JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 7155–7160 Vol. 187, No. 20
0021-9193/05/$08.00+0 doi:10.1128/JB.187.20.7155–7160.2005

YrxA Is the Transcriptional Regulator That Represses De Novo NAD Biosynthesis in *Bacillus subtilis*

Paola Rossolillo,† Ilaria Marinoni, Elisa Galli, Anna Colosimo, and Alessandra M. Albertini*

Dipartimento di Genetica e Microbiologia, Università degli Studi di Pavia, Pavia, Italy

Received 22 June 2005/Accepted 2 August 2005

The first genetic, in vivo, and in vitro evidences that YrxA is the regulator of NAD de novo biosynthesis in *Bacillus subtilis* are hereby reported. The protein is essential to the transcription repression of the divergent operons *nadBCA* and *nifS-yrxA* in the presence of nicotinic acid and binds to their shared operator-promoter region.

In eubacteria, NAD, the primary biological cofactor for oxidation-reduction reactions, is produced by a de novo pathway or recycled from exogenous or preformed nicotinic acid (NA) (2, 4, 22, 35). In the de novo biosynthetic pathway, NAD is synthesized from aspartic acid, which is converted to quinolinic acid (QA) by the concerted action of NadB, L-aspartate oxidase, and NadA, quinolinate synthetase (3, 6, 10, 18, 19, 31, 34); QA is then converted to nicotinic acid mononucleotide (NaMN) by NadC, QA phosphoribosyltransferase (14), and subsequently adenylated and amidated to NAD by NadD, NaMN adenylyltransferase, and NadE, deamido-NAD-ammonia ligase (NAD synthetase) (13, 20, 22, 30, 35). Finally, NADP is synthesized by phosphorylation of NAD catalyzed by the kinase NadF (8).

In the salvage or recycling pathway, NA, deriving from NAD dissociation or of exogenous origin, is reconverted to NaMN by a NA phosphoribosyltransferase (PncB in *Escherichia coli*; previously YueK in *Bacillus subtilis*) and reinserted in the enzymatic chain downstream of the reaction catalyzed by NadC (22).

In a comparative study in 1968, Saxton et al. revealed that the enzymatic activities of NadB, NadA, and PncB of *E. coli* are subject to inhibition by NA, while the inhibited enzymes for *B. subtilis* are NadB, NadA, and NadC but not PncB (29).

In *E. coli* and *Salmonella* species, the regulation of NAD biosynthesis is exerted by the trifunctional protein NadR, which transcriptionally represses the *nadB*, *nadA* (de novo pathway), and *pncB* (salvage pathway) genes and increases the uptake of nicotinamide-ribose and its conversion to nicotinamide mononucleotide and subsequently to NAD when the NAD concentration is low (7, 11, 12, 23, 25, 38).

A protein homologous to NadR is not present in *Bacillus subtilis*, in which, presumably, a different mechanism of NAD biosynthesis regulation is active. In the gram-positive bacterium, the *nadB*, *nadA*, and *nadC* genes are organized in an operon whose operator-promoter region is overlapping the operator-promoter region of the divergent dicistronic operon *nifS-yrxA* (17, 32) (http://genolist.pasteur.fr/SubtiList/).

NifS is a homologue of IscS cystein desulfurases and is supposed to be involved in the in vivo maturation of Fe-S clusters, possibly necessary to assemble an active form of the QA synthetase complex formed by NadB and NadA (21, 32). YrxA was classified as a potential transcriptional regulator (1), but the protein has no homologues in gram-negative bacteria, while genes for similar proteins are present in all the sequenced genomes of bacilli and other pathogenic gram-positive bacteria (genera *Listeria*, *Streptococcus*, and *Enterococcus*).

In 1993, Sun and Setlow (32) showed that in the presence of NA, the transcription of the *nadB* and *nifS* genes is abolished. The *pncB* gene (besides *nadD*, *nadE*, and *nadF*) was classified as essential for *Bacillus subtilis* by Kobayashi et al. (16): the authors speculated that the accumulation of NA, when *pncB* is inactivated, might repress NAD de novo synthesis and cause the bacterium's death.

We hypothesized that YrxA, due to its similarity with other transcriptional regulators able to bind small molecules as effectors (1), is, in the presence of NA, the repressor of *nadBCA* and *nifS-yrxA* transcription, allowing the recycling of the pyridinic ring from NA present in the medium and the availability of aspartic acid for protein synthesis.

Our genetic analysis shows that the *pncB* gene is essential only when *B. subtilis* is grown in the presence of NA and that the inactivation of *yrxA* bypasses its requirement. We show that YrxA represses the transcription of the two operons *nadBCA* and *nifS-yrxA* in vivo and that it binds in vitro to their overlapping promoters in the site of the putative operator sequence, using NA as a cofactor.

Construction of a *pncB* mutant. *pncB*, the gene previously named *yueK*, coding for NA phosphoribosyltransferase of the recycling pathway, was classified as essential in *B. subtilis* (16). We constructed a conditional-lethal mutant by transforming wild-type (wt) PB168 (*trp*C2) competent cells with the integrative plasmid pMUTIN4/pncB′, obtained by cloning into pMUTIN4 (37) the 410-bp sequence coding for the N terminus of PncB (from 3259589c to 3259999c, SubtiList coordinates [http://genolist.pasteur.fr/SubtiList/]).

Transformants were selected on tryptose blood agar base (TBAB) medium (Difco) with erythromycin (Ery) (0.3 μg/ml) in

* Corresponding author. Mailing address: Dipartimento di Genetica e Microbiologia, Università degli Studi di Pavia, 1, Via Ferrata, 27100 Pavia, Italy. Phone: (39) 0382-985549. Fax: (39) 0382-528496. E-mail: albert@ipvgen.unipv.it.

† Present address: Dipartimento Materno Infantile e di Biologia-Genetica, Università di Verona, Verona, Italy.

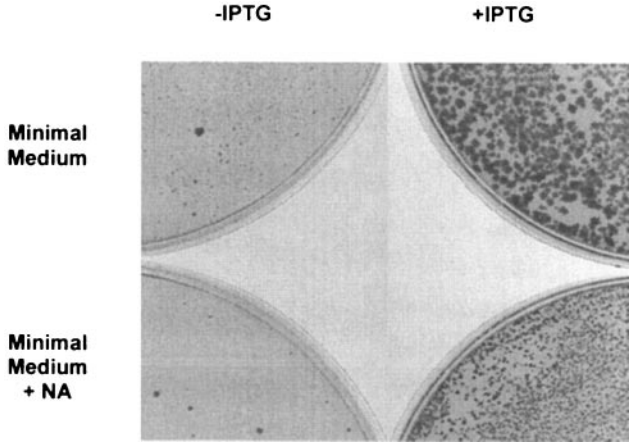

FIG. 1. Selection of Ery^r (0.3 μg/ml erythromycin) colonies using DNA of PB1934 (Ω*pncB′*::pMUTIN4) to transform PB168 (wt) competent cells on MM (5) in the presence or absence of IPTG (1 mM) and NA (50 μg/ml). Only sporadic spontaneous Ery^r mutants can be observed on the bottom left in MM with NA. Very small colonies can be observed in the absence of NA (top left) after 48 h of incubation at 37°C. When IPTG is added to the medium, the colonies observed in the presence of NA are smaller (bottom right) than the ones observed in absence of NA (top right).

the presence of 1 mM isopropyl-β-D-thiogalactoside (IPTG); the clones where Ery^r was brought from the vector were screened on 100 μg/ml Ery. One of the clones, verified by PCR, was named PB1934 (*trpC2* Ω *pncB′*::pMUTIN4, Ery^r). When PB168 competent cells were transformed with chromosomal DNA of PB1934, Ery^r colonies grew only on medium supplemented with 1 mM IPTG. This result confirmed that *pncB* is essential when *B. subtilis* is grown on rich medium (16). To investigate whether NA plays a role in causing *pncB* to be essential (as hypothesized in references 16 and 29), we selected Ery^r transformants on minimal medium (MM) (5) with or without NA (50 μg/ml). No colonies were visible in presence of NA (Fig. 1, bottom left), while very small colonies appeared on MM plates without NA after 48 h at 37°C (Fig. 1, top left) with roughly the same frequency observed in the plates where IPTG was added (Fig. 1, top right).

Interestingly, the colonies observed in the presence of IPTG and NA (Fig. 1, bottom right) were smaller than the ones observed without NA but significantly larger than the ones grown on MM without supplements. Both 50 and 0.5 μg/ml of NA inhibited the growth of PB1934 (Table 1), but when *pncB* expression was induced with IPTG, the growth was comparable to that of the Ery^r control strain BFS2652 (Ω*ynaB′*::pMUTIN4 *trp*C2 [16]). These in vivo experiments showed that *pncB* is essential only when NA is present, and this gene/metabolite combination suppresses de novo NAD biosynthesis and causes the bacterium's death. NAD cannot be synthesized due to the unavailability of NaMN, the end product of the de novo (*nadBCA*-dependent) or recycling (*pncB*-dependent) pathways. In particular, NA could act as a cofactor for a transcriptional regulator that represses *nadBCA* transcription.

Analysis of the YrxA primary sequence and of the genome map context of its gene. *yrxA* is the second gene of the dicistronic operon *nifS-yrxA*, located 150 bp away from the *nadBCA* operon and transcribed in the opposite direction (17). This gene, coding for a putative transcriptional regulator, is also present in *Bacillus anthracis* (26, 27), *Bacillus cereus* (15), *B. halodurans* (36), *B. licheniformis* (28), and *Listeria monocytogenes* and *Listeria innocua* (9). In these organisms, where YrxA shows a high degree of similarity (identity score, >50%; similarity score, >76%), the genomic organization of the two divergent *nadBCA* and *nifS-yrxA* operons is conserved. None of the YrxA-like proteins have been functionally characterized yet; database searches (InterPro [http://www.ebi.ac.uk/interpro/] and Pfam [http://www.sanger.ac.uk/Software/Pfam/index.shtml]) revealed that the protein's primary sequence can be divided into two functional domains. The first 80 amino acids are characterized by a strong similarity with DNA-binding proteins (winged-helix DNA binding domain), while the second domain, from residue 90 to the carboxy terminus, has been classified as a potential three-histidine binding site for small effector molecules (1). The similarity data, the localization and organization of the *nifS-yrxA* operon, and the fact that there's no homologue in *E. coli* make YrxA a good candidate to be the regulator that represses *nadBCA* and *nifS* transcription in the presence of NA.

In a Δ*yrxA* context, the *pncB* gene is no longer essential. To verify the possible role of YrxA in the regulation of NAD biosynthesis, a Δ*yrxA* mutant was constructed by substituting the central region of the gene with the *cat* (chloramphenicol acetyltransferase) cassette by a PCR-based technique. The *cat* gene was amplified from plasmid pJM105A (24) using the oligonucleotides 5′-cat (5′-TCTTCAACTAAAGCACCCATT-3′) and 3′-cat (3′-ACAGTCGGCATTATCTCATATT-3′). A 473-bp fragment from nucleotide (nt) 368 of the coding sequence (CDS) of *yrxA* to nt 607 of the CDS of the downstream gene *pheA* was amplified using the oligonucleotides yrxA-5′cat (5′-ATGGGTG CTTTAGTTGAAGAAGGCACCCGGAAAGAAGTT-3′; underlined linker sequence homologous to the 5′ region of *cat*) and 3′-pheA (5′-TTAATGGTCATGATGGTCCCGCA-3′), while a 585-bp fragment from nt 707 of the *nifS* CDS to nt 127 of the *yrxA* CDS was amplified with the oligonucleotides 5′-iscS (5′-TAAATGTTCCCGGGATCGGTG-3′) and yrxA-3′cat (5′-TATGAGATAATGCCGACTGTAACCTGTCTTGAGA

TABLE 1. Growth of single and double mutants on MM with or without NA and 1 mM IPTG[a]

Strain	Growth of strain on:			
	MM	MM + NA	MM + IPTG	MM + NA + IPTG
PB1934 (Ω*pncB*::pMUTIN4)	+−	−	++	+
PB1935 (Ω*pncB*::pMUTIN4, Δ*yrxA*)	++	++	++	++
BFS2652 (*ynaB*::pMUTIN4)	++	+	ND	ND

[a] The same results were obtained using 50 or 0.5 μg/ml of NA. ++, colonies appear after 24 h at 37°C; +, colonies appear after 48 h at 37°C; +−, very small colonies appear after 48 h; ND, not determined.

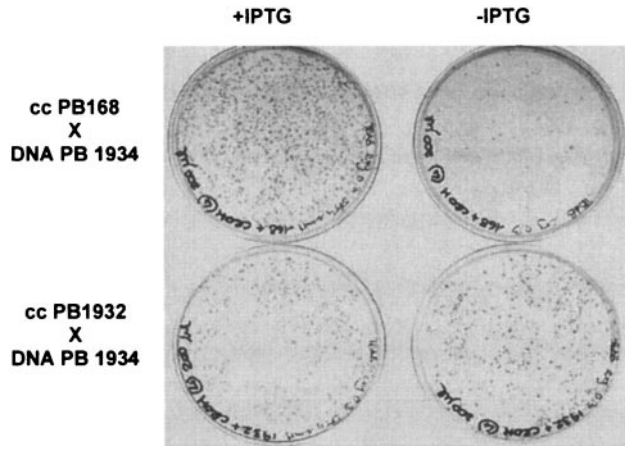

FIG. 2. Selection of Ery^r transformants (0.3 μg/ml erythromycin) on TBAB medium with or without IPTG (1 mM): in the top part of the figure, transformation of PB168 (wt) competent cells with chromosomal DNA of PB1934 (Ω*pncB′*::pMUTIN4), colonies are visible only on plates containing IPTG. When the same DNA was used to transform PB1932 (Δ*yrxA*) competent cells, the same number of colonies grew on TBAB plates with or without IPTG (lower part of the figure).

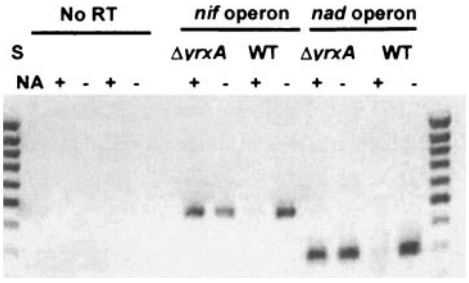

FIG. 3. RT-PCR experiments on the transcripts of the *nifS*/*iscS* and *nadB* genes in the PB1932 (Δ*yrxA*::*cat*) mutant and PB168 (wt) strains, grown in MM medium with or without NA (50 μg/ml). S, Mass Ruler DNA ladder (low range; Fermentas). Δ*yrxA*, RT-PCR on RNA extracted from PB1932 cells; WT, RT-PCR on RNA extracted from PB168 cells. A product of 297 bp indicates the presence of *nadBCA* operon transcripts, and a product of 480 bp indicates the presence of *nifS*/*iscS* transcripts.

CGTTCGCT-3′; underlined linker sequence homologous to the 3′ region of *cat*). The three PCR products were denatured together for three minutes at 94°C and slowly renatured by decreasing the temperature (6°C/min) to the annealing temperature of the 3′-pheA and 5′-iscS primers, used to amplify the mutagenic fragment. Five hundred nanograms of the *phe*A::*cat*::*isc*S mutagenic fragment were used to transform 0.5 ml of *B. subtilis* PB168 competent cells. Cm^r clones were obtained with a frequency of 1×10^2 per μg of DNA on nutrient broth (Difco) plates with 5 μg/ml chloramphenicol. One deletion-insertion Δ*yrxA*::*cat* mutant candidate, obtained by double crossover, was verified by PCR and named PB1932 (*trpC2* Δ*yrxA*::*cat*, Cm^r). To demonstrate that *yrxA* inactivation suppresses *pncB* essentiality and abolishes the repression role of NA, a double mutant was then created by transforming PB1932 competent cells with chromosomal DNA of PB1934. As expected, Ery^r clones grew on TBAB with erythromycin (0.3 μg/ml) with or without IPTG (Fig. 2). One of the Ery^r Cm^r clones obtained, verified by PCR, was named PB1935 (*trpC2* Ω*pncB′*::pMUTIN4 Δ*yrxA*::*cat*). The double mutant PB1935 also grew on MM with NA without IPTG: the addition of 0.5 or 50 μg/ml of NA did not impair the strain growth, since colonies were visible after 24 h (Table 1). PB1935 grew faster than the control strain BFS2652, suggesting that in a PncB$^+$ strain, NA repression of de novo synthesis reduces the total NAD availability. These in vivo experiments support the hypothesis that *yrxA*, probably in cooperation with NA, is the repressor of NAD de novo synthesis. Constitutive expression of the *nadBCA* operon supports NAD synthesis even in the absence of the recycling pathway (Ω*pncB′*::pMUTIN4).

YrxA and NA cooperate in *nadBCA* transcriptional repression. Since we hypothesized that the YrxA and NA repression action was performed at the transcriptional level, we analyzed the transcription of the *nadB* and *nifS* operons by reverse transcription (RT)-PCR under different conditions. PB168 (wt) and PB1932 (Δ*yrxA*) cells were grown at 37°C in PY broth (Penassay antibiotic medium 3; Difco), 0.5% glucose, to an optical density at 600 nm of 0.3; they were then washed and incubated for a further two hours at 37°C in MM with or without NA (50 μg/ml). RNA was extracted from cells by using the High Pure RNA isolation kit (Roche), and 1 μg of total RNA for each sample was retrotranscribed with the oligonucleotides NadBa2 (5′-CTCGTTCATTGCGGTCGAATG GAAATCC-3′) for the *nadB* transcripts and 3′-iscS (5′-TGA AGACAGGTGTTGAATGG-3′) for the *nifS* transcripts. cDNAs were then reamplified with primers NadBa2 and 3′NadB (5′-ATTGCAGTCATCGGTTCAGG-3′) for *nadB*, giving a 297-bp fragment, and with primers 3′-iscS and 5′-iscS (5′-TTATTTAGATTATGCAGCGACAACG-3′) for *nifS*, producing a 480-bp fragment. The *nadB* and *nifS* genes were not transcribed when the wt strain was grown in the presence of NA, while in strain PB1932 (Δ*yrxA*), both genes were transcribed even in the presence of NA (Fig. 3). This experiment confirms that YrxA is, in the presence of NA, the repressor of *nadBCA* and *nifS-yrxA* operons transcription. According to our results, the persistence of *nad* transcription regulation in the Δ*nifS*::*erm* mutant created by Sun and Setlow (32) could be explained by the absence of a polar effect on the downstream *yrxA* gene: the expression and the regulatory function of YrxA were evidently maintained. On the other hand, the authors did not specify if the disruption on *nifS* they performed introduced a strong transcription terminator: read-through from the *nif* promoter or from the *ermC* promoter itself cannot be ruled out.

YrxA binds to the overlapping promoter region of *nadB and nifS*. *nadB* and *nifS* are separated by a 150-bp region including a region of imperfect dyad symmetry encompassing the −35 and −10 sequences of both genes (32) that could act as an operator for the binding of the YrxA regulatory protein repressing *nadB* and *nifS* transcription. An 87-bp DNA fragment including the putative operator and the divergent promoter region common to *nadB* and *nifS* (OP) (SubtiList coordinates 2848699 to 2848786) was amplified from PB168 chromosomal DNA with primers BSS1 (5′-CCTCCTGTTGTTTACACCTG TCT-3′) and BSS2 (5′-CTTCCATCCGTTCTCCATAAAA-3′) by incorporating [α-^{32}P]CTP (3,000 Ci/mMol) in the PCR and used as a probe in an electrophoresis mobility shift assay (EMSA)

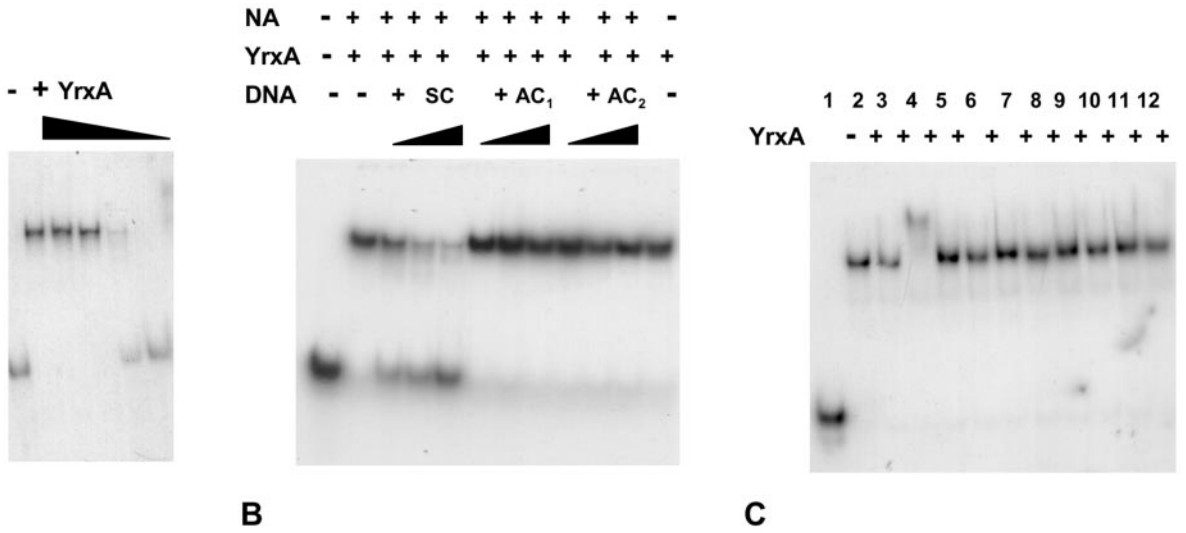

FIG. 4. Electrophoresis mobility shift assays. Panel A: band shift induced by YrxA. Increasing concentrations of the protein (0.003, 0.034, 0.34, 0.77, and 3.4 μM) induce an increasing retardation of the ^{32}P OP DNA-labeled fragment (1.7 nM) migration in the gel. Panel B: competition in EMSA between ^{32}P-labeled 87-bp OP fragment and cold specific SC_1 DNA (a 170-bp fragment spanning from just upstream to just downstream OP, including the 87-bp fragment) or nonspecific DNA (AC_1, a 318-bp fragment covering a region in *nadB* outside OP; and AC_2, a 330-bp fragment in *nifS*). The YrxA concentration was 50 nM, NA was 150 μM, and the increasing concentrations of competitors were, respectively, 2, 5, and 10 ng in 20 μl. Panel C: band shift and "supershift" in the presence of NA and analogues, intermediates, or end products of NAD synthesis (added at a concentration of 300 μM). Lanes: 1, no cofactors, no YrxA; 2, only YrxA; 3, 150 μM NA; 4, 300 μM NA; 5, nicotinamide; 6, quinolinic acid; 7, isoniazide; 8, nicotinic acid hydrazide; 9: pyrazynamide; 10, nicotinamide mononucleotide; 11, NAD; 12, NADH. A plus sign indicates the presence of 3.4 μM YrxA.

to test if YrxA binds to the promoter region. YrxA, glutathione *S*-transferase (GST) tagged at the N terminus, was overexpressed into BL21(DE3) *E. coli* cells after the cloning into the pGEX-6p1 expression vector (Amersham Biosciences) of the *yrxA* CDS, amplified from genomic wt DNA with primers 5′ByrxA-GEX1 (5′-CGGGATCCTTGACCGAAGAATTAAAGCTA-3′) and 3′yrxA-GEX (5′-CGGAATTCTTATTAATTAAAAATG CCGGCTTCT-3′), including the first of the two Met residues (Met-1 and Met-8) identified as a putative YrxA start codon (32). The YrxA-GST fusion protein was isolated by chromatography on glutathione Sepharose 4B columns (Amersham Biosciences), and after treatment with Prescission protease (Amersham Biosciences) and elution, a 90% pure YrxA protein was recovered. This 20.1-kDa YrxA protein (including five additional amino acids at the N-terminal end) was used in EMSA assays. All attempts made in expressing the untagged form of the protein failed, probably due to the instability of the protein in its native form.

The 87-bp OP labeled fragment, purified with a PCR purification kit (QIAGEN), was incubated (37°C for 20 min) at a concentration of 1.7 nM with increasing concentrations of the YrxA protein in a 20-μl reaction volume in binding buffer (20 mM Tris HCl [pH 8], 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 0.05 mg/ml bovine serum albumin, 0.05% NP-40, 10% [vol/vol] glycerol). The reactions were loaded on a 6% polyacrylamide non denaturing gel in 0.5× Tris-Borate EDTA. Electrophoresis was performed at 70 V for 90 min.

As shown in Fig. 4A, the mobility of the 87-bp OP fragment was shifted as a function of the YrxA concentration, increasing from 3 nM to 3.4 μM. The binding was specific, since the addition to the YrxA-probe reaction of increasing concentrations (5, 10, and 20 ng) of the specific cold competitor SC (a 150-bp fragment, including the 87-bp OP region, amplified with primers BSnadB 5′-CCCCTGAACCGATGACTGCA-3′ and BSnifS 5′-GGCGTTGTCGCTGCATAATC-3′), progressively eliminated the shift of labeled OP. The addition of the nonspecific cold competitors AC_1 (a 318-bp fragment internal to the *nadB* coding region, amplified with primers NadBa2, the same used for RT-PCR, and 5′nadB 5′-CACCAATGTCTA AAAAGACGATTG-3′) and AC_2, (330-bp fragment included in the *nifS* CDS, amplified with primers IscSex [5′-GGTTAACCA TATGATTTATTTAGATTATGCAG-3′] and Nif2 [5′-AAA GCAGCGCAATTATGAAT-3′]) had no effect on fragment mobility (Fig. 4B). Finally, to identify which molecule could act as an effector in the repression action of YrxA, NA, NA analogues, intermediates, or end products of NAD biosynthesis, were added to the reaction at a concentration of 300 μM. When 300 μM NA was added to the YrxA/DNA reaction mixture, the mobility of the labeled OP fragment had a supershift (Fig. 4C), while the addition of the other compounds did not alter OP mobility. Both a higher concentration of YrxA (6.8 μM) alone and the addition to the reaction of NA (>150 μM) in the presence of 3.4 μM YrxA caused a "supershift" in EMSA (data not shown), while addition of NAD up to a concentration of 1.3 mM had no effect. The way in which NA favors YrxA binding to the operator-promoter region suggests a multimeric cooperative interaction, which is to be investigated further.

In conclusion, we identified YrxA as the de novo NAD biosynthesis regulator in *B. subtilis*, and we propose to name it NadR. In the presence of NA, the protein represses transcription of the divergent operons *nadBCA* (for de novo biosynthesis) and *nifS-yrxA* (coding for the cystein desulfurase IscS, and YrxA itself) by binding to their common operator-promoter region. NA enhances the binding, as evidenced by the "supershift" shown in Fig. 4C.

This work first ascribes a biological function to the previously unknown *yrxA* gene and opens an interesting working hypothesis for developing new therapeutic and antimicrobial strategies for the gram-positive pathogenic bacteria (33) in which homologues of YrxA, and probably similar NAD regulation strategies, have been recognized in silico.

This work was financially supported by the University of Pavia and the Italian Ministry MIUR (Ministero dell'Istruzione, Università e Ricerca, COFIN 2002).

We thank Elisabetta Andreoli for expert technical assistance.

REFERENCES

1. **Anantharaman, V., E. V. Koonin, and L. Aravind.** 2001. Regulatory potential, phyletic distribution and evolution of ancient, intracellular, small-molecule-binding domains. J. Mol. Biol. **307:**1271–1292.
2. **Begley, T. P., C. Kinsland, R. A. Mehl, A. Osterman, and P. Dorrestein.** 2001. The biosynthesis of nicotinamide adenine dinucleotides in bacteria. Vitam. Horm. **61:**103–119.
3. **Ceciliani, F., T. Caramori, S. Ronchi, G. Tedeschi, M. Mortarino, and A. Galizzi.** 2000. Cloning, overexpression, and purification of *Escherichia coli* quinolinate synthetase. Protein Expr. Purif. **18:**64–70.
4. **Chen, J. L., and G. J. Tritz.** 1975. Isolation of a metabolite capable of differentially supporting the growth of nicotinamide adenine dinucleotide auxotrophs of *Escherichia coli*. J. Bacteriol. **121:**212–218.
5. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60:**17–28.
6. **Flachmann, R., N. Kunz, J. Seifert, M. Gutlich, F. J. Wientjes, A. Laufer, and H. G. Gassen.** 1988. Molecular biology of pyridine nucleotide biosynthesis in *Escherichia coli*. Cloning and characterization of quinolinate synthesis genes *nadA* and *nadB*. Eur. J. Biochem. **175:**221–228.
7. **Foster, J. W., E. A. Holley-Guthrie, and F. Warren.** 1987. Regulation of NAD metabolism in *Salmonella typhimurium*: genetic analysis and cloning of the *nadR* repressor locus. Mol. Gen. Genet. **208:**279–287.
8. **Garavaglia, S., A. Galizzi, and M. Rizzi.** 2003. Allosteric regulation of *Bacillus subtilis* NAD kinase by quinolinic acid. J. Bacteriol. **185:**4844–4850.
9. **Glaser P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gaultier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria species*. Science **294:**849–852.
10. **Griffith, G. R., J. L. Chandler, and R. K. Gholson.** 1975. Studies on the *de novo* biosynthesis of NAD in *Escherichia coli*. The separation of the *nadB* gene product from the *nadA* gene product and its purification. Eur. J. Biochem. **54:**239–245.
11. **Grose, J. H., U. Bergthorsson, and J. R. Roth.** 2005. Regulation of NAD synthesis by the trifunctional NadR protein of *Salmonella enterica*. J. Bacteriol. **187:**2774–2782.
12. **Holley, E. A., M. P. Spector, and J. W. Foster.** 1985. Regulation of NAD biosynthesis in *Salmonella typhimurium*: expression of *nad-lac* gene fusions and identification of a *nad* regulatory locus. J. Gen. Microbiol. **131:**2759–2770.
13. **Hughes, K. T., B. M. Olivera, and J. R. Roth.** 1988. Structural gene for NAD synthetase in *Salmonella typhimurium*. J. Bacteriol. **170:**2113–2120.
14. **Hughes, K. T., J. R. Roth, and B. M. Olivera.** 1991. A genetic characterization of the *nadC* gene of *Salmonella typhimurium*. Genetics **127:**657–670.
15. **Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, S. D. Ehrlich, R. Overbeek, and N. Kirpides.** 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature **423**(6935)**:**87–91.
16. **Kobayashi, K., S. D. Ehrlich, A. M. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron, K. Bunai, J. Chapuis, L. C. Christiansen, A. Danchin, M. Débarbouillé, E. Dervyn, E. Deuerling, K. Devine, S. K. Devine, O. Dreesen, J. Errington, S. Fillinger, S. J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C. R. Harwood, M. Hecker, D. Hosoya, M. F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauël, R. Meima, R. P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H. M. Pooley, G. Rapoport, J. P. Rawlins, L. A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H. H. Saxild, E. Scanlan, W. Schumann, J. F. M. L. Seegers, J. Sekiguchi, A. Sekowska, S. J. Séror, M. Simon, P. Stragier, R. Struder, H. Takamatsu, T. Tanaka, M. Takeuchi, H. B. Thomaides, V. Vagner, J. M. Van Dijl, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, and N. Ogasawara.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. USA **100:**4678–4683.
17. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, N. J. Cummings, R. A. Daniel, F. Denizot, K. M. Devine, A. Duesterhoft, S. D. Ehrlich, P. T. Emmerson, K. D. Entian, J. Errington, C. Fabret, E. Ferrari, D. Foulger, C. Fritz, M. Fujita, Y. Fujita, S. Fuma, A. Galizzi, N. Galleron, S.-Y. Ghim, P. Glaser, A. Goffeau, E. J. Golightly, G. Grandi, G. Guiseppi, B. J. Guy, K. Haga, J. Haiech, C. R. Harwood, A. Hénaut, H. Hilbert, S. Holsappel, S. Hosono, M.-F. Hullo, M. Itaya, L. Jones, B. Joris, D. Karamata, Y. Kasahara, M. Klaerr-Blanchard, C. Klein, Y. Kobayashi, P. Koetter, G. Koningstein, S. Krogh, M. Kumano, K. Kurita, A. Lapidus, S. Lardinois, J. Lauber, V. Lazarevic, S.-M. Lee, A. Levine, H. Liu, S. Masuda, C. Mauel, C. Medigué, N. Medina, R. P. Mellado, M. Mizuno, D. Moestl, S. Nakai, M. Noback, D. Noone, M. O'Reilly, K. Ogawa, A. Ogiwara, B. Oudega, S.-H. Park, V. Parro, T. M. Pohl, D. Portetelle, S. Porwollik, A. M. Prescott, E. Presecan, P. Pujic, B. Purnelle, G. Rapoport, M. Rey, S. Reynolds, M. Rieger, C. Rivolta, E. Rocha, B. Roche, M. Rose, Y. Sadaie, T. Sato, E. Scanlan, S. Schleich, R. Schroeter, F. Scoffone, J. Sekiguchi, A. Sekowska, S. J. Seror, P. Serror, B.-S. Shin, B. Soldo, A. Sorokin, E. Tacconi, T. Takagi, H. Takahashi, K. Takemaru, M. Takeuchi, A. Tamakoshi, T. Tanaka, P. Terpstr, A. Tognoni, V. Tosato, S. Uchiyama, M. Vandenbol, F. Vannier, A. Vassarotti, A. Viari, R. Wambutt, E. Wedler, H. Wedler, T. Weitzenegger, P. Winters, A. Wipat, H. Yamamoto, K. Yamane, K. Yasumoto, K. Yata, K. Yoshida, H.-F. Yoshikawa, E. Zumstein, H. Yoshikawa, and A. Danchin.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
18. **Mattevi, A., G. Tedeschi, L. Bacchella, A. Coda, A. Negri, and S. Ronchi.** 1999. Structure of L-aspartate oxidase: implications for the succinate dehydrogenase/fumarate reductase oxidoreductase family. Struct. Fold. Des. **7:**745–756.
19. **Nasu, S., F. D. Wicks, and R. K. Gholson.** 1982. L-Aspartate oxidase, a newly discovered enzyme in *Escherichia coli*, is the B protein of quinolinate synthetase. J. Biol. Chem. **257:**626–632.
20. **Nessi, C., A. M. Albertini, M. L. Speranza, and A. Galizzi.** 1995. The *outB* gene of *B. subtilis* codes for NAD synthetase. J. Biol. Chem. **270:**6181–6185.
21. **Park, J., P. C. Dorrestein, H. Zhai, C. Kinsland, F. McLafferty, and T. P. Begley.** 2003. Biosynthesis of the thiazole moiety of thiamin pyrophosphate (vitamin B1). Biochemistry **42:**12430–12438.
22. **Penfound, T., and J. W. Foster.** 1996. Biosynthesis and recycling of NAD. p. 721–730. *In* F. C. Neidhart, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter and H. E. Umarger (ed.), *Escherichia coli* and *Salmonella.* Cellular and molecular biology, 2nd ed. vol. 1 American Society for Microbiology, Washington, D.C.
23. **Penfound, T., and J. W. Foster.** 1999. NAD-dependent DNA-binding activity of the bifunctional NadR regulator of *Salmonella typhimurium*. J. Bacteriol. **181:**648–655.
24. **Perego, M.** 1993. Integrational vectors for genetic manipulation in *Bacillus subtilis*, p. 615–624. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington D.C.
25. **Raffaelli, N., T. Lorenzi, P. L. Mariani, M. Emanuelli, A. Amici, S. Ruggieri, and G. Magni.** 1999. The *Echerichia coli* NadR regulator is endowed with nicotinamide mononucleotide adenylyltransferase activity. J. Bacteriol. **181:**5509–5511.
26. **Read, T. D., S. L. Salzberg, M. Pop, M. Shumway, L. Umayam, L. Jiang, E. Holtzapple, J. D. Busch, K. L. Smith, J. M. Schupp, D. Solomon, P. Keim, and C. M. Fraser.** 2002. Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*. Science **296**(5575)**:**2028–2033.
27. **Read, T. D., S. N. Peterson, N. Tourasse, L. W. Baillie, I. T. Paulsen, K. E. Nelson, H. Tettelin, D. E. Fouts, J. A. Eisen, S. R. Gill, E. K. Holtzapple, O. A. Okstad, E. Helgason, J. Rilstone, M. Wu, J. F. Kolonay, M. J. Beanan, R. J. Dodson, L. M. Brinkac, M. Gwinn, R. T. DeBoy, R. Madpu, S. C. Daugherty, A. S. Durkin, D. H. Haft, W. C. Nelson, J. D. Peterson, M. Pop, H. M. Khouri, D. Radune, J. L. Benton, Y. Mahamoud, L. Yiang, I. R. Hance, J. F. Weidman, K. J. Berry, R. D. Plaut, A. M. Wolf, K. L. Watkins, W. C. Nierman, A. Hazen, R. Cline, C. Redmond, J. E. Thwaite, O. White, S. L. Salzberg, B. Thomason, A. M. Friedlander, T. M. Koehler, P. C. Hanna, A. B. Kolsto, and C. M. Fraser.** 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature **423**(6935)**:**81–86.
28. **Rey, M. W., P. Ramaiya, B. A. Nelson, S. D. Brody-Karpin, E. J. Zaretsky, M. Tang, A. Lopez de Leon, H. Xiang, V. Gusti, I. G. Clausen, P. B. Olsen,**

M. D. Rasmussen, J. T. Andersen, P. L. Jorgensen, T. S. Larsen, A. Sorokin, A. Bolotin, A. Lapidus, N. Galleron, S. D. Ehrlich, and R. M. Berka. 2004. Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparison with closely related *Bacillus* species. Genome Biol. **5:**R77.

29. **Saxton, R. E., V. Rocha, R. J. Rosser, A. J. Andreoli, M. Shimoyama, A. Kosaka, J. L. R. Chandler, and R. K. Gholson.** 1968. A comparative study of the regulation of nicotinamide-adenin dinucleotide biosynthesis. Biochim. Biophys. Acta **156:**77–84.
30. **Schneider, B. L., and L. J. Reitzer.** 1998. *Salmonella typhimurium nit* is *nadE*: defective nitrogen utilization and ammonia-dependent NAD synthetase. J. Bacteriol. **180:**4739–4741.
31. **Seifert, J., N. Kunz, R. Flachmann, A. Laufer, K. D. Jany, and H. G. Gassen.** 1990. Expression of the *E. coli nadB* gene and characterization of the gene product aspartate oxidase. Biol. Chem. Hoppe Seyler **371:**239–248.
32. **Sun, D., and P. Setlow.** 1993. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis nadB* gene and a *nifS*-like gene, both of which are essential for NAD biosynthesis. J. Bacteriol. **175:**1423–1432.
33. **Sutherland, S.** 2003. New antibiotics for anthrax? Drug Discov. Today **8:** 335–336.
34. **Suzuki, N., J. Carlson, G. Griffith, and R. K. Gholson.** 1973. Studies on the *de novo* biosynthesis of NAD in *Eschericha coli*. V. Properties of the quinolinic acid synthetase system. Biochim. Biophys. Acta **304:**309–315.
35. **Switzer, R. L., H. Zalkin, and H. H. Saxild.** 2002. Purine, pyrimidine, and pyridine nucleotide metabolism. p. 265–269. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
36. **Takami, H., K. Nakasone, Y. Takaki, G. Maeno, R. Sasaki, N. Masui, F. Fuji, C. Hirama, Y. Nakamura, N. Ogasawara, S. Kuhara, and K. Horikoshi.** 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res. **28:**4317–4331.
37. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
38. **Zhu, N., B. M. Olivera, and J. R. Roth.** 1991. Activity of the nicotinamide mononucleotide transport system is regulated in *Salmonella typhimurium*. J. Bacteriol. **173:**1311–1320.