp DNA fragment that included the entire intergenic region upstream of the *nupN* gene (Fig. 2) and showed that the fusion was highly repressed by CodY. Under conditions of maximal CodY activity, in the glucose-ammonium minimal medium containing ILV and a mixture of 13 other amino acids (referred to here as the 16-amino-acid-containing medium), fusion activity in the *codY*-null mutant strain BB2691 was >100-fold higher (97.5 Miller units [MU]) than in wild-type strain BB2686 (Table 3).

In the wild-type strain, activity of the fusion was 15-fold derepressed (to 14.2 MU) in 13-amino-acid-containing medium (i.e., when isoleucine, leucine, and valine were omitted), and was further increased 10-fold in the absence of all amino acids. Addition of ILV alone to glucose-ammonium medium reduced expression from the *nupN* promoter 5-fold to 28.6

TABLE 3. Expression of *nupN-lacZ* fusions[a]

Strain	Relevant genotype	Fusion genotype[b]	Motif(s) present[c]	Growth medium			
				TSS + 16 amino acids		TSS	
				β-Galactosidase activity (MU)	Repression ratio[d]	β-Galactosidase activity (MU)	Repression ratio[d]
BB2686	Wild type	*nupN503p*$^+$	1a, 1b, 1, 2	0.94	104	138.0	1.3
BB2691	*codY*			97.5		184.0	
BB3402	Wild type	*nupN503p12*	1a, 1b, 1, 2^{down}	2.73	64	178.0	1.3
BB3417	*codY*			176.0		236.0	
BB2809	Wild type	*nupN276p*$^+$	1a, 1b, 1	0.09	67	2.27	1.6
BB2819	*codY*			6.04		3.54	
BB2899	Wild type	*nupN347p*$^+$	2	1.61	6.5	16.1	0.99
BB2906	*codY*			10.4		15.9	
BB3401	Wild type	*nupN347p11*	2^{up}	0.04	50	0.73	3.3
BB3416	*codY*			2.01		2.42	
BB3493	Wild type	*nupN347p12*	2^{down}	10.8	1.3	20.3	0.93
BB3497	*codY*			13.6		18.8	
BB2900	Wild type	*nupN120p*$^+$	None	0.37	0.76		
BB2907	*codY*			0.28			

[a] Cells were grown in TSS glucose-ammonium medium with or without a mixture of 16 amino acids. β-Galactosidase activity was assayed and expressed in Miller units (MU). All values are averages of at least two experiments, and the mean errors did not exceed 30%.
[b] p^+ indicates the presence of an unmodified promoter sequence within a fusion; for mutant promoters, the allele number is indicated.
[c] The "up" and "down" notations indicate mutations that make CodY-binding motifs more or less similar to the consensus sequence.
[d] The repression ratio is the ratio of expression values for the corresponding fusions in the *codY*-null mutant and wild-type strain in each medium.

MU. This pattern of regulation is common for other CodY-dependent genes (5). Only a 1.9-fold effect of the medium composition on *nupN* expression was detected in cells defective for CodY, indicating that CodY itself or a CodY-dependent factor is the major regulator of the gene under the conditions tested (Table 3, strain BB2691). *nupN* expression in strain BB2686 was not affected by the presence of guanosine, inosine, adenosine, or uridine (200 μg/ml) or guanine or hypoxanthine (100 μg/ml) in the minimal glucose-ammonium medium (data not shown).

Transcription start point. A primer extension experiment established that the 5′ end of *nupN* mRNA (corresponding to a likely transcription start point) is 270 bp upstream of the initiation codon (Fig. 2B and 3A). The sequences TTTATT and TAAATT, with 3 and 2 mismatches with respect to the −35 and −10 regions of σ^A-dependent promoters, and a 17-bp spacer region can be identified upstream of the apparent transcription start point (Fig. 2B).

CodY binding to the *nupN* gene. Recently, we have shown that two *B. subtilis* genes, *bcaP* and *ybgE*, contain two CodY-binding sites each, as defined by gel mobility shift and DNase I footprinting assays. The upstream sites that overlap the corresponding promoters are apparently used for inhibiting transcription initiation, and binding of CodY to the downstream sites inhibits transcription elongation via a roadblock mechanism (5, 7). Sequence analysis revealed that the *nupN* regulatory region contains two sequences, called here motif 1 and motif 2, that strongly resemble the previously defined 15-bp CodY-binding consensus motif, AATTTTCWGAAAATT (6, 11, 16), and that are located at positions −64 to −50 and +169 to +183 with respect to the transcription start point (Table 5). (We define "motifs" as 15-bp sequences that are similar to the CodY-binding consensus and "sites" as experimentally determined regions of CodY binding.) In fact, we have already shown that the downstream motif 2 lies within a region where transcription elongation is blocked *in vitro* in the presence of CodY (7). Thus, it was important to assess the relationship between the motifs and CodY binding sites and the contribution of each motif to CodY-dependent regulation *in vivo*.

Using the entire 503-bp *nupN* regulatory region, two widely separated CodY binding sites were detected in a DNase I footprinting experiment in the presence of ILV and GTP (Fig. 3B). In order to establish more precisely the sites interacting with CodY, we performed separate DNase I footprinting experiments using two DNA fragments comprising the promoter-proximal and promoter-distal parts of the regulatory region. CodY protected with similarly high affinities two regions of the template DNA strand from positions −86 to −40 and from positions +164 to +204 with respect to the *nupN* transcription start point (Fig. 2 and 3B). The upstream (site I) and downstream (site II) regions of protection overlapped the previously recognized motifs 1 and 2, respectively (Fig. 2B).

The concentration dependence of CodY binding to site II was very similar whether this site was present on the same fragment of DNA with site I or on a separate fragment (Fig. 3B). Thus, binding of CodY to site II apparently occurs independently of its binding to site I. (The reverse experiment was difficult to perform because of the poor resolution of the site I footprint if present together with site II.)

In gel shift experiments, CodY bound to DNA fragments containing only site I or site II with an apparent K_D (equilibrium dissociation constant) of ≈10 or ≈5 nM, respectively, compared with ≈4 nM for the full-length fragment (Fig. 2A and 4). (We used the concentration of CodY at which 50% of the DNA molecules are bound as an approximation of K_D.) No binding of CodY to the *nupN120* fragment that lacked both CodY-binding sites was detected in a DNase I footprinting experiment (Fig. 2A and 3C). In gel-shift experiments, a similar *nupN* fragment lacking both CodY-binding sites was bound by CodY only at the concentration of 800 nM, apparently reflecting a nonspecific interaction between CodY and DNA (Fig. 4).

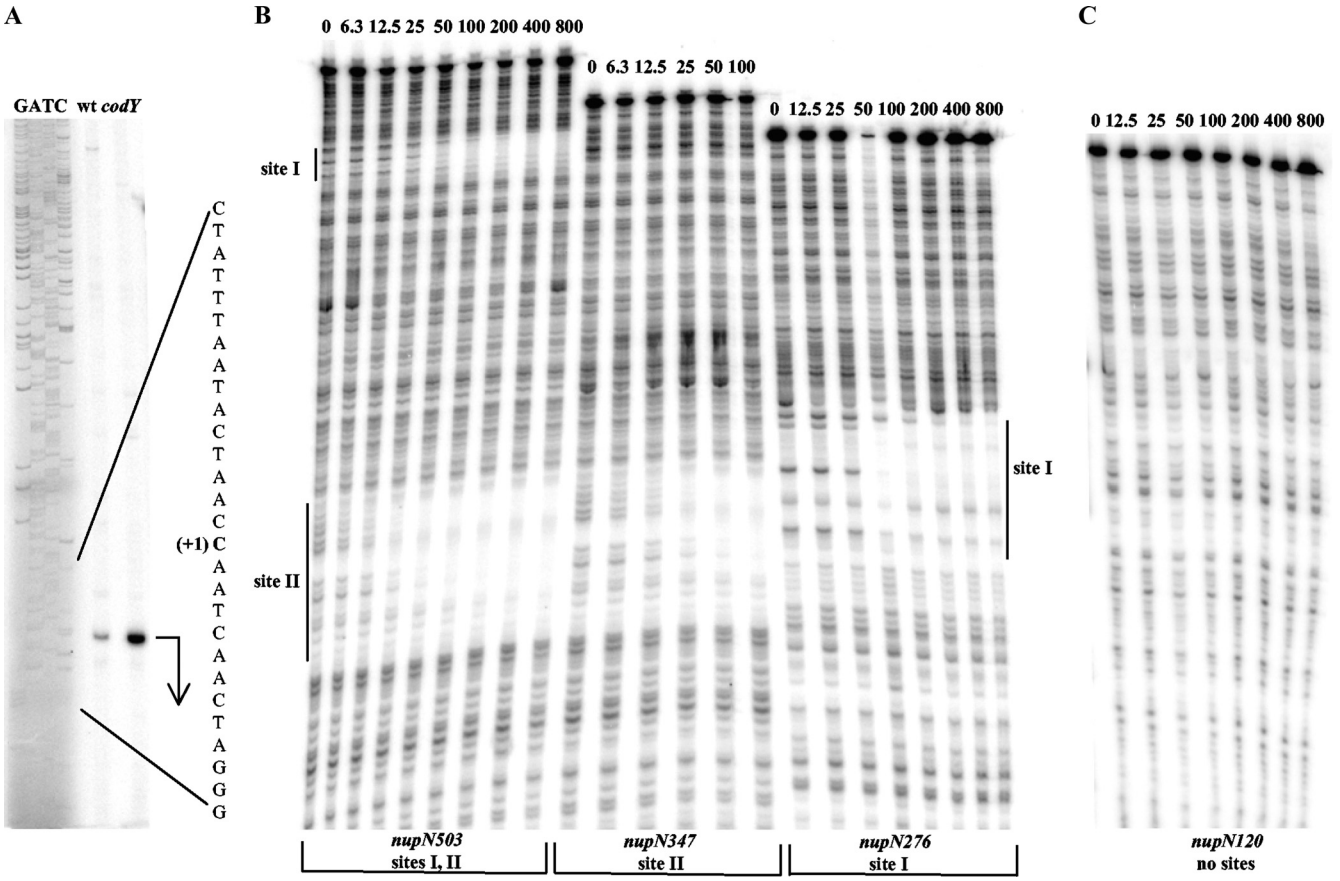

FIG. 3. Determination of the *nupN* transcription start point and CodY-binding regions. (A) Primer extension analysis of the *nupN* mRNA. Primer oBB102 annealing to the *lacZ* gene of the *nupN276-lacZ* fusion was extended with reverse transcriptase using as the template total RNA from fusion-containing strains BB2809 (wt) and BB2819 (*codY*) grown in the 16-amino-acid-containing medium. The sequence of the template strand of pBB1520 determined from reactions primed with oBB102 is shown to the left. The apparent transcription start site of the *nupN* gene is in bold and marked by the +1 notation. A bent arrow indicates the direction of transcription. (B) DNase I footprinting analysis of CodY binding to the *nupN* regulatory region. The *nupNp*$^+$ DNA fragments labeled on the template strand were incubated with increasing amounts of purified CodY in the presence of 10 mM ILV and 2 mM GTP and then with DNase I. The apparent transcription start site and direction of *nupN* transcription are shown by the bent arrow. The protected areas are indicated by the vertical lines. CodY concentrations used (nanomolar concentrations of monomers) are indicated above each lane. (C) Same as panel B, *nupN120p*$^+$ fragment.

Contribution of CodY-binding sites to *nupN* regulation. To determine the relative contributions of the two CodY-binding sites to regulation of *nupN*, we used *lacZ* fusions containing truncated versions of the *nupN* regulatory region lacking either the downstream or the upstream CodY-binding site (Fig. 2). The resulting fusions, *nupN276-lacZ* (site I only) and *nupN347-lacZ* (site II only), were both still repressed by CodY 67- and 6.5-fold, respectively (Table 3; compare strains BB2809 and BB2899 and their *codY* derivatives). No repression by CodY was observed for the *nupN120-lacZ* fusion that lacked both CodY-binding sites (Fig. 2 and Table 3, strains BB2900 and BB2907).

Unexpectedly, CodY-dependent regulation of the *nupN276-lacZ* fusion lacking the downstream site II was almost as efficient as regulation of the full-length fusion *nupN503-lacZ* (67-fold versus 104-fold repression in 16-amino-acid-containing medium) (Table 3, strains BB2809, BB2686, and their *codY* derivatives). Therefore, although sites I and II were both able to confer CodY-mediated repression independently, it was not clear to what extent site II contributes to CodY-mediated repression when site I is present.

Interestingly, removal of the sequence upstream of position −49 in the *nupN* regulatory region reduced the maximal level of expression >10-fold (compare the activities of the *nupN347-lacZ* [−49 to +298] and *nupN120-lacZ* [−49 to +71)] fusions to those of their respective counterparts, *nupN503-lacZ* [−205 to +298] and *nupN276-lacZ* [−205 to +71], in the *codY* mutant strain (Table 3). Part of the deleted sequence may play a functional role similar to that of an UP promoter element (18, 28). The upstream boundary of this apparent activating sequence lies downstream of position −69 (see the results for the *nupN367-lacZ* [−69 to +298] and *nupN140-lacZ* [−69 to +71] fusions in Table 4). As shown below, some point mutations in this region also reduced expression of the *nupN-lacZ* fusions.

It should be noted that truncation at position +71 also reduced the expression of the *nupN-lacZ* fusions (compare the *nupN503-lacZ* and *nupN276-lacZ* fusions or the *nupN347-lacZ* and *nupN120-lacZ* fusions in Table 3). Whereas this result

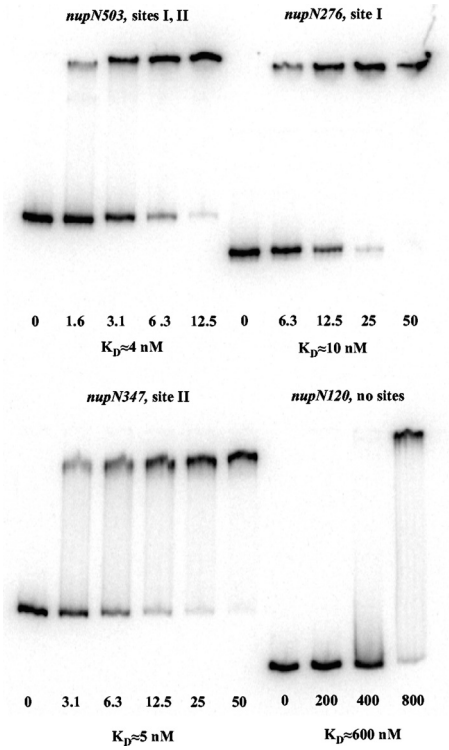

FIG. 4. Gel shift assay of CodY affinity for *nupN* DNA fragments. Different labeled *nupNp*$^+$ DNA fragments were incubated with increasing amounts of purified CodY in the presence of 10 mM ILV and 2 mM GTP. The CodY concentrations used (nanomolar concentrations of monomers) are indicated below each lane. K_D, the apparent equilibrium dissociation constant, was estimated as the protein concentration needed to shift 50% of DNA fragments under conditions of vast protein excess over DNA.

might suggest the existence of an activating element downstream of position +71, it is well known that *lacZ* fusions with different junctions between the promoter-containing sequence and the reporter gene can yield very different activities of β-galactosidase, apparently due to differences in stability or translatability of the corresponding mRNA. To restrict our analysis to the role of CodY in *nupN* gene expression, we have compared the fold differences (repression ratio) between the activities of each fusion, rather than their absolute activities, in a *codY* mutant and wild-type cells under conditions in which CodY is maximally active (in the 16-amino-acid-containing glucose-ammonium medium).

The roles of multiple 15-bp CodY-binding motifs in *nupN* site I function. Two CodY-binding motifs, 1a and 1b, with poor adherence to the consensus sequence (4 and 5 mismatches, respectively) could be recognized upstream of CodY-binding motif 1 (Fig. 2; Table 5). (We did not consider motifs with 6 mismatches that can be found very frequently in A+T-rich regulatory regions.) Moreover, the 22-bp region upstream of *nupN* motif 1 that was protected by CodY in DNase I footprinting experiments included the entire motif 1b and part of motif 1a (Fig. 2C). To analyze the potential roles of these sequences in CodY-mediated regulation, we constructed a series of *lacZ* fusions containing truncated versions of CodY-binding site I with or without site II. The *nupN380-lacZ* and *nupN153-lacZ* fusions that had most of motif 1a removed (Fig. 2) were repressed 170- to 380-fold by CodY in the 16-amino-acid-containing medium (Table 4, strains BB2953 and BB2955), i.e., to an even greater extent than the parent *nupN503-lacZ* and *nupN276-lacZ* fusions (Table 3, strains BB2686 and BB2809). However, the *nupN367-lacZ* fusion, in which all but 5 bp upstream of motif 1, including most of motif 1b, was removed, was repressed by CodY 13-fold less that the full-length fusion (Table 4, strain BB2935; and Table 3, strain BB2686) and at a level almost identical to that of the fusion that lacked site I entirely (Table 3, strain BB2899). When the *nupN* CodY-binding site II was deleted from the *nupN367-lacZ* fusion, the resulting *nupN140-lacZ* fusion (Fig. 2), retaining motif 1 but lacking motifs 1a, 1b, and 2, completely lost the ability to be regulated by CodY (Table 4, strain BB2936). Because a similar fusion, *nupN153-lacZ*, containing only 13 additional upstream nucleotides, was highly regulated by CodY, we conclude that the sequence within the *nupN* CodY-binding site I located upstream of motif 1 and overlapping motif 1b (from position −82 to position −70 with respect to the transcription start point) contains a region that is required for repression by CodY.

To analyze the role of motif 1b, we introduced an up mutation, p21, and down mutations, p22, p23, and p24, that made this motif more or less similar to the consensus sequence, respectively (Table 5). p21 increased the ability of CodY to repress the *nupN380-lacZ* or *nupN153-lacZ* fusions; all down mutations, including p24, which affects the least-conserved position of the consensus, had the opposite effect (Table 4, strains BB2972 to BB2975, BB3597, and BB3598). The p23 mutation affecting the most conserved position of the consensus motif almost completely eliminated regulation by CodY at site I. Taken together, these data strongly indicate that motif 1b, located 3 bp upstream of motif 1, is a functional regulatory element essential for CodY-dependent repression at site I.

To confirm our initial assumption that the 15-bp CodY-binding motif 1 is also important for the contribution of site I to *nupN* repression, we introduced single down mutations (p2 and p3) and a double up mutation (p1) into this sequence (Table 5). As expected, both down mutations greatly reduced the ability of CodY to repress, and the p1 up mutations increased the ability of CodY to repress (Table 4, strains BB2952, BB2954, BB3014, BB3015, and BB3491). The p1 mutation also eliminated the requirement for the motif 1b sequence for CodY-mediated repression (Table 4, strains BB2952 and BB2954). Conversely, the up mutation p21 in motif 1b was able to compensate partly for the defect in the adjacent motif 1 imposed by the down mutation p2 (Table 4, strains BB3014 and BB3599).

Mutational analysis of site II contribution to *nupN* regulation. Since CodY binds to site II with higher affinity than to site I, it was surprising that deletion of site II, as in the *nupN276-lacZ* and *nupN153-lacZ* fusions (Fig. 2), affected the extent of *nupN* regulation by CodY only ≤2-fold. We were concerned that this result could be an artifact caused by extensive deletions used to construct the *nupN276-lacZ* and *nupN153-lacZ* fusions that led to their reduced expression. Therefore, to analyze further the actual role of site II in the regulation of *nupN* in the presence of site I, we introduced a single down mutation, p12, in the CodY-binding motif 2 (Table 5). As

TABLE 4. Expression of *nupN-lacZ* fusions[a]

Strain	Relevant genotype	Fusion genotype	Motifs present	Growth medium			
				TSS + 16 amino acids		TSS	
				β-Galactosidase activity (MU)	Repression ratio	β-Galactosidase activity (MU)	Repression ratio
BB2953	Wild type	*nupN380p*$^+$	1b, 1, 2	0.91	381	171.0	2.7
BB2960	*codY*			347.0		468.0	
BB3491	Wild type	*nupN380p3*	1b, 1^{down}, 2	0.78	105	67.1	1.2
BB3495	*codY*			81.8		77.4	
BB3492	Wild type	*nupN380p11*	1b, 1, 2^{up}	0.20	1,525	88.8	5.3
BB3496	*codY*			305.0		471.0	
BB3431	Wild type	*nupN380p3/11*	1b, 1^{down}, 2^{up}	0.77	326	166.0	2.7
BB3435	*codY*			251.0		449.0	
BB2972	Wild type	*nupN380p21*	1b^{up}, 1, 2	0.38	982	28.0	18
BB2976	*codY*			373.0		492.0	
BB2973	Wild type	*nupN380p22*	1b^{down}, 1, 2	9.25	41	377.0	1.3
BB2977	*codY*			379.0		496.0	
BB2955	Wild type	*nupN153p*$^+$	1b, 1	0.09	174	4.01	2.4
BB2962	*codY*			15.7		9.71	
BB3014	Wild type	*nupN153p2*	1b, 1^{down}	2.27	1.6	2.07	0.60
BB3019	*codY*			3.54		1.24	
BB3015	Wild type	*nupN153p3*	1b, 1^{down}	2.41	6.8	12.5	0.81
BB3020	*codY*			16.3		10.1	
BB2974	Wild type	*nupN153p21*	1b^{up}, 1	0.04	423	0.27	47
BB2978	*codY*			16.9		12.8	
BB2975	Wild type	*nupN153p22*	1b^{down}, 1	1.30	13	12.3	0.97
BB2979	*codY*			17.0		11.9	
BB3597	Wild type	*nupN153p23*	1b^{down}, 1	9.30	1.5	8.96	0.82
BB3600	*codY*			13.6		7.36	
BB3598	Wild type	*nupN153p24*	1b^{down}, 1	0.16	99	6.14	1.7
BB3601	*codY*			15.9		10.3	
BB3599	Wild type	*nupN153p2/21*	1b^{up}, 1^{down}	0.09	43	1.14	0.80
BB3602	*codY*			3.91		1.42	
BB2935	Wild type	*nupN367p*$^+$	1, 2	29.8	8.0	396.0	0.93
BB2943	*codY*			238.0		368.0	
BB2952	Wild type	*nupN367p1*	1^{up}, 2	0.31	623	30.6	8.2
BB2959	*codY*			193.0		250.0	
BB2936	Wild type	*nupN140p*$^+$	1	12.9	0.95	12.3	0.78
BB2944	*codY*			12.3		9.64	
BB2954	Wild type	*nupN140p1*	1^{up}	0.05	172	0.37	16
BB2961	*codY*			8.60		5.88	

[a] Cells were grown, β-galactosidase activity was assayed, and repression ratios were calculated as described in Table 3.

expected, in the context of a fusion that lacked site I (*nupN347-lacZ*), this mutation reduced CodY-dependent repression in the 16-amino-acid-containing medium >5-fold (Table 3, strains BB2899 and BB3493). However, the highly expressed *nupN503p12-lacZ* fusion, containing wild-type site I and a down mutation in site II, was repressed only 1.6-fold less strongly than the *nupN503p*$^+$*-lacZ* fusion, consistent with the results of our deletion analysis and confirming that under the conditions tested site II does not contribute significantly to repression if site I is present (Table 3, strains BB3402 and BB2686).

To determine whether the strength of site II is limiting its ability to contribute to regulation, we introduced a two-nucleotide substitution (the up mutation p11) in motif 2 that made it a perfect match to the consensus. The efficiency of repression in the 16-amino-acid-containing medium of the *nupN347p11-lacZ* fusion, containing the improved site II as the only CodY-binding region, was increased 8-fold over that of the *nupN347p*$^+$*-lacZ* fusion (Table 3, strains BB3401 and BB2899). Similarly, the *nupN380p3/11-lacZ* fusion, containing a partially inactive version of site I in addition to the improved site II, was repressed by CodY 4-fold more strongly than the *nupN380p3-lacZ* fusion (Table 4, strains BB3431 and BB3491). Finally, even the *nupN380p11-lacZ* fusion, containing both the intact site I and improved site II, was 4-fold more repressed by CodY in 16-amino-acid-containing medium than a similar fusion without the p11 mutation (Table 4, strains BB3431 and BB2953). We conclude that though site I is adequate for the full physiological extent of repression of *nupN* by CodY, increasing the similarity of motif 2 (within the CodY-binding site II) to the consensus sequence can cause more efficient repression.

Possible autoregulation of *nupN*. It was previously suggested that the *nupN* promoter may be subject to positive autoregulation and cannot be transcribed if *nupN* or the downstream *nupOPQ* genes are not expressed (29). The suggestion was based on the lack of expression of an *nupN*::pMutin (*lacZ*) fusion when it was integrated at the *nupN* locus of the *B. subtilis* chromosome, thus disrupting the *nupN* gene and likely preventing expression of the downstream *nupOPQ* genes due to a polar effect on their transcription. However, expression of the ectopic *nupN503-lacZ* fusion in the strain containing a

TABLE 5. CodY-binding motifs of the *nupN* gene

Motif[a]	Sequence[b]	No. of mismatches	Score[c]	Location with respect to transcription start point
Consensus	AATTTTCWGAAAATT	0	13.8–14.1	
1a	AtTTTTaAGcAAATa	4	4.0	−94–−80
1b	AtaaTTCTaAAAATa	5	4.6	−82–−68
1b p21	AtaaTTCT**G**AAAATa	4	6.6	
1b p22	AtaaTT**g**TaAAAATa	6	1.9	
1b p23	AtaaTTCTa**c**AAATa	6	0.85	
1b p24	Ataa**a**TCTaAAAATa	6	3.7	
1c[d]	tAaTTTtTaAAAAaT	5	4.6	−65–−51
1 p$^+$	AATTTTtAaAAAATT	2	10.1	−64–−50
1 p1	AATTTT**C**A**G**AAAATT	0	14.1	
1 p2	AATTTTtAa**c**AAATT	3	6.4	
1 p3	AA**a**TTTtAaAAAATT	3	8.3	
2 p$^+$	tATTaTCAGAAAATT	2	12.0	+169–+183
2 p11	**A**ATT**T**TCAGAAAATT	0	14.1	
2 p12	tATTaTCA**c**AAAATT	3	8.0	

[a] Shown are strong CodY-binding motifs 1 and 2 and weaker motifs found upstream of motif 1 and analyzed in this work. Additional weak motifs can be found downstream of motifs 1 and 2 (see Discussion).

[b] Mismatches to the proposed CodY-binding consensus are indicated by lowercase letters. Mutations are in boldface. Overlapping parts of motifs 1a and 1b and the part of motif 1c that overlaps motif 1 are underlined.

[c] The scores for individual CodY-binding motifs have been generated using the position-specific weight matrix, as described in reference 6.

[d] Motif 1c almost completely overlaps strong motif 1 and therefore is unlikely to have a separate functional role. This is supported by the fact that the p1 mutation that increased CodY-mediated repression is a strong down mutation in motif 1c.

nupN in-frame deletion or the *nupN*::pMutin allele proceeded unimpeded (data not shown). We conclude that the *nupN* gene is unlikely to be subject to autoregulation.

DISCUSSION

Role of *nupNOPQ* in nucleoside uptake. The results presented here show that the *nupNOPQ* genes encode an uptake system for guanosine and perhaps other nucleosides. At least two other transporters, NupG (19) and YutK (as mentioned in reference 27), have been found to have similar or overlapping specificities (none of them characterized in detail). NupG is the major adenosine and guanosine permease in cells grown in minimal medium (19). However, *nupG* expression is low in the presence of guanine or hypoxanthine in the medium (19) and is apparently subject to a guanine riboswitch-mediated control (25a). Thus, it is likely that the role of the NupNOPQ transporter in purine nucleoside uptake becomes more important in cells grown in the presence of guanine and related compounds, such as guanosine, hypoxanthine, or inosine. Our data indicate that NupNOPQ may also be a low-affinity transporter of pyrimidine nucleosides. NupC serves as the major transporter of pyrimidine nucleosides in *B. subtilis* (33); NupNOPQ may be the previously unidentified minor transporter of these nucleosides. NupNOPQ-like transporters in other bacteria take up both purine and pyrimidine nucleosides (9, 26, 41). A more detailed analysis of the contributions of NupG, NupNOPQ, YutK, and NupC to the uptake of different nucleosides in cells grown under different conditions was beyond the scope of this work.

Nucleosides, especially guanosine, are poor nitrogen and carbon sources (34, 40). Therefore, it makes physiological sense that their utilization under conditions of nutrient abundance is delayed by CodY until other, preferred sources of nitrogen and carbon have been consumed. Inhibition of guanosine uptake under growth conditions when CodY is highly active may also serve to keep the already high cellular pool of GTP, a positive effector of CodY, in check.

CodY-dependent regulation of the *nupN* promoter. Two high-affinity CodY-binding sites were identified within the regulatory region of *B. subtilis nupN*. Each site was shown to bind CodY independently of the other.

CodY-binding site I, a major contributor to *nupN* repression, is located only 3 bp upstream of the −35 region of the promoter. Thus, CodY binding to this site is likely to prevent RNA polymerase binding and may specifically target binding of the σ-subunit or the α-subunit of RNA-polymerase; an A+T-rich sequence located downstream of position −69 and overlapping position −49 could potentially have a function similar to that of an α-interacting UP element (18, 28).

The downstream site II is, if anything, a more efficient CodY binder than site I *in vitro*, but only a minor role for this site in *nupN* regulation (≤2-fold) could be detected when the upstream site I was present. Nonetheless, site II serves as a roadblock for transcription elongation in *in vitro* transcription experiments (7) and negatively regulates expression *in vivo* of *nupN-lacZ* fusions that lack site I. Moreover, when we improved the similarity of motif 2 within site II to the CodY-binding consensus motif, the modified site II highly increased repression by CodY, even in the presence of site I. Given our inability to demonstrate a significant contribution of native site II to CodY-mediated repression *in vivo*, site II is likely to be a minor player in *nupN* regulation under most growth conditions. On the other hand, with its apparent higher affinity for CodY, site II could potentially contribute more prominently to *nupN* repression under growth conditions when CodY activity is low. Also, we cannot exclude the possibility that additional regulators operate at the *nupN* promoter and mask or modify effects of CodY in some of our experiments. The existence of such regulators could be consistent with our observation that some deletions and mutations affect *nupN* expression in a CodY-independent but medium-dependent and sequence context-specific manner.

In addition to *nupN*, the *B. subtilis* genes *bcaP* and *ybgE* were shown to possess two CodY-binding sites, of which one is located >60 bp downstream of the transcription start site. In all three cases, the downstream site has a higher affinity for CodY than does the upstream site and causes transcription elongation pausing or arrest due to a CodY-imposed roadblock *in vitro* (5, 7). However, the contributions of the downstream sites to CodY-mediated repression *in vivo* are drastically different: the *ybgE* downstream site is the major contributor to regulation (7), the *bcaP* downstream site contributes nearly as much as the upstream site (5), and the *nupN* downstream site is apparently a weak contributor. The efficiency of a transcription roadblock may depend both on the strength of the corresponding promoter and the sequence context of the roadblock site (13, 24).

The roles of multiple motifs within a single CodY-binding site. To our surprise, we found that efficient CodY-mediated regulation at *nupN* site I requires the presence of two 15-bp motifs separated by 3 bp. This is reminiscent of the regulation of the *B. subtilis putBCP* operon (3). In the case of the *putB*

promoter, the two motifs, separated by 2 bp, have 3 to 4 mismatches each with respect to the consensus sequence, and we surmised that it was their relative weakness that required the presence of both of them for efficient regulation by CodY. In two other cases, the *dpp* operon and the *ybgE* gene, strong CodY binding is associated with the presence of 3 to 4 adjacent or overlapping motifs (with 4 to 5 mismatches each), though their exact roles have not been characterized (6, 7). We presumed that weaker motifs are required to be in groups of at least two to provide effective binding and regulation by CodY. In the case of *nupN*, however, the native version of motif 1 has only 2 mismatches to the consensus, yet its contribution to regulation is fully dependent on the presence of the upstream motif 1b. The requirement for motif 1b in regulation is abolished if motif 1 is made stronger.

Binding of CodY to two adjacent motifs may be a more widespread phenomenon than currently assumed. Many strong motifs with 2 to 3 mismatches—the only ones that can be predicted to participate in CodY binding with some degree of confidence—are frequently associated with weaker motifs (with 4 to 5 mismatches) that are either adjacent to or overlap with the stronger motif (our unpublished analysis), consistent with the hypothesis that several nearby motifs can improve CodY binding to DNA or may be essential for such binding. Moreover, experimentally determined CodY-binding sites typically cover more extended regions than just a single 15-bp motif. Interestingly, additional 15-bp sequences with 5 mismatches to the consensus CodY-binding motif can be found within *nupN* site I (overlapping 14 or 4 promoter-proximal nucleotides of motif 1 from positions −63 to −49 and −53 to −39, respectively) and within *nupN* site II (5 bp downstream of motif 2 from positions +189 to +203). The roles of these sequences in regulation remain unknown. It should be noted that 15-bp sequences with 5 mismatches to the consensus CodY-binding motif are common in the (A+T)-rich *B. subtilis* genome and even more so in the (A+T)-enriched regulatory regions.

The data showing that efficient CodY-mediated regulation requires sequences outside a single 15-bp motif were also obtained for the *bcaP* gene (B. R. Belitsky and A. L. Sonenshein, unpublished data). Recently, the participation of two overlapping motifs was proposed to be a general rule for CodY binding (42). The accumulated results suggest a more complex mechanism of CodY-DNA interaction than previously thought.

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