Strategy for Efficient Importing and Function Characterizing of Nicotinamide Adenosine Dinucleotide Analogs in *Escherichia coli* Cells

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ABSTRACT

Nicotinamide adenosine dinucleotide (NAD) and its phosphorylated form (NADP) are essential metabolites that are key cofactors for many redox enzymes and co-substrates for several protein modification enzymes. It is difficult to study and manipulate the functions of NAD(P), because these cofactors participate in complex metabolic network. NAD analogs can reduce the complexity by bioorthogonalization of metabolic modules and enhance our ability to understand and regulate the bioenergetic and signaling pathways mediated by NAD. But the design and application of NAD analogs is limited because these compounds cannot be easily delivered into cells. Here we explored the strategy to import those adenine-replaced NAD analogs (NXDs) into *Escherichia coli* cells. We showed that the transporter NTT4 derived from *Protochlamydia amoebophila* was efficient for NXDs import. By constructing an *ushA*-deletion mutant, we improved extracellular stability of NXDs significantly and realized continuous import upon concurrent expression of NTT4. The *in vivo* functions of NXDs were then characterized in *E. coli* cells. Nicotinamide guanine dinucleotide was identified as an inhibitor of NAD synthesis and can partially support cell growth of an NAD-auxotrophic *E. coli* strain. Nicotinamide cytosine dinucleotide was proved an excellent energy transporter with distinct bioorthogonality biocompatibility. The efficient importing system will stimulate developing and screening of functional NXDs.

IMPORTANCE NAD analogs are importante tools for manipulating the bioenergetic and signaling pathways mediated by NAD, but they have to be imported into cells as these compounds are membranes impermeable. The efficiency of importing NAD analogs into cells can be conspicuously improved by limiting extracellular degradation while expressing an efficient NAD importer. Then the potential application of the analogs can be preliminarily forecasted according to their *in vivo* characters. That is, the analog similar to NAD is candidate regulator of NAD metabolism, while analog has bare interference with natural system may serve as bioorthogonal energy carrier. In summary, we explore a strategy for continuous and efficient importing of NAD analogs and this work will facilitate the characterization and utilization of NAD analogs.

KEYWORDS NAD analog, nicotinamide guanine dinucleotide, nicotinamide cytosine dinucleotide, cofactor manipulation, cofactor transporter, cofactor stability

INTRODUCTION

Nicotinamide adenosine dinucleotide (NAD, Fig. 1a) and its phosphorylated form (NADP) are natural pyridine nucleotide cofactors, which play a central role in biology. They are essential metabolites participating in both bioenergetic and signaling pathways (1, 2). But efforts to study the roles of NAD(P) have proven difficult because these cofactors are subjected to tight regulation and participate in complex metabolic network. Cofactor analogs are general tools for study and alteration of cofactor functions, yet such efforts are limited because these compounds cannot be easily delivered into cells (3-5).

As a pivotal class of cofactors in bioenergetic pathways, pyridine nucleotide cofactors such as NAD and its reduced form NADH function as essential electron acceptors or donors in numerous catabolic and anabolic reactions. They also play critical roles in maintaining intracellular redox homeostasis. When modification or introduction of metabolic pathways affects the level of NAD/NADP or redox state, the cellular metabolism will be dramatically influenced, resulting in decreased robustness and biosynthetic capacity (2, 6, 7). One promising way for avoiding interference between the target cofactor dependent system and natural systems is constructing bioorthogonal redox metabolic circuits based on NAD analogs. For example, nicotinamide cytosine dinucleotide (NCD, Fig. 1a) wired metabolic circuits can transfer energy pathway-selectively and shift the reaction equilibrium with little interference with natural systems (4, 5).

NAD metabolism plays important roles in the maintenance of NAD pools and has beneficial effects on regular calorie restriction on health span (1, 8). The correlated enzymes are potential drug target for anticancer or antipathogen therapy (9, 10). However, characterizing the molecular mechanisms that underlie the complex regulation of NAD metabolism remain unclear. Many efforts are focused on the development of pyridine nucleotide cofactor analogs to serve as effective functional and mechanistic probes in the cellular environment. Recombinant human nicotinamide adenylyl transferase 1 (Nmnat1) was employed to condense nicotinamide mononucleotide and tzATP to yield NtzAD (11). Nmnat3 could synthesize nicotinamide guanine dinucleotide (NGD, Fig. 1a) (12). However, the biological functions of these analogs are difficult to be investigated by exogenously addition, because pyridine nucleotide cofactors are membranes impermeable (13). Our ability to experimentally control *in vivo* specific pyridine nucleotide cofactor

concentrations could enhance our understanding and application of cofactor-dependent reactions.

Synthesis of pyridine nucleotide cofactors using corresponding membranes permeable nucleosides via NAD salvage pathway is one possible route to the introduction of pyridine nucleotide cofactors into cells. For example, *in vivo* NGD has been verified in mice overexpressing Nmnat3 (13). However, recognition of corresponding nucleosides by the salvage pathway may not be sufficient, and the salvage pathways relying on the activation of free nucleosides to produce the desired pyridine nucleotide cofactor is less than optimal (13). This will increase the challenge of achieving controlled intracellular concentrations of the pyridine nucleotide cofactor, which is likely to limit many applications. In general, study and application of pyridine nucleotide cofactor analogs desires general means to directly introduce the cofactors into cells.

Many NAD transporters have been discovered in both prokaryote and eukaryote organisms, such as the first NAD transporter reported NTT4 derived from the prokaryote chlamydial endosymbiont *Protochlamydia amoebophila* UWE25 (14), and NAD transporters discovered in the eukaryotes *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* located at mitochondria or chloroplasts (12, 15). These transporters import intact NAD(H) in counter exchange with ADP for cells or organelles, and they are potential candidates for importing pyridine nucleotide cofactor analogs. The characterization of cofactor transporters are generally carried out in model organism *Escherichia coli* (5, 14, 16-18). Recombinant expression of the NAD transporter from *A. thaliana* mitochondria (AtNDT2) in *E. coli* functions to import exogenously added NCD, but it can only get 0.059 mM intracellular NCD in the presence of 0.1 mM exogenous NCD (5). More efficient importing strategy is desired for further application of pyridine nucleotide cofactor analogs.

Stability is also a key character for efficiency of cofactors import. In many prokaryote and eukaryote organisms, the extracellular pyrophosphatase hydrolyzes the pyrophosphate bond of pyridine nucleotide cofactors and catalyzes further hydrolysis to permeable nicotinamide riboside (NR) and nucleosides (19, 20). To limit or compensate extracellular decomposition of the cofactors, import of cofactors is generally carried out by decreasing operating time (14, 17, 21) or supplying excess cofactors to the cultural medium (17, 22). As the main pyrophosphatases are generally coded by one or two genes (19, 20, 23, 24), simple gene deletion may significantly

improve cofactor stability.

Here we optimize the importing efficiency of pyridine nucleotide cofactors in *E. coli* by choosing an efficient transporter and deleting *ushA* gene encoding the main pyrophosphatase for the decomposition of exogenous cofactors. Then pyridine nucleotide cofactors can be directly introduced in a continuous and efficient way. We import NAD analogs with the adenine replaced by other bases (NXDs, Fig. 1A) via the strategy, and then characterize biological functions of NXDs.

RESULTS

Identification of efficient NAD analog transporter

Among the well characterized NAD transporters, AtNDT2 derived from *A. thaliana* mitochondria shows the widest substrate spectrum and the highest activity (12, 15). AtNDT2 has been applied to importing NCD for construction of *in vivo* metabolic circuits, but the importing efficiency is insufficient for further study and application (5).To date, *P. amoebophila* UWE25 derived NTT4 has been proved an excellent NAD transporter by heterologous expression in *E. coli*, but its substrate spectrum has not been well studied (14, 22).

To evaluat the importing efficiency of NAD analogs by AtNDT2 and NTT4, four NXDs was designed by substituting the adenine group of NAD with natural bases (Fig. 1A). The NXD with natural base might have excellent biocompatibility and can be synthesized via modified endogenetic NAD salvage pathways (13), which will facilitate *in vivo* applications of characterized NXDs. Based on the structure similarity, NAD and NGD with purine group might have similar character, and possess different properties toward NCD, NTD and NUD with pyrimidine group. NAD and NCD were chosen for preliminary design and characterization of efficient NXD import strategy.

The cofactor transporting efficiency of NTT4 and AtNDT2 was compared by expressing the transporters with constitutive promoter gntT105P of the plasmid pBCTD in the strains WL023 and WL024, respectively. To correct for endogenetic synthesis of NAD, we subtracted the background NAD concentration from the control strain incubated without exogenous cofactors. Compared with AtNDT2, NTT4 was a more efficient transporter for both NAD and NCD (Fig. 1B). With 0.1 mM exogenously added cofactors, NTT4 increased the cellular NAD and NCD level by

1.8 mM and 1.2 mM, respectively, while AtNDT2 only increased the cellular NAD and NCD level by 0.5 mM and 0.1 mM, respectively. Both NTT4 and AtNDT2 preferred NAD to NCD. NTT4 could concentrate exogenous cofactor into cells via counter exchange with ADP, and made an 12 fold higher intracellular NCD concentration than the exogenously added 0.1 mM NCD.

NCD is primarily designed for pathway specific energy transformation in cellular environment, and its influence on cell growth will affect the *in vivo* application. NCD had no detectable influence on cell growth of WL023 when exogenously added at 0.1 mM, which indicated that NCD has an excellent biocompatibility.

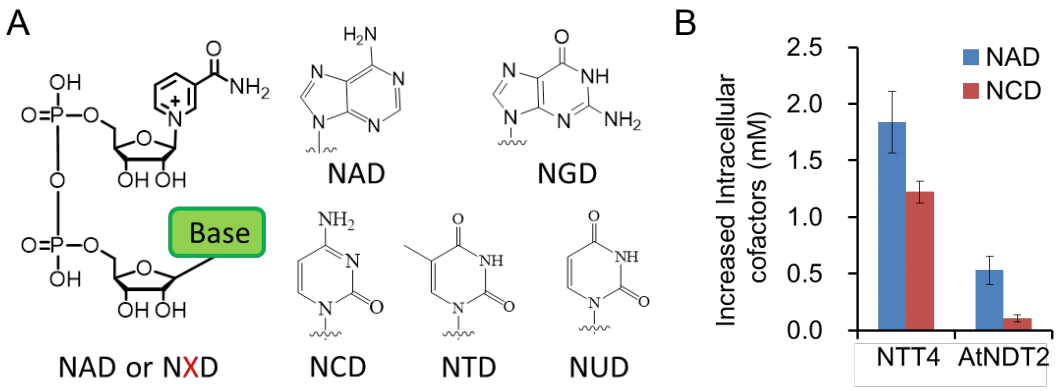


FIG 1 Identification of efficient NAD analog transporter. (A) Structure of pyridine nucleotide cofactors used for the importing assay. Nucleobases are shown separately for clarity. NAD, nicotinamide adenosine dinucleotide; NGD, nicotinamide guanine dinucleotide; NCD, nicotinamide cytosine dinucleotide; NTD, nicotinamide thymine dinucleotide; NUD, nicotinamide uracil dinucleotide. (B) Cellular cofactor level increments of *E. coli* WL023 or WL024 having NTT4 or AtNDT2 overexpressed. Cells were incubated in the presence of 0.1 mM cofactor at 30 °C for 8 h. The data represent the average standard deviations of three independent experiments.

Reducing extracellular cofactor degradation

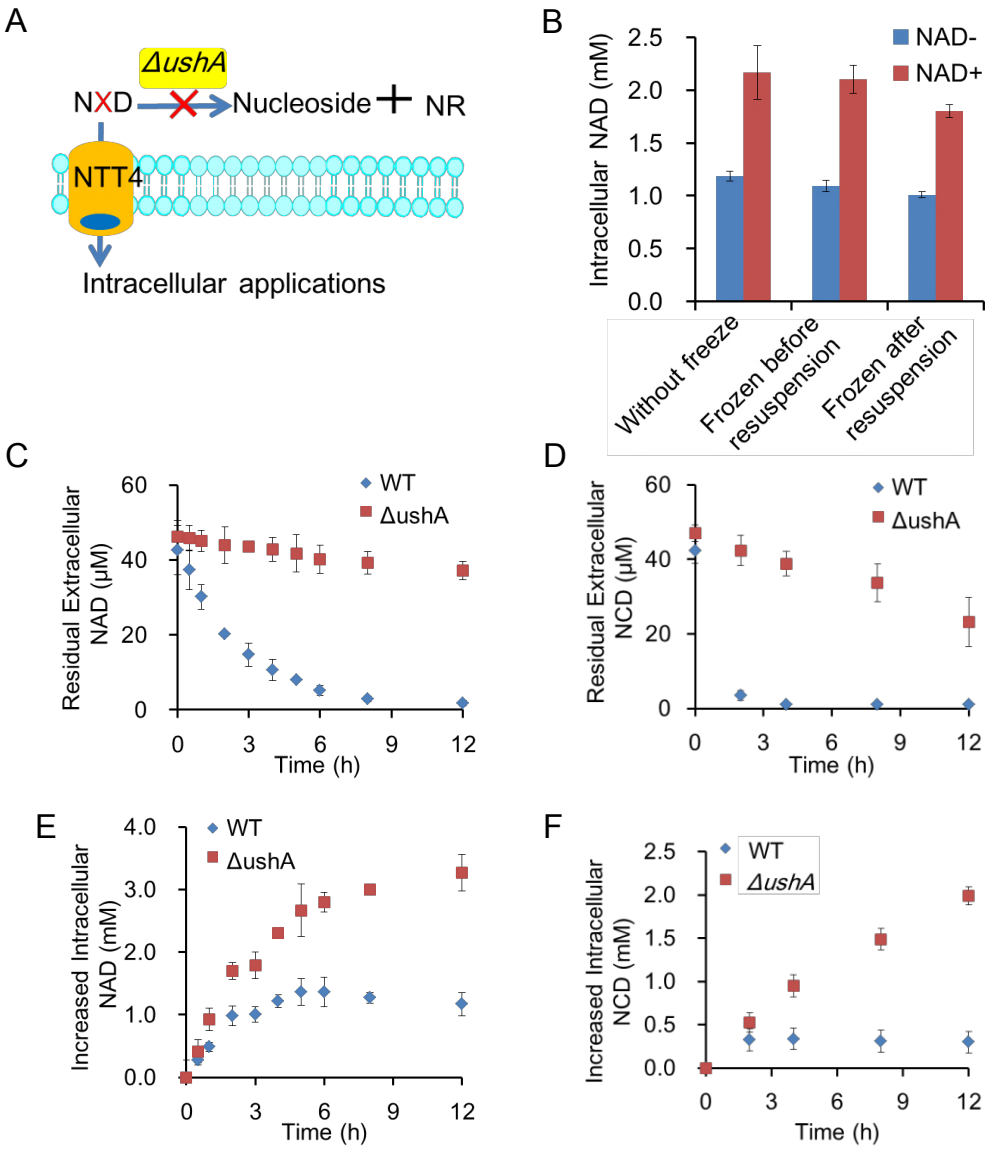
Because NAD analogs are added extracellularly for further *in vivo* utilization, their stability in the environment has to be evaluated. As UshA is a major periplasmic enzyme for NAD degradation in *E. coli*, it is expected that cofactor degradation should be significantly reduced of the *ushA* deletion mutant (Fig. 2A). As storage at −80 °C had little influence on intracellular cofactor and cofactor uptake (Fig. 2B), the frozen cells were used for investigating cofactor uptake.

Extracellular NAD and NCD stability in the presence of wild type strain cells

(YJE004) and *ushA*-deletion mutant cells (WL023) was compared by determining the time course of NAD and NCD degradation (Fig. 2C, D). When NAD and NCD were incubated with WL023 cells for 12 h, the cofactors kept stable and the residual NAD and NCD concentration kept at 37 μM and 23 μM, respectively. When incubated with YJE004 cells, NAD and NCD were almost completely degraded within 6 h and 4 h, respectively. These data demonstrated that the deletion of the *ushA* gene improved extracellular NAD stability, which should be beneficial to the efficiency of pyridine nucleotide cofactor analogs import. NAD was more stable than NCD in both cells with or without UshA, because a bigger group at nucleotide side enhanced stability of NAD (19).

Corresponding to extracellular NAD and NCD degradation, detectable import of NAD and NCD by YJE004 cells were stopped at 5 h and 2 h, respectively. Meanwhile, WL023 cells could import NAD and NCD in a continuous and efficient way (Fig. 2E, F). Variation of NADH during the importing course had little influence on total NAD(H) level, so only the levels of oxidized cofactors were considered. For NAD, YJE004 cells maximally increased the intracellular NAD concentration by 1.4 mM at 6 h, while WL023 cells increased intracellular NAD concentration by 2.8 mM at the same time, and the concentration reached to 3.3 mM at 12 h. For NCD, YJE004 cells maximally increased intracellular NCD concentration by 0.34 mM at 4 h, while WL023 cells increased intracellular NCD concentration by 0.95 mM at the same time, and the concentration reached to 2.0 mM at 12 h. Though there were residual gene(s) responsible for extracellular NAD analog degradation outside WL023 cells, the present stability of NAD analog is sufficient for continuous import.

As NCD can not be synthesized by *E. coli*, NCD was employed as an indicator of intracellular degradation activity in rest cells. The intracellular NCD decreased from 0.34 mM at 4 h to 0.30 mM at 12 h, which suggest cofactors is more stable in rest cell cytoplasm than in extracellular environment.



199 **FIG 2** Reducing extracellular cofactor degradation. (A) Reducing cofactor degradation by *ushA*
200 deletion. (B) Influence of storage at −80 °C on cofactor uptake by WL023. *E. coli* cells at an
201 initial OD_{600} of 1 in MOPS medium were prepared as followed: freshly (Without freeze), stored at
202 −80 °C for 7 days before being suspended (Frozen before resuspension) and suspended in MOPS
203 medium then stored at −80 °C for 7 days (Frozen after resuspension). Then the cells were mixed
204 with or without 50 μM of NAD for 8 h at 30 °C. (C, D) Time course of cofactor degradation by
205 YJE004 and WL023 cells. (E, F) Time course of cofactor import by YJE004 and WL023 cells.
206 YJE004, wild type; WL023, *ushA*-deletion mutant. Cells were incubated in the presence of 50 μM
207 cofactor at 30 °C. The data represent the average standard deviations of three independent
208 experiments.

209 **Apparent kinetics of cofactor transportation by NTT4**

210 The impact of cofactor stability on apparent cofactor affinity of NTT4 (Km) was

characterized (Table 1). Cofactor stability enhanced apparent affinity of cofactor transportation by NTT4 and guaranteed sustainable import. Importing time had little effect on uptake of NAD by WL023 cells, and NAD uptake for 4 h and 8 h occurred at a high apparent affinity (Km of 6 μM). With the cofactor stability decreasing, the apparent affinity became sensitive to importing time. When the uptake time of NAD by YJE004 extended from 4 h to 8 h, the apparent Km rose from 8 μM to 12 μM. As the stability of NCD was much lower than that of NAD, uptake of NCD by WL023 cells was also sensitive to importing time, and the Km rose from 23 μM to 84 μM when importing time changed from 4 h to 8 h. According to the data, cofactor stability is critical for sustainable cofactor import.

TABLE 1 Apparent kinetic parameters of NTT4 [a]

Cofactor	Strain	Time (h)	Km (μM)	V max (mM/h)
NAD [b]	YJE004 [c]	4	8.0±1.0	0.53±0.050
		8	12±2.0	0.80±0.050
	WL023 [c]	4	6.0±0. 20	0.40±0.030
		8	6.1±0. 40	1.0±0.10
NCD [b]	WL023 [c]	4	23±5.1	0.30±0.090
		8	84±17	0.63±0.070

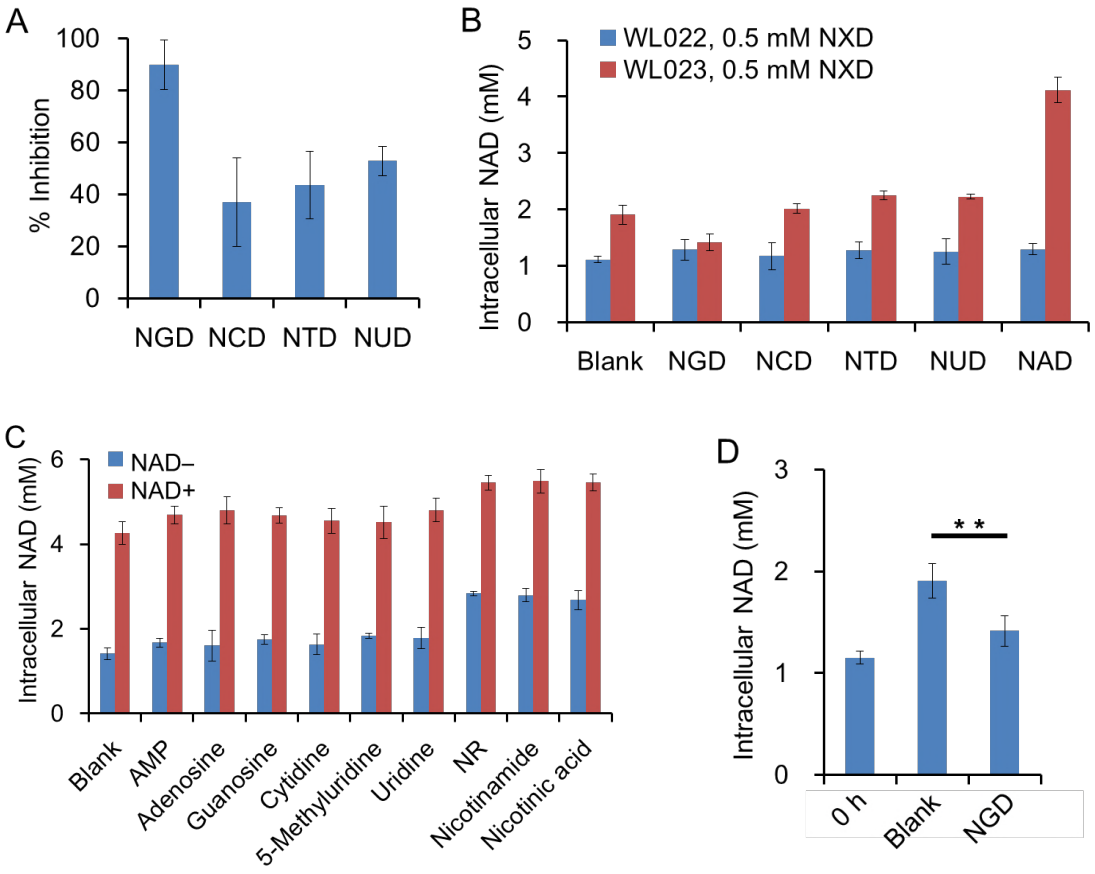
[a] Cells were incubated with varying concentrations of NAD or NCD for 4 h or 8 h at 30 °C, and then were pelleted and washed before extracting intracellular cofactor. The resulting curves were fit to the Michaelis−Menten equation to determine apparent Km and apparent Vmax values. Assays were done in triplicate and the data represent the average ± standard deviation. [b] NAD and NCD concentration ranged from 5 μM to 1000 μM. [c] YJE004, wild type; WL023, *ushA*-deletion mutant.

Characterizing NTT4 binding of NXD

The efficient cofactor importing strain WL023 may be also applicable to import of other NXDs with purine group or pyrimidine group. The binding of NXDs to NTT4 was characterized by inhibition of the uptake of NAD (Fig. 3A) (14, 25). WL023 cells were incubated with 50 μM of NAD and a 10 fold excess of the NXD. NGD potently inhibited NAD uptake (89.9% inhibition), while NCD, NTD and NUD inhibited uptake much less efficiently (37.0%, 43.6% and 52.8% inhibition, respectively). Based on this competitive inhibition data, NTT4 had broad affinity to NXDs, and preferred NXDs with purine group (NAD and NGD) to NXDs with pyrimidine group (NCD, NTD and NUD).

The impact of NXD on intracellular NAD concentration was assayed by strains with (WL023) or without (WL022) NTT4 (Fig. 3B). When supplemented with 0.5 mM of NXD, little impact on the intracellular NAD concentration of WL022 cells was detected, and the concentration varied between 1.1 mM and 1.3 mM. A possible reason is the degradation of NXDs to membranes permeable precursors was slow, and the following stimulation for NAD synthesis by passive uptake of the precursors was negligible. When supplemented 0.5 mM of NXD to WL023 cells, NCD, NTD and NUD increased intracellular NAD concentration approximately by 42%. The increase of intracellular NAD concentration might due to NR or nucleosides produced by intracellular degradation of NXD, and the hypothesis was confirmed by the impact of NXD precursors on intracellular NAD concentration (Fig. 3C). NR, nicotinamide and nicotinic acid stimulated synthesis of NAD, and increased intracellular NAD concentration approximately by 1 fold, which is consistent with the phenomenon in mice and humans (26). Meanwhile, the NAD precursors had no impact on NAD uptake (Fig. 3C), which suggested the nucleotide precursors had little impact on NAD metabolism.

When supplemented WL023 cells with 0.5 mM of NGD, the intracellular NAD concentration was lower than the sample without NGD (Fig. 3D, p-value=0.002). This suggested NGD potently inhibited intracellular NAD synthesis. Intact NGD was necessary for the inhibition of NAD synthesis, and its degradation products (guanosine and NR) did not impress NAD synthesis (Fig. 3D).



259 **FIG 3** Effects of NXD on NAD import. (A) Percent inhibition of NAD import by the NXDs.
260 WL023 cells were mixed with 500 μM of NXD and 0 or 50 μM of NAD. (B) Influence of NXD
261 import on intracellular NAD concentration. *E. coli* WL023 or WL022 with or without NTT4 were
262 mixed with 0.5 mM of NXD. (C) Impact of NXD precursors on intracellular NAD concentration
263 and uptake of NAD. WL023 cells were mixed with 500 μM of precursors and 0 or 50 μM of NAD.
264 NR, nicotinamide riboside. (D) Impact of NGD on intracellular NAD concentration and uptake of
265 NAD. Statistical significance testing was carried out by paired two-sample t-tests with Excel.
266 **p-value<0.01. WL023 cells were mixed with or without 0.5 mM of NGD. All the assays were
267 carried out at an initial OD_{600} of 1 in MOPS medium, and incubated with agitation at 30 °C for 8 h.
268 The data represent the average standard deviations of three independent experiments.

269 **Utilization of NXD by *E. coli***

270 Besides NAD and NADP, *in vivo* NCD and NGD have also been reported
271 recently (5, 13), but the physiological functions of NCD and NGD have not been
272 studied.

273 The potential of NXD to substitute some functions of NAD was determined in
274 NAD auxotrophic mutant YJE003. With proliferation of YJE003 cells, the preloaded
275 NAD was distributed into daughter cells, and the proliferation terminated until the

276 cellular NAD concentration was insufficient for supporting cell growth (22). If a
277 NXD can substitute some functions of NAD, YJE003 cells supplemented with the
278 NXD may generate more daughter cells than cells without additional cofactor
279 supplied. Though YJE003 still possessed the *ushA* gene, the importing efficiency of
280 YJE003 would be sufficient for the experimental purpose.

281 The YJE003 cells were cultivated with 50 μM of exogenous NXD at an initial
282 OD_{600} of 0.3 (Fig. 4A). The cell density of YJE003 increased to 2.5 fold in LB
283 medium without cofactor supplementation, and grew to an OD_{600} of 1.5 with NAD.
284 YJE003 cells supplied with NCD, NTD and NUD had similar cell density with the
285 control without supplement of cofactors, so the NXDs with pyrimidine group barely
286 participated in cell metabolism. The cell density of YJE003 with NGD were 0.12
287 higher than the control without cofactor (p-value=0.04). The data suggested NGD
288 could participate in some growth metabolism and substitute some functions of NAD
289 during cell proliferation. The intracellular NAD concentration of cells with an initial
290 OD_{600} of 0.3 was detected. NGD and NXDs with pyrimidine group had no
291 contribution to intracellular NAD concentration (less than 0.069 mM, Fig. 4B), while
292 the NAD supplied cells had an intracellular NAD concentration as high as 0.8 mM.

293 NXD may also influence uptake of NAD by YJE003 cells. YJE003 cells were
294 cultured with 50 μM NAD and 500 μM NCD or NGD at an initial OD_{600} of 0.3, and
295 the growth and intracellular NAD concentration was monitored (Fig. 4C, D). As
296 demonstrated above, NXD could not directly influence intracellular NAD
297 concentration of YJE003, whose NAD salvage pathway was blocked. NXD might
298 affect NAD utilization by adjusting uptake and stability of exogenously added NAD.
299 NGD inhibited the uptake of NAD, which resulted in lower cell density (p-value=0.02)
300 and intracellular NAD concentration. In contrast, NCD enhanced cell growth
301 (p-value=0.05) and utilization of NAD. As NCD is an inhibitor of NAD uptake, NCD
302 might protect NAD from being degraded by YJE003 cells. According to the residual
303 NAD in culture medium (Fig. 4E), there remained 13 μM of NAD in the medium
304 supplemented with excess 0.5 mM NCD besides 50 μM NAD. Meanwhile, the
305 cultures supplemented with NGD or without NXD kept less than 6 μM of NAD. NGD
306 was not a good protector for NAD, because the guanine group of NGD is bigger than
307 adenine, and UshA expressed by YJE003 preferred NAD to NGD.

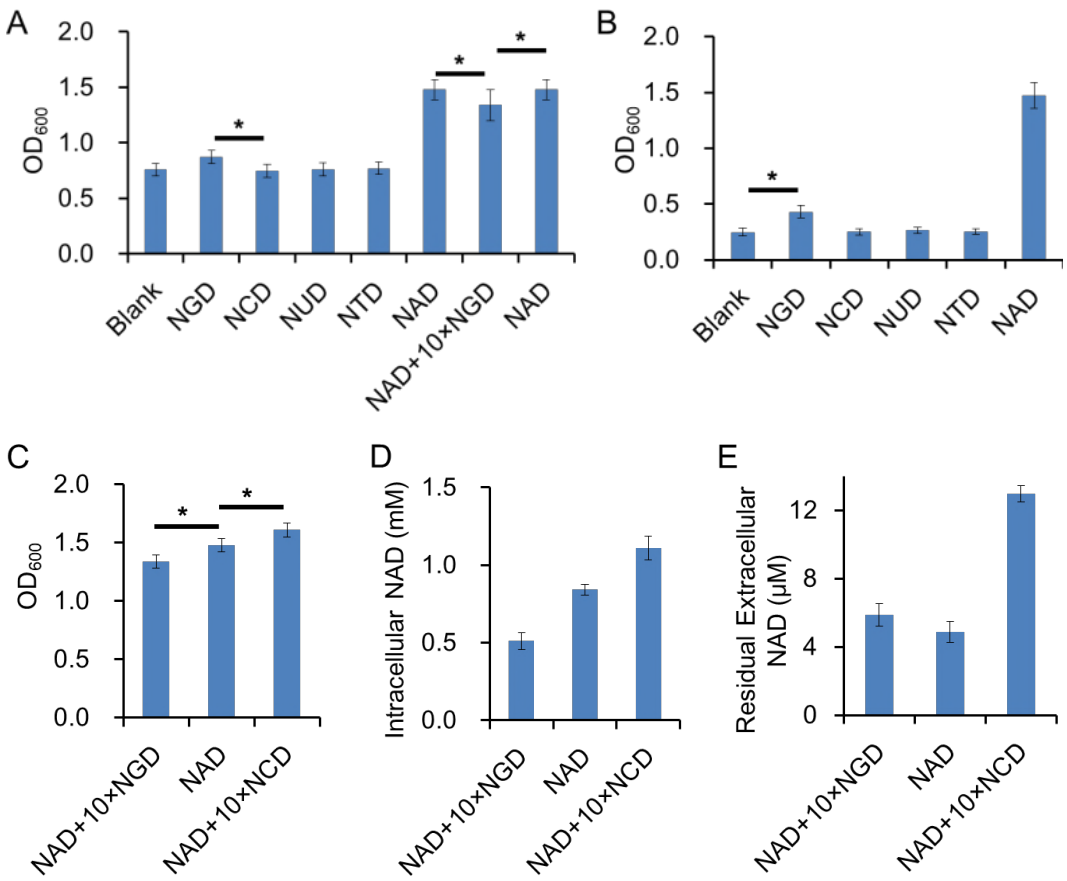


FIG 4 Effects of NXD on cell growth and NAD import of *E. coli* YJE003. A) Cell density of YJE003 cells grown for 12 h in the presence of 50 μM of NAD or NXD. Statistical significance testing was carried out by paired two-sample t-tests with Excel. *p-value<0.05. B) Intracellular NAD concentration of YJE003 incubated with 50 μM of NAD or NXD. C) Cell density of YJE003 cells grown for 12 h in the presence of 50 μM of NAD and 0.5 mM NGD or NCD. Statistical significance testing was carried out by paired two-sample t-tests with Excel. *p-value<0.05. D) Intracellular NAD concentration of YJE003 incubated with 50 μM of NAD and 0.5 mM NGD or NCD. E) Residual extracellular NAD concentration of YJE003 incubated with 50 μM of NAD and 0.5 mM NGD or NCD. YJE003 cells were incubated into LB medium with an initial OD of 0.3 and cultivated with shaking for 12 h at 30 °C. The data represent the average standard deviations of three independent experiments.

DISCUSSION

Efficient import of pyridine nucleotide cofactor analogs will enhance our ability to control *in vivo* modified pyridine nucleotide cofactor concentrations. To expand the strategy to other organisms, the optimal transporter may be varying, but NTT4 may be practical for most bacteria. Single deletion of *ushA* in *E. coli* greatly increased

stability of exogenous NXDs, and such strategy may also be practical for other organisms, for example deletion of CD37 in human cells may decrease the degradation of extracellular NAD analogs (20). Cofactor decomposition prefers cofactors with bigger group at nucleotide side (19). So NAD was more stable than NCD and excess amount of NCD could improve stability of exogenous added NAD. Meanwhile NGD with bigger nucleotide group has little improvement on NAD stability. That is, we have to design cofactor analogs with bigger group at nucleotide side for higher stability or design analogs with smaller group as protectors of target cofactor. According to *in vivo* NCD degradation, there is little pyrophosphatase activity inside cell, so efficient import of NXDs is an alternative strategy for improving NXD stability.

The NAD auxotrophic mutant YJE003 can be employed to screening different NAD analogs for studying or regulating NAD relevant metabolism. The analogs promoting proliferation of YJE003 cells should substitute some functions of NAD for cell growth, so they may have similar characters with NAD. Such analogs are candidate regulator of NAD metabolism. For example, NGD is an inhibitor of NAD synthesis, and it supplies a new tool for understanding NAD metabolism and regulating NAD-dependent reactions. As tumor cells are more dependent on the NAD salvage pathways (10), NGD may serve as anticancer therapy with low toxicity. The analogs having little effect on proliferation of YJE003 cells may have bare interference with natural systems. Such analogs may be applied to set bioorthogonal energy transfer system for metabolic engineering and may provide additional control mechanism for life. For example, NCD has been proved an excellent bioorthogonal cofactor with good biocompatibility and bioorthogonality, and it has already been used as a pathway specific energy carrier with little interference toward natural systems (5).

The efficient importing strategy can stimulate developing and screening of functional modified pyridine nucleotide cofactors, and it will facilitate the general utility of the analogs in the field of cofactor metabolism studies and synthetic biology applications.

354 # MATERIALS AND METHODS

355 ## Reagents

356 Pyridine nucleotide cofactors were chemically synthesized as previously reported
357 (4). All other reagents and enzyme substrates were from Sigma. Recombinant
358 His-tagged Mae* (L310R/Q401C mutant of *E. coli* derived malic enzyme) was
359 expressed and purified as previously reported (5, 28).

360 ## Bacterial strains and plasmids

361 The strains and plasmids used are listed in Table 2. Deletion of *ushA* was
362 performed originally from the strain JW0469 with *ushA::kan* mutant as described
363 previously (29, 30). The colonies of *E. coli* strains were cultivated for 12 h with
364 agitation at 30 °C, 200 rpm in LB broth, and appropriate antibiotics were added
365 (Kanamycin sulfate, 50 μg/mL; ampicillin, 100 μg/mL) if necessary. The cells from
366 100 mL of cultures were harvested by centrifugation at 4000 × g for 6 min at 4 °C,
367 washed twice and suspended with MOPS medium (5) to an optical density at 600 nm
368 (OD_{600}) of 20. The cells were stored at −80 °C before use.

369

370 **TABLE 2** Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Reference or source
E. coli Strains		
BW25113	*rrnB3*, Δ*lacZ4787*, *hsdR514*, Δ*(araBAD)567*, Δ*(rhaBAD)568 rph-1*	CGSC
BL21(DE3)	F–, *dcm*, *ompT*, *hsdS* (rB–, mB–), *gal*, *λ*(DE3)	Novagen
JW0469	BW25113/*ushA*:: *Kan*	[5]
WL020	BW25113/*ΔushA*	This study
YJE003	BW25113/pET15K-NTT4, *nadE::cat*	[8]
YJE004	BW25113/pBCTD-NTT4	[9]
WL022	WL020/pBCTD	This study
WL023	WL020/pBCTD-NTT4	This study
WL024	WL020/pBCTD-AtNDT2	This study
Plasmids		
pET24b–Mae*	With Mae* inserted between NdeI and XhoI sites	[1]
pBCTD	Expressing gene by gnt105p promoter	[9]
pBCTD-AtNDT2	*AtNDT2* under gnt105p promoter, *AtNDT2* expression, *kan*	[2]
pBCTD-NTT4	*ntt4* under gnt105p promoter, NTT4 expression, *kan*	[9]

371

Import of pyridine nucleotide cofactors

E. coli samples were assayed at an initial OD_{600} of 1 in MOPS medium. For each sample, cells were mixed with 100 μM or 50 μM of NXD. The mixture was incubated with agitation at 30 °C for certain time course. The cells were collected and washed with ice–cold PBS buffer and used for intracellular NAD or NCD measurements. Supernatants were quenched by adding 0.1 volume of 2 M HCl and incubated at 50 °C for 10 min, then the mixtures were neutralized by 0.1 volume of 1 M NaOH. All the samples were stored at –80 °C before NAD or NCD concentration analysis. The cell density was correlated to the intracellular volume with 0.63 mL/L equivalent to an OD_{600} of 1 (31).

Analytic methods

NAD and NCD were assayed by enzymatic cycling assays. NAD concentration was assayed by ADH as described previously [31]. NCD was assayed by Mae*, and the mixture contained 1 M Tris-Cl (pH 7.5), 0.4 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 1 mM phenazine ethosulfate, 5 mM malate, 10 mM $MgCl_2$ and 1.7 U/L Mae*. The reaction was started by mixing 10 μL of sample with 90 μL of above mixture. Reaction rates were determined by monitoring the increase of absorbance at 570 nm. One unit of enzymatic activity was defined as 1 μmol NADH or NCDH produced per minute.

Inhibition based uptake assay

E. coli samples were assayed at an initial OD of 1 in MOPS medium. For each sample, 0.5 mL of cells was mixed with NAD (50 μM) and an excess of the NXD being tested for uptake (500 μM) to a final volume of 1 mL. The mixture was incubated at 30 °C for 8 h. Then the intracellular NAD was extracted as described above. All the samples were stored at −80 °C before NAD concentration analysis.

Utilization of NXD by YJE003

The colonies of NAD auxotrophic mutant YJE003 were picked into LB medium containing 0.1 mM NAD and cultivated overnight at 37 °C, 200 rpm. Then, the cells were diluted to OD_{600} ~ 0.1 or 0.3 in LB supplemented with 50 μM NXD and

cultivated with shaking for 12 h. Then OD_{600} was measured and 1 mL samples were collected for measuring intracellular NAD. The cells were collected and washed with ice-cold PBS buffer and used for intracellular NAD measurements. 1 mL of culture supernatants were quenched by adding 0.1 mL of 2 M HCl and incubated at 50 °C for 10 min, and then the mixtures were neutralized by 0.1 mL of 1 M NaOH. All the samples were stored at −80 °C before NAD concentration analysis.

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