Genomics-driven Reconstruction of Acinetobacter NAD Metabolism

INSIGHTS FOR ANTIBACTERIAL TARGET SELECTION

Enzymes involved in the last steps of NAD biogenesis, nicotinate mononucleotide adenyltransferase (NadD) and NAD synthetase (NadE), are conserved and essential in most bacterial species and are established targets for antibacterial drug development. Our genomics-based reconstruction of NAD metabolism in the emerging pathogen Acinetobacter baumannii revealed unique features suggesting an alternative targeting strategy. Indeed, genomes of all analyzed Acinetobacter species do not encode NadD, which is functionally replaced by its distant homolog NadM. We combined bioinformatics with genetic and biochemical techniques to elucidate this and other important features of Acinetobacter NAD metabolism using a model (nonpathogenic) strain Acinetobacter bayllyi sp. ADP1. Thus, a comparative kinetic characterization of PncA, PncB, and NadV enzymes allowed us to suggest distinct physiological roles for the two alternative, deamidating and nondeamidating, routes of nicotinamide salvage/recycling. The role of the NiaP transporter in both nicotinate and nicotinamide salvage was confirmed. The nondeamidating route was shown to be transcriptionally regulated by an ADP-ribose-responsive repressor NrtR. The NadM enzyme was shown to possess dual substrate specificity toward both nicotinate and nicotinamide mononucleotide substrates, which is consistent with its essential role in all three routes of NAD biogenesis, de novo synthesis as well as the two salvage pathways. The experimentally confirmed unconditional essentiality of nadM provided support for the choice of the respective enzyme as a drug target. In contrast, nadE, encoding a glutamine-dependent NAD synthetase, proved to be dispensable when the nondeamidating salvage pathway functioned as the only route of NAD biogenesis. Acinetobacter baumannii is an emerging pathogen that belongs to a relatively underexplored branch of γ-proteobacteria. It can cause severe pneumonia and infections of the urinary tract, bloodstream, and other parts of the body. Some isolates of A. baumannii display resistance to many known antibiotics (1, 2), emphasizing the importance of pursuing new therapeutic targets for drug development. Biogenesis of nicotinamide adenine nucleotide (NAD), an indispensable cofactor involved in a multitude of biochemical transformations in metabolic networks of all species, was recently established as a target pathway for the development of new antibiotics (3–7). Beyond its main function as a redox cofactor, NAD is consumed as a co-substrate by a number of nonredox enzymes such as bacterial DNA ligase and protein deacetylase of the CobB/Sir2 family (8, 9). A degradative consumption of NAD by these and, likely, other (not fully elucidated) enzymes demands continuous replenishing of the NAD pool, providing further rationale for targeting essential enzymes involved in its biogenesis and recycling. Among these enzymes, nicotinate mononucleotide adenyllyltransferase (NaMNAT)4 of the NadD family and NAD synthetase of the NadE family are widely recognized as the most promising drug targets (6, 7). First, small-molecule inhibitors targeting bacterial NadD and NadE enzymes and showing antibacterial activity were recently reported by several research groups (4, 5, 10–14). Second, our recent genomic survey of NAD metabolism (captured as “NAD(P) biosynthesis” subsystem in the SEED data base (15)) confirmed that these two enzymes are conserved in the overwhelming majority of >800 diverse bacterial species with completely sequenced genomes (9, 16). NadD and NadE enzymes together comprise a two-step conversion of the committed precursor nicotinic acid mononucleotide (NaMN) to NAD. Alternative de novo and salvage routes leading to NaMN synthesis converge at this nearly universal downstream pathway, explaining the conservation and essentiality of nadD and nadE genes. Nevertheless, a few groups of bacteria (including Acinetobacter spp.) appear to deviate from a common pattern lacking orthologs for one or both of these.

4 The abbreviations used are: NaMNAT, nicotinate mononucleotide adenyllyltransferase; NaAD(P), nicotinate adenine dinucleotide (phosphate); Nm, nicotinamide; Na, nicotinate; NaMN, nicotinate mononucleotide; ADPR, AMP-ribose; PRPP, phosphoribosylpyrophosphate.
genes and pointing to the existence of alternative routes of NAD biogenesis as well as to the necessity of alternative targeting strategies. Thus, obligate intracellular pathogens Chlamydia and Rickettsia have lost the entire NAD biosynthetic machinery, replacing it by a unique capability to salvage NAD from the host cell (9). Haemophilus influenzae lacking both nadD and nadE as well as most other common genes of NAD biosynthesis is entirely dependent on a relatively rare PnuC-NadR pathway of nicotinamide riboside (so-called V-factor) salvage (17). Another alternative route of NAD biosynthesis was recently discovered in Francisella tularensis, the causative agent of tularemia or rabbit fever (16). In this species lacking nadD, the NaMN intermediate is first amidated to NMM by NMM synthetase, a divergent member of the NadE family. In the second step, NMM is converted to NAD by NMM synthetase of the NadM family (16, 18). Members of this family, which also belongs to a large superfamily of HIGH nucleotidyltransferases (19), are commonly present in Archaea as a functional replacement of NadD (20). They are scarcely distributed in bacteria, typically as fusion proteins with ADP-ribose pyrophosphatase. In most cases they are present in addition to (rather than instead of) NadD in the context of a dispensable nicotinamide salvage/recycling pathway, which is initiated by the conversion of nicotinamide to NMM by nicotinamide phosphoribosyltransferase (NMPRT) of the NadV family (21). The recently established essential role of NadM in F. tularensis first highlighted this family as an alternative drug target in NAD biosynthesis of bacterial pathogens. This choice is additionally supported by the fact that members of NadM family are only distantly homologous to their human counterparts (22).

To evaluate the last enzymatic steps of NAD biosynthesis as antibacterial targets in A. baumannii, we combined bioinformatics with biochemical and genetic techniques to systematically analyze the complete NAD metabolic subnetwork in the non-pathogenic model organism Acinetobacter baylyi (previously called Acinetobacter sp. ADP1) (23). This analysis led to the discovery of unique aspects of NAD metabolism in the Acinetobacter group providing new guidelines for antibacterial discovery efforts.

EXPERIMENTAL PROCEDURES

Bioinformatics Tools and Resources—Genome analysis and metabolic reconstruction tools implemented in the SEED database (15) were used to analyze NAD metabolism in A. baumannii ATCC 17978 and A. baylyi ADP1 in comparison with ~800 other completely sequenced bacterial genomes. Full results are available at The SEED Viewer in the “NAD and NADP cofactor biosynthesis global” and “NAD regulation” subsystems. Genomes of additional (recently sequenced) orphans lacking nadD were analyzed using NCBI and MicrobesOnline data base (24).

Strains, Growth, and Media—Escherichia coli DH5α (Invitrogen) and BL21(DE3) (Stratagene) were used as general strains for gene cloning and protein overexpression, respectively. All other strains used in this study are listed and briefly described in supplemental Table S1. A. baylyi ADP1 and its mutant derivatives were grown at 30 °C either in rich (Luria broth) or defined minimal Acinetobacter medium (25) (31 mM Na2HPO4, 25 mM KH2PO4, 18 mM NH4Cl, 41 mM nitritotriacetic acid, 2 mM MgSO4, 0.45 mM CaCl2, 3 μM FeCl3, 1 μM MnCl2, 1 μM ZnCl2, 0.3 μM (CrCl3, H2BO3, CoCl2, CuCl2, NiCl2, Na2MoO4, Na2SeO3). Unless otherwise stated, glycerol was used as a carbon source at a final concentration of 0.4% (w/v). E. coli was grown in M9 minimal medium (26) at 37 °C (i.e. for purposes of niaP in trans experiment). The growth rates of bacterial cultures were monitored using Bioscreen C apparatus (Growth Curves USA, minimal Acinetobacter).

Mutant Construction—Strain construction was performed as described in Metzgar et al. (27). Briefly, genes or markers were deleted from the chromosome by a double crossover recombination event after the transformation of a linear PCR product using positive or negative selection markers as previously extensively described (27). Oligonucleotides used for gene deletion and insertion protocols are listed in supplemental Table S2.

Cloning—Primers and plasmids used for molecular cloning are listed in supplemental Tables S1 and S2. A. baylyi pncB, nadV, nadD, and nadE were amplified by polymerase chain reaction from genomic DNA and cloned into the Nhel and EcoRI sites of pET28a (Novagen). A. baylyi niaP was cloned into the Ncol and PstI sites of pBAD24 (Invitrogen). A. baylyi pncA was amplified by polymerase chain reaction from genomic DNA and cloned into the Ncol and PstI sites of pPROEX-HTb (Invitrogen). AbpncA was also amplified by PCR from pPROEX-HTb-abpncA construct and cloned into a PET-derived vector (28) containing the T7 promoter, His tag, and tobacco etch virus protease cleavage site. AbrnR was amplified by polymerase chain reaction from genomic DNA and cloned into the BamH1 and Bpu1102I sites of pET15b (Novagen). All constructs were sequence-verified for accuracy.

Protein Expression and Purification—Recombinant A. baylyi proteins PncA, PncB, NadV, NadM, NadE, and NrtR were produced by overexpression of the corresponding cloned genes as N-terminal fusions with His6 tags in E. coli BL21(DE3) with isopropyl-β-D-galactopyranoside induction. Proteins were purified to homogeneity from 1–2-liter cultures by chromatography on a TALON affinity resin (Clontech) or HisTrap HP column (Amersham Biosciences) followed by gel filtration on a HiLoad Superdex 200 16/60 column (GE Healthcare) or Superose 12 HR 10/30 column (Amersham Biosciences) using AKTA FPLC system as described (29, 30).

Enzyme Assays and Kinetics—Nicotinate phosphoribosyltransferase activity for abPncB enzyme was measured with an end-point HPLC-based assay relying on direct quantitation of NaMN product. The reaction mixtures were incubated for 20 min at 30 °C and analyzed on an ion-paired analytical Supelco C18 column (3 μm, 4.6 × 150 mm) as described (16). Reaction mixtures contained 50 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM DTT, 2 mM ATP, 5 mM PRPP, and 0.5 mM nicotinic acid.
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in the presence of 1.2 nm abPncB. For initial rate measurements, nicotinic acid concentration was 0.1–300 μM. To test abPncB specificity for nicotinamide, higher concentrations of enzyme (25 μM) and substrate (0.03–20 mM) were used. Nicotinamide phosphoribosyltransferase activity for abNadV enzyme was measured as above. Reaction mixtures contained 50 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2 mM ATP, 5 mM phosphoribosyl pyrophosphate (PRPP), and 0.5 mM nicotinamide in the presence of 10 nM abNadV. For initial rate measurements, nicotinamide concentration was 0.003–1 mM, whereas a higher concentration of enzyme (15 μM) and substrate (0.03–100 mM) was used to test abNadV specificity toward nicotinic acid. Nicotinamide deamidase activity was assayed in 100 mM Hepes, pH 7.5, and 1–300 μM nicotinamide in the presence of 2 nM abPncA. The same end-point HPLC method as above was used to monitor nicotinic acid formation. In an alternative continuous assay modified from Boshoff and Mizrahi (31) ammonia release by PncA was coupled to the conversion of α-ketoglutarate to l-glutamate by glutamate dehydrogenase in the presence of NADPH. The coupled reaction was monitored by the decrease of NADPH fluorescence at 465 nm (excitation at 340 nm). The assay mixture contained 20 mM potassium phosphate, pH 7.5, 0.2 mM NADPH, 3.3 mM α-ketoglutarate, 2 units of bovine liver glutamate dehydrogenase, 1–300 μM nicotinamide (Nm), and appropriate amounts of abPncA. NaMN/NMN adenylyltransferase-coupled assays were performed as described (16). The NaMN adenylyltransferase activity of abNadM (15 nM) was assessed by in situ conversion of the NaAD product to NAD (by excess of Bacillus anthracis NAD synthetase) coupled to the reduction of NAD to NADH (by alcohol dehydrogenase) and monitored at 340 nm. NMN adenylyltransferase activity measurements were performed under the same conditions without adding NAD synthetase. NAD synthetase activity was tested using a similar coupled assay in mixtures containing 50 mM Hepes, pH 7.5, 2 mM ATP, 1 mM NaAD, 5 mM ammonia (or glutamine), 10 mM MgCl₂, 20 mM semicarbazide (or 2 mM Na₂SO₄), 6 units/ml alcohol dehydrogenase, 60 mM ethanol, and 60 mM abNadE. For kinetic measurements, concentration of NaAD was varied in the range 0.005–2 mM. NMN synthetase activity was measured with a HPLC-based end-point assay as described (16). Reaction mixtures consisted of 50 mM Hepes, pH 7.5, 2 mM ATP, 1 mM NaMN, 5 mM ammonia, 10 mM MgCl₂, and 22 μM abNadE. NaMN was varied from 0.1 to 25 mM for initial rate studies. Steady-state kinetic parameters were determined using Prism software (GraphPad) by fitting to standard Michaelis-Menten equation or its modification for substrate (nicotinamide) inhibition (32) as observed for abNadV.

Electrophoretic Mobility Shift Assay (EMSA)—A 90-bp region upstream of pros-nadV operon containing predicted tandem regulatory sites was PCR-amplified from genomic DNA using primers shown in supplemental Table S2. One of the primers was 5′-biotinylated (Sigma). The labeled DNA fragment (1 nM) was incubated for 20 min at 25 °C with 2 nM purified abNrtR in 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 0.05% Nonidet P-40, and 0.50 mg/ml bovine serum albumin. The complexes were analyzed by native PAGE (5%) in 0.5 × Tris borate-EDTA followed by electrophoretic transfer onto a nylon membrane (Pierce) and UV cross-linking. Biotin-labeled DNA was detected with the LightShift Chemiluminescent EMSA kit (Pierce). To control the specificity of abNrtR binding, a competitive EMSA assay was performed with a 100-fold molar excess of non-biotinylated target DNA (specific competitor) or 1 μg of poly(dl-dC) (nonspecific competitor). To evaluate ADP-ribose and other NAD-related metabolites effects on the protein-DNA binding, the protein and metabolite were preincubated at the indicated concentration for 10 min at 25 °C before biotinylated DNA was added.

Quantitative RT-PCR Analysis—total RNA was isolated from A. baylyi cells grown in LB and collected at midlog phase using the SV Total RNA Isolation System (Promega). The RNA was subjected to DNase I digestion to remove genomic DNA and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). The reaction mix was then subjected to quantitative real-time PCR to detect levels of the corresponding nadV and nrtR cDNAs using primers listed in supplemental Table S2. The levels of expression (relative to 16 S rRNA) were analyzed using MJ Research Opticon™ 2 software (MJ Research, Watertown, MA).

RESULTS

Bioinformatics Predictions—The results of a comparative genomic reconstruction of NAD metabolism in A. baylyi and A. baumannii performed using a subsystems-based approach (15) implemented in the SEED genomic platform are illustrated in Fig. 1 and Table 1. Although most of the reconstructed sub-network could be seamlessly inferred by analogy with previously studied species, certain key aspects appeared to be unique and, thus, required additional experimental analysis. Most notably, an apparent functional replacement of NadD by NaDM could have two alternative interpretations. Based on similarity with previously characterized bacterial representatives of the NaDM family (16, 18, 33), abNaDM could be assigned a functional role of MNAT acting on the NNM intermediate. This assignment would be consistent with its role in the nondeamidating salvage of nicotinamide via NMPRT enzyme (NaDv). However, utilization of a canonical MNAT for the processing of NaMN intermediate generated by de novo (NaB-NaA-NaC) or deamidating salvage (PncA-PncB) routes (Fig. 1) would be extremely inefficient or require the presence of an additional enzyme, NNM synthetase, as previously shown for F. tularensis (ft-NadE) (16). The amino acid sequence of abNaD did not show appreciable similarity with the NNM synthetase subfamily but was rather characteristic of NAD synthetase with the additional glutamine transferase domain responsible for the utilization of glutamine as in eukaryotes (34) and some bacteria (e.g. Mycobacterium tuberculosis) (35). Nevertheless, this possibility could not be excluded without additional experimental analysis (see below).

A possible alternative interpretation would be that abNaD possesses dual MNAT/NaMNAT activity. Such dual specificity is characteristic of eukaryotic enzymes from a distinct (albeit evolutionarily and structurally related) PNAT family (36, 37). Indeed, both PNAT activities are physiologically relevant for
NAD biosynthesis in human cells, which includes de novo and salvage routes proceeding via NaMN or NMN intermediates (36). Moreover, enzymes of NadM, and not NadD, family are universally present in Archaea where they are expected to play the NaMNAT role in the de novo synthesis of NAD (38). To distinguish between these two possibilities, in this study we set to experimentally determine the actual substrate preference of the purified recombinant abNadM and to use genetic techniques to address yet another (albeit unlikely) possibility; that is, the existence of an alternative NaMNAT nonhomologous to previously characterized enzymes.

Another unprecedented feature of Acinetobacter spp. is the simultaneous presence of two alternative routes of nicotinamide salvage inferred from genome analysis and raising questions about their actual physiological roles and regulation. Our approach to addressing these questions combined the enzymatic characterization of respective gene products with the phenotype analysis in a series of engineered A. baylyi mutants.

Two additional bioinformatics predictions related to niacin salvage/recycling were deemed a subject of experimental testing. Gene abNiaP was tentatively assigned a role of Nm

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In our recent study we directly confirmed the dual NaMNAT/NMNAT specificity of NadM from Methanocaldococcus jannaschii, L. Sorci et al., unpublished results.

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FIGURE 1. Genomic reconstruction of NAD biosynthesis, uptake, and regulation in Acinetobacter spp. A, biochemical transformations directly involved in the downstream, salvage, and recycling biosynthetic routes are represented in solid black arrows. Dotted lines relate to uptake and de novo synthesis. Metabolite abbreviations are described in the footnotes. Functional roles are shown by gene names (see also Table 1). B, regulation of NtrR transcription factor is shown. A cluster of three genes are co-regulated by abNrtR: prs, a paralog of phosphoribosyl pyrophosphate synthetase, nadV, and nrtR. Two tandem NtrR-binding sites are shown by red spheres. Corresponding consensus motifs are shown on the right. Positions (−34, −59) are shown as distance from prs gene (ACIAD0964). A diagram for the non-deamidating recycling of Nm is provided in supplemental Fig. S5.
and/or nicotinic acid (Na) transporter based on homology with the previously characterized distant member of this family from *Bacillus subtilis* (39). Gene deletion and phenotype analysis experiments were designed to verify this functional assignment and to test whether *ab*NiaP is essential for Nm and/or Na salvage or (as in *B. subtilis*) there are additional mechanisms of their uptake.

Expression of *nadV* gene was predicted to be under control of a transcriptional regulator from the NrtR family (29). Regulators controlled by the members of NrtR family, which typically include genes involved in various aspects of NAD metabolism, have been recently described in many diverse bacteria as captured in RegPrecise data base (40). Two representatives of this family from *Shewanella oneidensis* and *Synechocystis* sp. have been characterized in vitro, and their DNA binding activity was shown to be negatively regulated by ADP-ribose (ADPR), a byproduct of NAD degradative utilization. However, at the time of this study, NrtR activity has not yet been demonstrated in vivo in any species. Therefore, in this study we aimed to test the predicted *ab*NrtR function both by characterization of the purified recombinant *ab*NrtR and by the analysis of *nadV* gene expression in *ΔnrtR* mutant. The brief description and interpretation of experimental results addressing all of the questions formulated above is provided in the following sections.

**De Novo Synthesis of NaMN—Acinetobacter spp.** possess readily detectable orthologs of all three genes, *nadB*, *nadA*, and *nadC*, of the *de novo* synthesis of NaMN from L-aspartate (Table 1). All three respective enzymes have been characterized from a variety of species (for a recent overview, see Ref. 9). We have confirmed the *in vivo* functional activity of this pathway by the comparison of growth phenotypes of *A. baylyi* wild-type and *ΔnadB* mutant (Table 2). As expected, the *nadB* strain could not grow on minimal medium without added Nm or Na (see also supplementary Fig. S1).

### TABLE 1

| Symbol       | Functional role | Gene name (A. baylyi) | Validation method | Gene ID (A. baumannii) |
|--------------|-----------------|-----------------------|-------------------|------------------------|
| ASPOX       | *De novo*       | *nadB*                | G                 | ABAYE0935              |
| QASYN       | 1-Aspartate oxidase (EC 1.4.3.16) | *nadA*                | G                 | ABAYE3104              |
| QAPRT       | Quinolinate phosphoribosyltransferase (EC 2.4.2.19) | *nadC*                |                   | ABAYE8232              |
| NIAT        | Niacin transporter | *niaP*                | B + G             | ABAYE680               |
| NMASE       | Nicotinamide deamidase (EC 3.5.1.19) | *pncA*                |                   | ABAYE0059              |
| NAPRT       | Nicotinphosphate phosphoribosyltransferase (EC 2.4.2.11) | *pncB*                |                   | ABAYE0104              |
| NMPRT       | Nicotinamide phosphoribosyltransferase (EC 2.4.2.12) | *nadV*                |                   | ABAYE1970              |
| NNM/NaMNAT  | NNM/NaMN adenylyltransferase (EC 2.7.7.18) | *nadM*                | B + G             | ABAYE9016              |
| NADSYN      | NAD synthetase (EC 6.3.1.5) | *nadE*                | B + G             | ABAYE2955              |
| NRTTR       | NAD-related transcriptional regulator | *nrtR*                |                   | ABAYE1791              |

*No viable knockout strain could be obtained, possibly due to the cytotoxic effect of quinolinate accumulation.

*Experimentally characterized by Fyfe et al. (51).*

### TABLE 2

| Strains | Media |
|---------|-------|
|         | MM    | MM + Na | MM + Nm |
| Wild type | +     | +       | +       |
| ΔnadB     | –     | +       | +       |
| ΔnadBΔpncB | –     | –       | –       |
| ΔnadBΔnadV | –     | +       | +       |
| ΔnadBΔpncA | –     | +       | +       |
| ΔnadBΔpncBΔnadE | – | – | – |

Conversion of Nm (or Na) to NaMN via Deamidating Salvage Route—Although the orthology-based functional assignment of genes encoding both enzymes of this common pathway, nicotinamidase (gene *pncA*) and nicotinic acid phosphoribosyl transferase (gene *pncB*), was straightforward (Table 1), their experimental characterization was essential for the assessment of physiological roles of the two alternative salvage routes of Nm (Fig. 1). The analysis of steady-state kinetic parameters revealed a high (physiologically relevant) catalytic efficiency of both purified recombinant enzymes (*kₐₜ/Kₘ* values, see Table 3). The physiological role of this enzyme and the functional *in vivo* activity of the entire pathway were directly confirmed by genetic analysis (Table 2). The *A. baylyi ΔnadBΔpncB* strain lost the ability to grow on minimal medium supplemented by 0.1 mM Nm. However, this strain could grow normally in the presence of 0.1 mM Nm, confirming the physiological activity of the alternative non-deamidating route (see below). The physiological activity of *ab*-PncA is supported by the normal growth of the *A. baylyi ΔnadBΔnadV* strain in the presence of 0.1 mM Nm.
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Conversion of Nm to NMN via Nondeamidating Salvage Route—As already mentioned, nondeamidating salvage of Nm is initiated by the NMPRT enzyme encoded by gene nadV (Fig. 1). Previously described members of NadV family share a fold and a general catalytic mechanism of phosphoribosyl transfer with the PncB family (41) but display a distinct substrate specificity for Nm (21, 42). The same strict substrate preference for Nm over Na (∼5,000-fold) was observed by us for the recombinant purified abNadV enzyme (Table 3), confirming its role in the nondeamidating route. Notably, both phosphoribosyltransferases, abPncB and abNadV, are strongly activated in the presence of 2 mM ATP (∼100- and ∼10-fold, respectively) as previously reported for other representatives of these families (43, 44). Comparison of catalytic efficiencies of abNadV and abPncA (Table 3) potentially competing for the Nm substrate suggests that the deamidating route is likely to prevail, especially at low concentrations of Nm (as the $K_{cat}$ value of abNadV is ∼20-fold higher than of abPncA). The biological significance of the coexistence of two alternative Nm salvage routes in Acinetobacter spp. is not entirely clear. The observed difference in catalytic efficiency of the respective enzymes together with precedents in other species (21) points to a likely role of nondeamidating route in recycling of endogenous Nm (product of intracellular NAD degradation). However, our genetic data (Table 2) proved that A. baylyi can sustain a normal growth with the nondeamidating (NadV-mediated) salvage of Nm as the only route of pyridine nucleotide synthesis.

Uptake of Nm and Na—Two complementary approaches were used to assess the physiological role of the predicted abNiaP transporter similar to how it was previously described for NiaP from B. subtilis (39). In the first “knock-in” approach the overexpression of the niaP gene from A. baylyi in E. coli BW25113 ΔnadA strain led to a notable increase in its growth rate on minimal medium supplemented with a limiting concentration of either Nm or Na (0.16 μM) (Fig. 2, A and B). This strain cannot produce NAD via the de novo route, and its growth is entirely dependent on the uptake and salvage of exogenous niacin. The observed increase in growth rate provided the first experimental verification of the abNiaP functional assignment as high affinity niacin (Nm and Na) transporter. In the second “knock-out” approach, the growth of an A. baylyi ΔnadB strain (with blocked de novo route) was compared with a double-mutant ΔnadBΔniaP derivative in minimal medium supplemented with varying concentrations of either Nm or Na (0–100 μM). Whereas the supplementation of 0.5 μM Nm or Na was sufficient for the growth of the ΔnadB strain (Fig. 2, C and D), at least 200-fold higher niacin concentration was required to maintain the normal growth of the ΔnadBΔniaP strain (Fig. 2, E and F). This observation suggests that NiaP substantially contributes to niacin transport when its concentration in the medium is very low (<0.5 μM, see supplemental Fig. S3 for dose dependence data). As in case of B. subtilis (39), an alternative (NiaP-independent) uptake mechanism is operational at higher niacin concentration (>100 μM).

Transcriptional Regulation of Nondeamidating Salvage/Recycling Route—In addition to the regulation of flux distribution between the two salvage/recycling routes through differential catalytic efficiency of the respective enzymes (as described above), the nondeamidating salvage route is regulated at the level of transcription by a predicted ADPR responsive transcriptional repressor abNrtR (29). The results of in vitro characterization of the recombinant purified abNrtR protein are illustrated in Fig. 3. EMSA confirmed the specific binding of abNrtR to the DNA region containing previously predicted DNA motif sites located upstream of the prs-nadV operon (Fig. 3A). The observed binding was efficiently suppressed in the presence of 100 μM ADPR or phospho-ADPR (Fig. 3B), confirming a proposed role of these NAD(P) degradations products as antirepressors. None of the intermediary metabolites associated with NAD biosynthesis, Nm, Na, quinolinate, NMN, NaMN, NAD, NaADP, NaAD, NADP, tested at 100 μM interfered with abNrtR-DNA binding (some of them are shown in Fig. 3B). The in vivo activity of abNrtR was confirmed by the comparison of nadV expression levels in wild-type A. baylyi and in a ΔnrtR mutant using RT-PCR. The basal level of nadV expression in the wild-type strain grown in rich medium was very low, whereas it increased ∼150-fold upon the deletion of nrtR gene (supplemental Fig. S2). These results are consistent with abNrtR acting as a negative regulator of nadV expression providing in vivo functional verification for a member of NrtR family.
NMNAT type with nearly identical catalytic efficiency ($k_{\text{cat}}/K_m$) for both physiological substrates (Table 3). To our knowledge it is the first documented example of a dual specificity NaMNAT/NMNAT enzyme in bacteria. Most importantly, the dual specificity of $\text{abNadM}$ is fully consistent with its universal and essential role in all routes of NAD biogenesis via each of the two pyridine nucleotide intermediates.

The essentiality of $\text{abNadM}$ is supported by the fact that the deletion of the respective gene in $A.\ baylyi$ could be accomplished only with complementation by another NaMNAT or NMNAT encoding gene. Thus, we were able to obtain a viable $A.\ baylyi\ \Delta\text{nadM}$ knock-out mutant only after introduction of $E.\ coli\ \text{nadD}$ or the $F.\ tularensis\ \text{nadM}$ genes into an ectopic site on the chromosome under the control of a constitutive promoter (see supplemental Fig. S4 and Table S1). As of today, *Acinetobacter* is the second example of bacterial species (after *F.\ tularensis* (16)) where the member of NadM family was shown to be an indispensable enzyme functionally replacing NadD in NAD biogenesis and homeostasis.

**Conversion of NaAD to NAD by NadE**—The enzymatic characterization of the purified recombinant $\text{abNadE}$ confirmed its proposed functional role of NAD synthetase in the last step of a two-step conversion of NaMN to NAD (Fig. 1). As expected from the presence of an additional glutamine transferase domain, $\text{abNadE}$ can efficiently utilize l-glutamine (as well as ammonia) for the amidation of NaAD precursor (specific activities are quite similar for both ammonia donors; not shown). No appreciable amidation was observed using NaMN instead of NaAD as a substrate, which effectively excluded a possibility of $\text{abNadE}$ having an alternative NMN synthetase activity (Table 3). Based on the confirmed physiological activity of the $\text{abNadV-NadM}$ nondeamidating salvage route (Fig. 1), $\text{abNadE}$ enzyme was expected to be dispensable in *Acinetobacter* in the presence of Nm in the growth me-
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Targeting essential enzymes involved in the biosynthesis of NAD(P), the indispensable redox cofactor, has been recognized as a promising strategy for the development of novel antibiotics (4, 5, 10, 12). However, the rational choice of actual drug targets requires better understanding of pathways that comprise NAD metabolic subnetworks, especially in divergent and poorly explored organisms such as pathogenic species of Acinetobacter. Indeed this study revealed that among the two nearly universal target enzymes, NadD and NadE, the former is functionally replaced in Acinetobacter by a member of a distinct NadM family, whereas the latter is potentially dispensable.

Our approach to the reconstruction of NAD metabolism in Acinetobacter was based on comparative genomics analysis combined with focused experimental testing and refinement of the key bioinformatics predictions. The bioinformatics analysis included all Acinetobacter species with completely sequenced genomes (7 genomes were publicly available), whereas the experimental testing was performed using a non-pathogenic strain, A. baylyi ADP1. This strain provided us with an ideal model system due to its remarkable genetic versatility (27, 45) and availability of a genome-wide collection of knock-out mutants by Genoscope (25). Moreover, understanding of NAD metabolism in A. baylyi is of additional importance for its biotechnological applications in biodegradation and manufacturing of biopolymers (46). Indeed, maintenance and regeneration of the NAD cofactor pool is a known bottleneck in many microbial fermentations (47, 48).

In silico genomic reconstruction performed in this study revealed an extensive network of pathways leading to NAD, which is conserved in all analyzed Acinetobacter spp. and includes de novo synthesis and two alternative salvage/recycling routes (Fig. 1 and Table 1). This analysis pointed us to several unique and previously unexplored features, and targeted biochemical and genetic experiments were designed to test the key bioinformatics inferences and reconcile ambiguities and possible alternative scenarios.

In the upstream part of Acinetobacter NAD subnetwork, the most unusual feature is the simultaneous presence of the two alternative (and potentially competing) Nm salvage routes, deamidating (via PncA-PncB) and nondeamidating (via NadV). A widely different phylogenomic distribution of these two alternative salvage pathways and possible evolutionary implications have been previously discussed (8, 21), and they are further illustrated in supplemental Table S4.

Through in vitro characterization of all respective enzymes (Table 3) and growth phenotype analysis of engineered knock-out mutants (Table 2), we have confirmed that both salvage routes are operational in Acinetobacter. Indeed, each of them could support the normal growth of A. baylyi as a sole source of pyridine nucleotides on the minimal medium supplemented with Nm. Although the biological significance of this duality is not completely clear, some experimental data obtained here allow us to speculate that the first route is predominantly responsible for the salvage of exogenous Nm (as well as Na), especially when its concentration in the environment is very low (Table 2 and Fig. 2). On the other hand, the second, abNadV-mediated route is likely to contribute largely to the recycling of endogenous Nm, which may accumulate due to degradative utilization of NAD by CobB/Sir2 NAD-dependent protein deacetylase and, possibly, by other (yet unknown) NAD-glycohydrolases. Indirect evidence in favor of this interpretation was provided by the experimentally confirmed in vivo transcriptional regulation of nadV gene by ADPR-responsive repressor abNrtR. The ability of ADPR to disrupt binding of abNrtR to the predicted operator site in the upstream of prs-nadV operon was confirmed in vitro by EMSA analysis (Fig. 3). These observations are consistent with the hypothesis that accumulation of ADPR resulting from NAD degradation may be interpreted by the cell as a signal to activate recycling of Nm. The latter is released as a byproduct of this degradation, and the abNadV-mediated nondeamidating route appears to be the most efficient way of its conversion to NAD with the help of NMNAT activity of abNadM (see below). Notably, the first gene of the prs-nadV operon encoding a paralog of PRPP synthetase may also directly contribute to Nm recycling by feeding PRPP as a second substrate for NadV reaction (Fig. 1). It is tempting to speculate that ribosyl 5-phosphate required for the synthesis of PRPP may be at least partially supplied by the hydrolysis of ADPR (supplemental Fig. S5) by the product of nudF gene.
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(ACIAD0275 in A. baylyi) tentatively assigned an ADPR pyrophosphatase function based on homology with other characterized members of Nudix family (49).

The remarkable plurality of the upstream pathways in the NAD subnetwork leading to the formation of pyridine nucleotide precursors (NaMN and NMN) emphasizes the importance of NAD biogenesis and homeostasis in Acinetobacter. At the same time, a demonstrated functional redundancy of these pathways (at least in laboratory conditions) argues that none of the respective enzymes would constitute a sustainable drug target. Moreover, of the two enzymes abNadM and abNadE involved in downstream conversion of these precursors to NAD, only the former is truly indispensable, as supported by combined genetic and kinetic evidences (Table 1–3). The abNadM is the first documented example of dual NMNAT/NaMNAT specificity among bacterial members of the NadM family. As noticed before, this family is only sparsely represented in bacteria where NadM is usually present in addition to (and not instead of) the housekeeping NadD enzyme and contributes to an optional Nm salvage/recycling together with NadV enzyme (21). In most cases these two genes, nadV and nadM, are encoded in one operon. Importantly, all previously characterized bacterial NadM enzymes have two features distinguishing them from abNadM; (i) strong (at least 50–100-fold) substrate preference for NMN over NaMN and (ii) the presence of an additional C-terminal domain harboring ADPR pyrophosphatase of Nudix family. Both enzymatic activities are relevant for Nm salvage as discussed above for A. baylyi where the ADPR pyrophosphatase activity is encoded by a remotely located nudF gene. The structural and enzymatic analysis of the two representative bacterial NadM-NudF fusion proteins did not reveal any functional interactions between the two domains that would affect the NadM substrate preference (18). At the same time, the NadM (and not NadD) family is universally present in nearly all Archaea where it appears to function as the housekeeping NaMNAT/NaMNAT specificity among bacterial members of the NadM family. None of the analyzed archael genomes revealed a presence of the bacterial-like NadM-NudF fusion protein. These observations as well as characteristic variations in gene patterns associated with deamidating and nondeamidating Nm salvage/recycling routes point to a likely archael origin of bacterial NadM (see supplemental Table S4 and the accompanying comments suggesting a possible evolutionary scenario).

Overall, the most important practical implication of this study is the identification of abNadM as an attractive target for the development of novel antibiotics against pathogenic strains of A. baumannii. This is the second case (after F. tularensis (16)) where a member of NadM family functionally replaces NadD (which is otherwise present in nearly all pathogenic bacteria) and constitutes an alternative drug target. In both cases, members of NadE family were shown to be dispensable (at least in laboratory conditions). The latter observation can be tentatively projected to some other bacterial species (including pathogenic strains of Burkholderia) containing the complete nondeamidating salvage pathway comprised of NMPRT (NadV family) and NMNAT (NadM or NadR family). More generally, it reflects a fundamental difference between the two types of biochemical transformations associated with the downstream pathways of NAD biosynthesis. At least one of the two adenyl transferase reactions converting pyridine mononucleotides (NaMN or/and NMN) to respective dinucleotides (NaAD or/and NAD) is present and unconditionally essential in all life forms (including all bacterial pathogens) except for some intracellular endosymbionts. On the other hand, the ATP-dependent amidation of the carboxyly moiety of NaAD by NAD synthetase (or, rarely, of NaMN by NMy synthetase) in some species may be bypassed (as in Francisella) or even replaced (as in Haemophilus) by the nondeamidating salvage of amidated precursors (Nm or Nm-ribose, respectively). NAD metabolism of Acinetobacter analyzed in this study is another example that illustrates this fundamental principle providing important guidelines for drug targets selection and prioritization.

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