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ORIGINAL PAPER

Comparative genomics and functional analysis of the NiaP family uncover nicotinate transporters from bacteria, plants, and mammals

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Abstract The transporter(s) that mediate uptake of nicotinate and its *N*-methyl derivative trigonelline are not known in plants, and certain mammalian nicotinate transporters also remain unidentified. Potential candidates for these missing transporters include proteins from the ubiquitous NiaP family. In bacteria, *niaP* genes often belong to NAD-related regulons, and genetic evidence supports a role for *Bacillus subtilis* and *Acinetobacter baumannii* NiaP proteins in uptake of nicotinate or nicotinamide. Other bacterial *niaP* genes are, however, not in NAD-related regulons but cluster on the chromosome with choline-related (e.g., *Ralstonia solanacearum* and *Burkholderia xenovorans*) or thiamin-related (e.g., *Thermus thermophilus*) genes, implying that they might encode transporters for these compounds. Radiometric uptake assays using *Lactococcus lactis* cells expressing NiaP proteins showed that *B. subtilis, R. solanacearum*, and *B. xenovorans* NiaP transport nicotinate via an energy-dependent mechanism. Likewise, NiaP proteins from maize (GRMZM2G381453, GRMZM2G066801, and GRMZM2G081774), *Arabidopsis* (At3g13050), and mouse (SVOP) transported nicotinate; the *Arabidopsis* protein also transported trigonelline. In contrast, *T. thermophilus* NiaP transported only thiamin. None of the proteins tested transported choline or the thiazole and pyrimidine products of thiamin breakdown. The maize and *Arabidopsis* NiaP proteins are the first nicotinate transporters reported in plants, the *Arabidopsis* protein is the first trigonelline transporter, and mouse SVOP appears to represent a novel type of mammalian nicotinate transporter. More generally, these results indicate that specificity for nicotinate is conserved widely, but not absolutely, among pro- and eukaryotic NiaP family proteins.

Keywords Comparative genomics · Membrane transport · Nicotinate · Thiamin · Trigonelline

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Introduction

The metabolic pathways involved in synthesis and salvage of B vitamins and cofactors are well characterized in prokaryotes and eukaryotes but relatively few of the transporters involved in these pathways are known. Niacin (vitamin B3), a collective term for nicotinate and nicotinamide, and the corresponding cofactor NAD are typical in this regard. Thus, two alternative routes for de novo NAD synthesis (from aspartate or from tryptophan) are well defined at enzyme and gene levels, as are salvage pathways that reclaim nicotinate, nicotinamide, and other NAD metabolites for re-use in NAD synthesis (Sorci et al. 2010b).

While there is evidence for nicotinate uptake in bacteria (Neujahr and Varga 1966; McPheat and Wardlaw 1982; Rowe et al. 1985), fungi (Llorente and Dujon 2000), plants (Zheng et al. 2005), and mammals (Takanaga et al. 1996; Nabokina et al. 2005; Shimada et al. 2006; Said et al. 2007), only two nicotinate transporters have so far been characterized biochemically: TNA1 from yeast (Llorente and Dujon 2000; Ma et al. 2009) and SMCT1 from mammals (Gopal et al. 2005, 2007). Two lines of evidence show that mammals have at least one other nicotinate transporter. Firstly, intestine and liver take up nicotinate but do not express SMCT1; secondly, this uptake is Na^+-independent whereas SMCT1 is Na^+-dependent (Gopal et al. 2005; Nabokina et al. 2005; Said et al. 2007).

Recent comparative genomics analyses have predicted two novel families of niacin transporters. One, the NiaX family, is confined to Firmicute bacteria (Rodionov et al. 2009). The other, the NiaP family—a subgroup of the major facilitator superfamily (Pao et al. 1998)—occurs widely in eubacteria and also in animals and plants (Rodionov et al. 2008b). Analysis of *niaP* family genes in bacterial genomes showed that around half belong to regulons associated with NAD synthesis (Rodionov et al. 2008a, b). Consistent with this genomic evidence, an *Escherichia coli* strain auxotrophic for niacin showed improved growth on low concentrations of nicotinate or nicotinamide when expressing NiaP from *Bacillus subtilis* or *Acinetobacter baumannii* (Rodionov et al. 2008b; Sorci et al. 2010a), and ablating *B. subtilis niaP* decreased sensitivity to the toxic nicotinamide analog 6-aminonicotinamide (Rodionov et al. 2008b). Such evidence for niacin transport, while indirect, makes NiaP family proteins strong candidates for "missing" niacin transporters in certain bacteria and perhaps also in animals and plants.

This study combined comparative genomics predictions with experimental validation using NiaP proteins expressed in *Lactococcus lactis* cells. *L. lactis* is an efficient platform for functional expression of pro- and eukaryotic membrane proteins (Kunji et al. 2003; Frelet-Barrand et al. 2010) and a niacin auxotroph (Neves et al. 2002). We first obtained direct evidence that *B. subtilis* NiaP transports nicotinate, and explored mechanism and substrate range. We then characterized bacterial NiaP family members whose genes are not in NAD regulons. Finally, we demonstrated nicotinate transport by plant and mammalian NiaP family members.

Materials and methods

Bioinformatics

For the comparative genomic context analysis of *niaP* genes, we used bacterial genomes integrated in the SEED database (Overbeek et al. 2005). Functional coupling of genes via clustering on the chromosome and distribution of genes in the genomes were analyzed using SEED tools. The content of NadR, NrtR, and NiaR regulons was analyzed in the RegPrecise database (Novichkov et al. 2010). Bacterial, plant, and mammal NiaP sequences were obtained from GenBank and aligned with ClustalX (Thompson et al. 1997). The maximum likelihood phylogenetic tree of the NiaP family was constructed by the PHYLIP package (Felsenstein 1981) and visualized with Phylodendron software (Huson et al. 2007).

Radiochemicals

[Carboxyl-^{14}C]nicotinate (specific radioactivity 58.3 mCi/mmol) and [methyl-^{14}C]choline (55 mCi/mmol) were obtained from Amersham Biosciences. [^{14}C]Trigonelline (*N*-methylnicotinate) (58.3 mCi/mmol) was synthesized from [^{14}C]nicotinate according to Sarett et al. (1940) and purified by ion exchange (Hitz and Hanson 1980). [^{3}H(G)] Thiamin (10 Ci/mmol) was obtained from American Radiolabeled Chemicals and hydrolyzed to 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 5-(2-hydroxyethyl)-4-methylthiazole (thiazole) using recombinant thiaminase II (TenA) from *B. subtilis* (Fig. S1) (Toms et al. 2005). Substrates used in transport assays were at the specific activities above.

Bacterial strain and medium

L. lactis strain NZ9000 was used as an expression system for transport activity measurements (Kuipers et al. 1998). Cells were grown statically at 30°C in M17 medium (Oxoid) supplemented with 1% (*w/v*) glucose and, when cells carried pNZ8048 constructs, 5 μg/ml chloramphenicol.

Expression constructs

Sequences encoding NiaP family proteins were amplified from genomic DNA for *B. subtilis*, *Burkholderia xenovorans*, *Ralstonia solanacearum*, and *Thermus thermophilus* and from cDNAs for mouse (*Mus musculus*) SVOP, *Arabidopsis thaliana* At3g13050, and maize (*Zea mays*) GRMZM2G381453, GRMZM066801, and GRMZM081774. The cDNAs came from Open Biosystems (mouse), the *Arabidopsis* Biological Resource Center (*Arabidopsis*), or the Arizona Genomics Institute (maize). The primers are listed in Table S1. The amplicons were cloned using appropriate restriction enzymes into the nisin-regulated expression vector pNZ8048 (Kuipers et al. 1998). All constructs were sequenced. Plasmid DNA was prepared with the Wizard® Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's protocol except that the resuspension buffer

contained 10 mg/ml lyzozyme, resuspended cells were incubated for 10 min at 55°C to partly digest cell walls, and the column was washed with 40% (*v/v*) isopropanol containing 4.2 M guanidine.HCl before the wash buffer step.

Radiometric uptake assays

L. lactis cells transformed with the pNZ8048 vector alone or harboring a *niaP* gene were inoculated at an OD_{600} of 0.05 in the medium described above. When cultures reached an OD_{600} of 0.6 (about 3 h), *nia*P expression was induced by adding 0.1% (*v/v*) of a culture supernatant of the nisin A-producing strain NZ9700 to the medium. After 16 h of induction, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in this buffer at an OD_{600} of 20. Uptake assays were made at 30°C with magnetic stirring. Cells (500 µl) were preincubated with glucose or 2-deoxyglucose (25 mM final concentration) as specified in the figure legends. Assays were started by adding 500 µl of PBS containing 1.7 µM [^{14}C]nicotinate, 1.7 µM [^{14}C]trigonelline, 1.7 µM [^{14}C]choline, or 10 nM [^{3}H] thiamin (or the equivalent amount of [^{3}H]thiamin breakdown products). Samples (150 µl) were withdrawn at intervals and passed through a 0.45 µm cellulose nitrate membrane filter (Whatman), which was washed twice with 2 ml of ice-cold PBS. The radioactivity retained on filters was determined by liquid scintillation counting. Uptake data were expressed relative to the total protein content (determined with the Pierce BCA assay kit) of the 1-ml reaction mixture. The protein contents of *L. lactis* cells harboring pNZ8048 alone or containing various *niaP* coding sequences were not significantly different, with a mean value of 4.49± 0.11 mg/ml for an OD_{600} of 20. To estimate intracellular [^{14}C]nicotinate concentration, the plateau value uptake at 120 s (in picomoles per milligram of protein) of cells expressing *B. subtilis* NiaP was divided by the specific internal volume coefficient (i.e., cytoplasmic volume) of 3.61 µl/mg protein determined for *L. lactis* (Poolman et al. 1987).

Subcellular localization

To fuse *Arabidopsis* NiaP (*At*NiaP) to the C terminus of green fluorescent protein (GFP), the *AtNiaP* ORF was amplified using primers 5′-GGACTAGTATGGCAGATGG GAACACAAGATTC-3′ and 5′-GCCTCGAGCTATAC GGAGGCTGAAGGTGGTTC-3′, and the *Spe*I-*Xho*I fragment was cloned in the corresponding sites of pUC-GFP5-Sp (Komarova and Rentsch, unpublished data). The construct was verified by sequencing. Transient expression in tobacco (cv. SRI) leaf protoplasts was performed as described (Dietrich et al. 2004; Weichert and Rentsch, unpublished data). The excitation wavelength for GFP and FM4-64 was 488 nm. GFP emission was detected at 492–511 nm and FM4-64 emission was detected at 600 nm. Chlorophyll autofluorescence was detected at 603–752 nm.

Results

The NiaP protein family

NiaP proteins occur in prokaryotes, plants, and animals (Fig. S2). The *niaP* genes in *B. subtilis* and many other bacteria belong to NAD metabolism regulons (Rodionov et al. 2008b) and/or cluster on the chromosome with genes for NAD metabolism or NAD-associated transcription factors (Fig. 1a). As chromosomal gene clustering and co-regulation by common transcription factors are strong predictors of gene function (Galperin and Koonin 2000; Osterman and Overbeek 2003; Rodionov 2007), such *niaP* genes are predicted to encode transporters for the NAD precursors nicotinate or nicotinamide (Rodionov et al. 2008b). In bacteria whose *niaP* genes lack NAD associations, genomic context evidence points to other transport substrates. Thus, the *niaP* genes of *R. solanacearum, B. xenovorans*, and *B. multivorans* are clustered with genes of choline catabolism, *betA* (choline dehydrogenase), *betB* (betaine aldehyde dehydrogenase), and *soxABDG* (sarcosine oxidase) (Fig. 1a). These arrangements suggest possible choline transport. Similarly, the *T. thermophilus niaP* gene is embedded in a predicted thiamin synthesis operon (Fig. 1a). This clustering pattern suggests transport of thiamin or a thiamin precursor.

The mammalian NiaP family protein, SVOP, is located in synaptic vesicles (Janz et al. 1998); it is reported to bind nucleotides but its function is not known (Yao and Bajjalieh 2009). One possibility is transport of choline or acetylcholine, since both are metabolized in synaptic vesicles; transporters for these compounds are, however, already known (Prado et al. 2002; Ferguson and Blakely 2004). Plant NiaP proteins are of unknown function and subcellular location; *Arabidopsis* has one and maize has three (Fig. S2).

NiaP proteins from *B. subtilis*, *R. solanacearum*, *B. xenovorans*, *T. thermophilus*, mouse, *Arabidopsis*, and maize were expressed in *L. lactis* and tested for transport of nicotinate, choline, and thiamin. Trigonelline, a plant nicotinate metabolite, and the cleavage products/precursors of thiamin, HMP and thiazole, were also tested, the latter together. Fig. 1b shows the structures of these compounds.

Uptake experiments confirm nicotinate transport by *B. subtilis* NiaP

L. lactis cells expressing *B. subtilis* NiaP showed rapid [^{14}C]nicotinate uptake that reached a plateau after 60 s. This value was >20 times higher than that in control cells

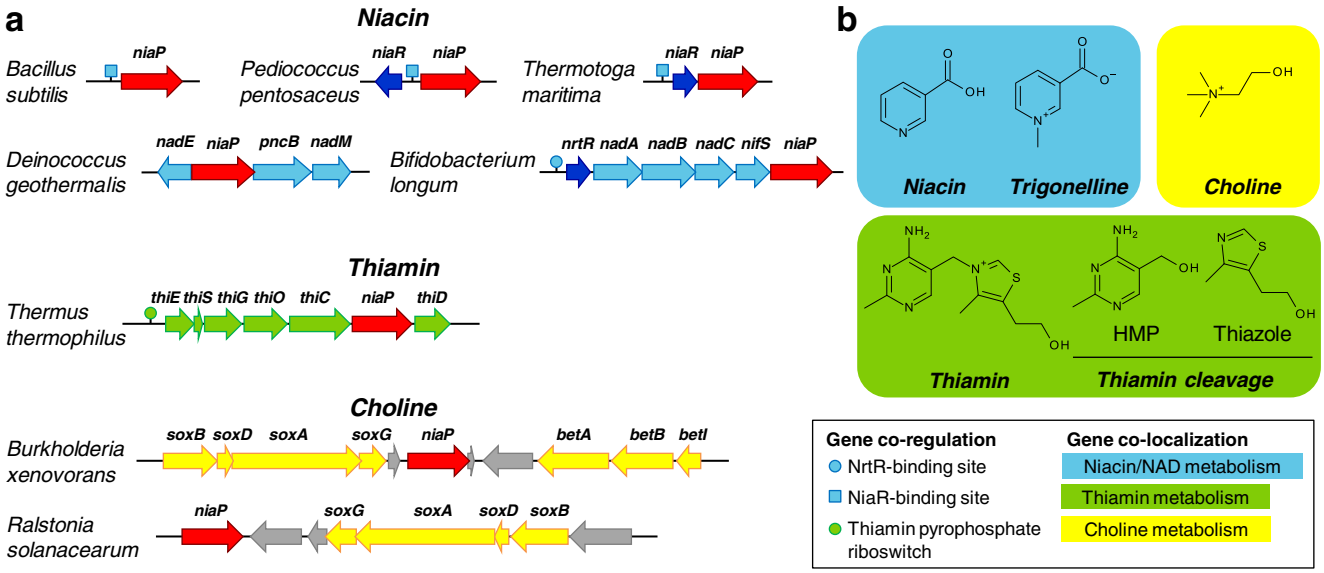

Fig. 1 Genomic context of *niaP* genes in representative bacteria, and potential substrates of the corresponding NiaP proteins. **a** Genomic context analysis. Genes are represented by *arrows* colored in *red* for *niaP* genes, in *blue*, *green*, or *yellow* for genes related to metabolism of niacin, thiamin, or choline, respectively, and in *gray* for unrelated genes. *Blue circle* or *squares* indicate the position of known or predicted NrtR- or NiaR-binding sites, respectively. The *green circle* indicates the position of a predicted thiamin pyrophosphate riboswitch. **b** Structures of potential substrates, with *background colors* matching (**a**)

transformed with the expression vector alone (Fig. 2a, b). The transport was concentrative (38-fold) since the medium contained 1.7 μM [^{14}C]nicotinate and the plateau uptake value (233±31 pmol/mg protein) corresponds to an intracellular concentration of 65 μM (based on a cytoplasmic volume of 3.61 μl/mg protein; Poolman et al. 1987). Consistent with accumulation against a concentration gradient, the uptake process was energy-dependent; [^{14}C]nicotinate uptake was stimulated by glucose and inhibited by 2-deoxyglucose, and this inhibition was overcome by glucose (Fig. 2b). The accumulation of [^{14}C]nicotinate was reversed by adding a 30-fold excess of unlabeled nicotinate, the initial rate of label efflux being similar to the initial rate of influx (Fig. 2a (inset)). These data are consistent with a bidirectional transport mechanism, and also indicate that little metabolism of [^{14}C]nicotinate occurred in the short time frame (60–120 s) of the assay.

No uptake occurred when radiolabeled choline, thiamin, thiamin breakdown products, or trigonelline replaced [^{14}C]nicotinate (not shown). As trigonelline differs from nicotinate only in an *N*-methyl substituent that confers a positive charge on the ring (Fig. 1b), the lack of trigonelline uptake implies a high degree of substrate specificity. Also consistent with discrimination among similar structures, adding unlabeled nicotinamide, nicotinamide mononucleotide, NAD, or NADP in 120- or 1,200-fold excess had little or no effect on [^{14}C]nicotinate uptake rate although, as expected, excess unlabeled nicotinic acid strongly reduced it (Fig. 2c). That nicotinamide had no effect is at first sight surprising, as expressing NiaP in an *E. coli* niacin auxotroph increased growth at a limiting concentration of nicotinamide (Rodionov et al. 2008b). However, our competition experiments with unlabeled compounds only point towards substrate preferences relative to nicotinate, and do not exclude a low capacity for nicotinamide transport. The depression of [^{14}C]nicotinate uptake by a 1,200-fold excess of nicotinic acid mononucleotide (Fig. 2c) may reflect its extracellular hydrolysis to nicotinate.

Testing of predictions for other bacterial NiaP proteins

Although genomic context evidence (Fig. 1a) suggests that the NiaP proteins of *R. solanacearum* and *B. xenovorans* might transport choline, no [^{14}C]choline transport activity was detected in *L. lactis* cells expressing either protein (data not shown). However, [^{14}C]nicotinate transport was readily detected for both proteins (Fig. 3a). As with *B. subtilis* NiaP, *R. solanacearum* NiaP-mediated [^{14}C]nicotinate uptake was inhibited by 2-deoxyglucose and the inhibition was reversed by glucose (Fig. 3a (inset)). Also as with *B. subtilis* NiaP, trigonelline and thiamin were not taken up and nor were thiamin breakdown products (data not shown).

T. thermophilus NiaP was predicted to transport thiamin or its breakdown products (Fig. 1a). *L. lactis* has a thiamin transporter of the ECF family (Rodionov et al. 2009) and cells transformed with vector alone showed relatively high basal [^{3}H]thiamin uptake (Fig. 3b); this uptake was not suppressible by growth on medium containing a high thiamin level (59 μM). Notwithstanding the high basal uptake, significantly increased (P<0.05) [^{3}H]thiamin uptake

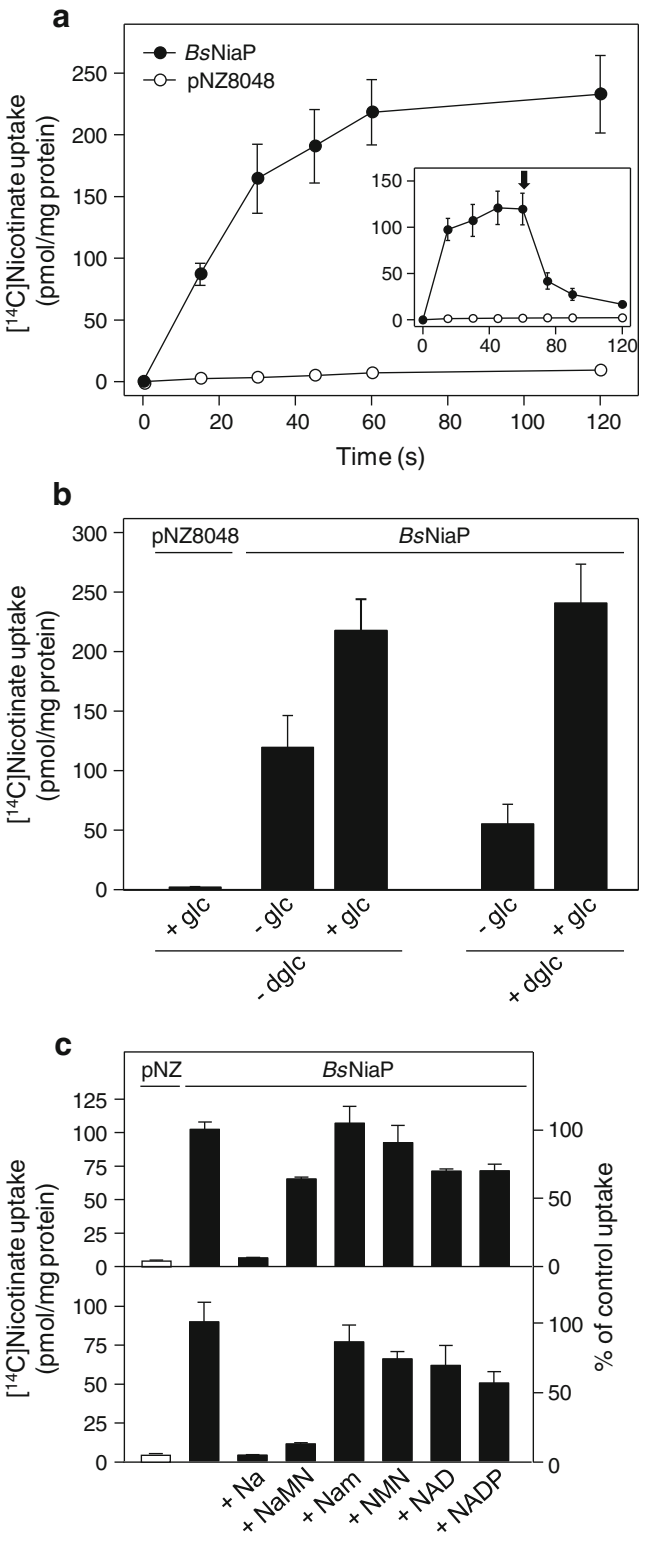

Fig. 2 Transport of [^{14}C]nicotinate by *Bacillus subtilis* NiaP expressed in *L. lactis*. **a** Time-course of [^{14}C]nicotinate uptake by cells harboring the pNZ8048 vector alone or expressing *B. subtilis* NiaP (*Bs*NiaP). Uptake assays used 1.7 μM [^{14}C]nicotinate; cells were preincubated with 25 mM glucose for 5 min. *Inset* shows the effect of adding 50 μM unlabeled nicotinate at 60 s (*arrow*). *Symbols* and *axes* are as in the main panel. **b** [^{14}C]Nicotinate uptake at 60 s in cells carrying pNZ8048 alone or expressing *Bs*NiaP. Cells were preincubated without or with 25 mM deoxyglucose (*dglc*) for 30 min and then without or with 25 mM glucose (*glc*) for 5 min. **c** Initial rate of [^{14}C] nicotinate uptake (measured at 15 s) in the presence of 200 μM (*upper panel*) or 2 mM (*lower panel*) of unlabeled compounds: *Na* nicotinate, *NaMN* nicotinic acid mononucleotide, *Nam* nicotinamide, *NMN* nicotinamide mononucleotide, *NAD* β-NAD, *NADP* β-NADP. Cells were preincubated with 25 mM glucose for 5 min. The right-hand axis shows uptake as a percentage of that in the control without unlabeled compounds. Data are means and SE from three replicates

was detected in cells expressing *T. thermophilus* NiaP when substrate concentration was 10 nM (Fig. 3b) or 1.7 μM (not shown). To test whether thiamin breakdown products are substrates, HMP and thiazole were produced by hydrolyzing [^{3}H]thiamin with *B. subtilis* TenA (Fig. S1). No label uptake was detected from the HMP/thiazole mixture (data not shown). Nor was uptake of trigonelline or choline detected.

Testing of mammalian and plant NiaP proteins

In conformity with expectation, *L. lactis* cells expressing mouse SVOP did not take up [^{14}C]choline (data not shown) but showed readily detectable [^{14}C]nicotinate uptake (Fig. 3c). As with bacterial NiaP proteins, label uptake via SVOP was glucose-dependent (Fig. 3c (inset)). No transport activity was found with trigonelline, thiamin, or its breakdown products (data not shown).

All four plant NiaP proteins tested (*Arabidopsis* At3g13050 and maize GRMZM2G381453, GRMZM2G066801, and GRMZM2G081774) conferred the ability to take up [^{14}C]nicotinate, albeit at lower rates than bacterial proteins, and with an initial lag in the cases of the maize proteins (Fig. 4a, b). As with the bacterial proteins, *Arabidopsis* NiaP-mediated [^{14}C]nicotinate uptake was glucose-dependent (Fig. 4a (inset)). No uptake of choline, thiamin, or thiamin breakdown products was detected for any plant protein.

Surprisingly, the *Arabidopsis* protein transported [^{14}C] trigonelline at a rate comparable to [^{14}C]nicotinate (Fig. 5a), but no such transport was detected with any of the maize proteins (not shown). The transport of trigonelline was glucose-dependent (Fig. 5a (inset)). Adding a 30-fold excess of unlabeled trigonelline stopped label accumulation but did not reverse it (Fig. 5b). This is unlike the pattern seen for [^{14}C]nicotinate (Fig. 2a (inset)), but as these molecules differ in net charge at physiological pH (trigonelline is neutral whereas nicotinate is an anion), differences in their influx/efflux behavior might be anticipated.

Besides being a nicotinate analog, trigonelline is a betaine, i.e., it has a quaternary ammonium group and a carboxyl group (Rhodes and Hanson 1993). We therefore tested two common plant betaines (glycine betaine and

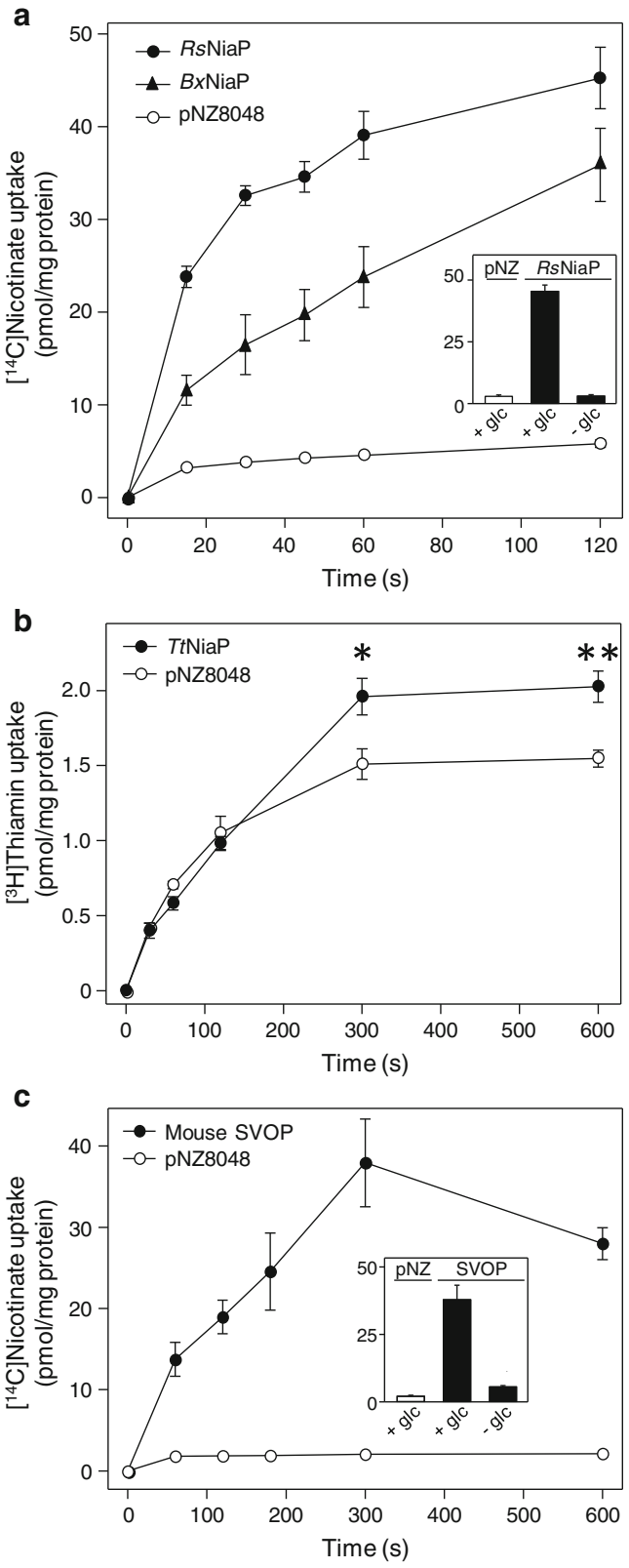

Fig. 3 Transport activities of bacterial and mammalian NiaP proteins expressed in *L. lactis*. **a** Uptake of 1.7 μM [^{14}C]nicotinate by cells harboring pNZ8048 alone or expressing *R. solanacearum* NiaP (*Rs*NiaP) or *B. xenovorans* NiaP (*Bx*NiaP); cells were preincubated with 25 mM glucose for 5 min. *Inset* shows uptake at 60 s in cells carrying pNZ8048 alone (*pNZ*) or expressing *Rs*NiaP. Cells were preincubated in 25 mM deoxyglucose for 30 min and then without or with 25 mM glucose (*glc*) for 5 min. **b** Uptake of 10 nM [^{3}H]thiamin by cells harboring pNZ8048 alone or expressing *T. thermophilus* NiaP (*Tt*NiaP); cells were preincubated with 25 mM glucose for 5 min. **P*<0.05; ***P*<0.02, statistically significant differences. **c** Uptake of 1.7 μM [^{14}C]nicotinate by cells harboring pNZ8048 alone or expressing mouse SVOP; cells were preincubated with 25 mM glucose for 5 min. *Inset* shows uptake at 300 s in cells carrying pNZ8048 alone or expressing SVOP. Cells were preincubated in 25 mM deoxyglucose for 30 min and then without or with 25 mM glucose for 5 min. Data are means and SE from three replicates

proline betaine) as well as nicotinate and related compounds for ability to reduce [^{14}C]trigonelline uptake. In 120-fold excess (Fig. 5c (upper panel)), only trigonelline itself reduced label uptake, indicating that *Arabidopsis NiaP* is not a general betaine carrier, that it prefers trigonelline to nicotinate as substrate, and that it has little or no capacity to transport nicotinamide or nicotinamide mononucleotide. In 1,200-fold excess (Fig. 5c (lower panel)), the two betaines reduced label uptake substantially and similarly to nicotinate, suggesting that they are either poor transport substrates or inhibitors. Nicotinamide and nicotinamide mononucleotide only modestly depressed [^{14}C]trigonelline uptake, confirming that they are likely not physiological substrates.

The subcellular localization of the *Arabidopsis* NiaP protein was investigated by transient expression of a GFP fusion protein in tobacco protoplasts. Confocal analyses indicated that 15% of protoplasts showed GFP fluorescence at the plasma membrane, as evidenced by the fluorescence overlap of GFP and the plasma membrane marker FM4-64, although fluorescence was predominantly localized in internal membranes (Fig. S3).

Discussion

The NiaP proteins from *B. subtilis* and *A. baumannii* were previously shown by genetic approaches to transport nicotinamide and/or nicotinate (Rodionov et al. 2008b; Sorci et al. 2010a). Here, we present biochemical evidence that *B. subtilis* NiaP is indeed a nicotinate transporter. While our results do not establish a transport mechanism, they are consistent with the possibility that *B. subtilis* NiaP is a secondary transporter that utilizes the electrochemical proton gradient (inside negative and alkaline) to drive nicotinate uptake via H^+ symport (Konings et al. 1989). Such a mechanism is common among members of the major facilitator superfamily (Pao et al. 1998).

Like the NiaP protein of *B. subtilis*, those of *B. xenovorans* and *R. solanacearum* were found to transport nicotinate but not the other labeled substrates tested. That their transport

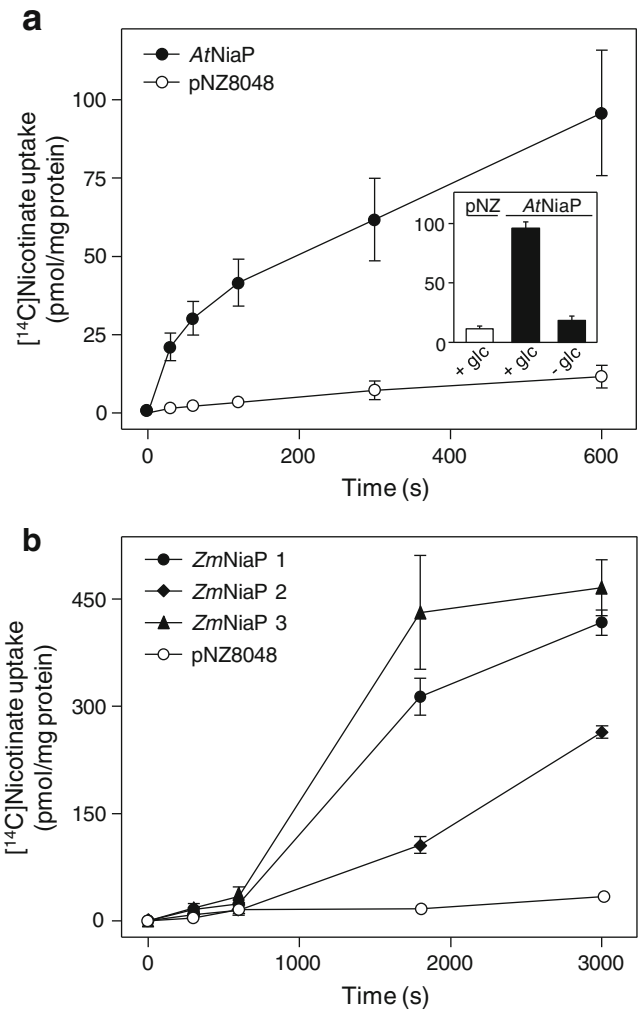

Fig. 4 Transport of [^{14}C]nicotinate by *Arabidopsis* and maize NiaP proteins expressed in *L. lactis*. **a** Uptake of 1.7 μM [^{14}C]nicotinate by cells harboring pNZ8048 alone or expressing *Arabidopsis* NiaP (*At*NiaP); cells were preincubated with 25 mM glucose for 5 min. *Inset* shows uptake at 600 s in cells carrying pNZ8048 alone (*pNZ*) or expressing *At*NiaP. Cells were preincubated with 25 mM deoxyglucose for 30 min and then without or with 25 mM glucose (*glc*) for 5 min. **b** Uptake of 1.7 μM [^{14}C]nicotinate by cells expressing a maize NiaP protein (*Zm*NiaP 1=GRMZM2G381453, *Zm*NiaP 2=GRMZM2G081774, and *Zm*NiaP 3=GRMZM2G066801); cells were preincubated with 25 mM glucose for 5 min. Data are means and SE from three replicates

rates were lower than *B. subtilis* NiaP may reflect levels of expression of transport-active proteins in the *L. lactis* host, to which *B. subtilis* is taxonomically close but *B. xenovorans* and *R. solanacearum* are not. The lack of choline transport did not bear out the prediction based on gene clustering (Fig. 1a). However, the *niaP* genes in *B. xenovorans* and *R. solanacearum* are neighbors of choline-related genes, but not in operons with them, making the prediction of functional coupling of *niaP* and choline genes a relatively weak one.

The prediction of a thiamin-related function for *T. thermophilus* NiaP was stronger because the *niaP* gene is embedded in an operonic structure containing six thiamin-related genes that is downstream from a thiamin pyrophosphate riboswitch (Mironov et al. 2002; Winkler et al. 2002). Consistent with this prediction, the only transport activity detected was for thiamin. Although the activity was partially masked by endogenous thiamin uptake, it was observed consistently. That the amount rather than the rate of uptake was increased by NiaP may reflect mechanistic differences between NiaP—a secondary transporter, see above—and the endogenous activity, which is most probably due to an ECF family ATP-driven primary transporter (Rodionov et al. 2009).

Our data show that the mammalian NiaP family protein, SVOP, can transport nicotinate. SVOP proteins are specifically expressed in the central nervous system, in synaptic vesicles (Janz et al. 1998; Cho et al. 2009). It is interesting in this connection that synaptic vesicles release NAD, and that NAD may function as a neurotransmitter (Breen et al. 2006; Yamboliev et al. 2009).

Although plant cells have long been known to take up nicotinate and trigonelline (Heeger et al. 1976; Zheng et al. 2005), no transporter for nicotinate has (to our knowledge) been reported, and there is only indirect evidence for trigonelline transport via proline/compatible solute transporters (Breitkreuz et al. 1999). The *Arabidopsis* and maize NiaP proteins thus appear to be the first nicotinate transporters identified in plants, and the *Arabidopsis* protein the first trigonelline transporter. The GFP fusion results indicate that plant NiaP is more likely a plasmalemma-localized than an organellar transporter, and so may contribute to the reported cellular uptake. The high capacity for trigonelline transport by the *Arabidopsis* protein but not the maize proteins might seem remarkable. However, *Arabidopsis* NiaP shares only 52–62% amino acid identity with the maize proteins, so that there is more than enough structural divergence to account for functional divergence. The physiological significance of this functional divergence is not clear. It is not simply related to the occurrence of trigonelline, which is a natural metabolite in both maize and *Arabidopsis*, as well as many other higher plants (Rhodes et al. 1989; Allen et al. 2010).

A final, striking aspect of our data is the conservation of function within the NiaP family (Fig. 6). It is not very common for substrate specificity to be retained among members of a family from organisms as diverse as bacteria, animals, and plants since substrate specificity appears to be readily altered during evolutionary history (Saier 2000). The conservation of function in this case is not absolute, however, as attested by the addition of trigonelline to the substrate range of *Arabidopsis* NiaP, and also by a shift to specificity for thiamin in *T. thermophilus* (Fig. 6). A parallel situation exists in the folate-biopterin transporter (FBT) family, which—like NiaP—belongs to the major

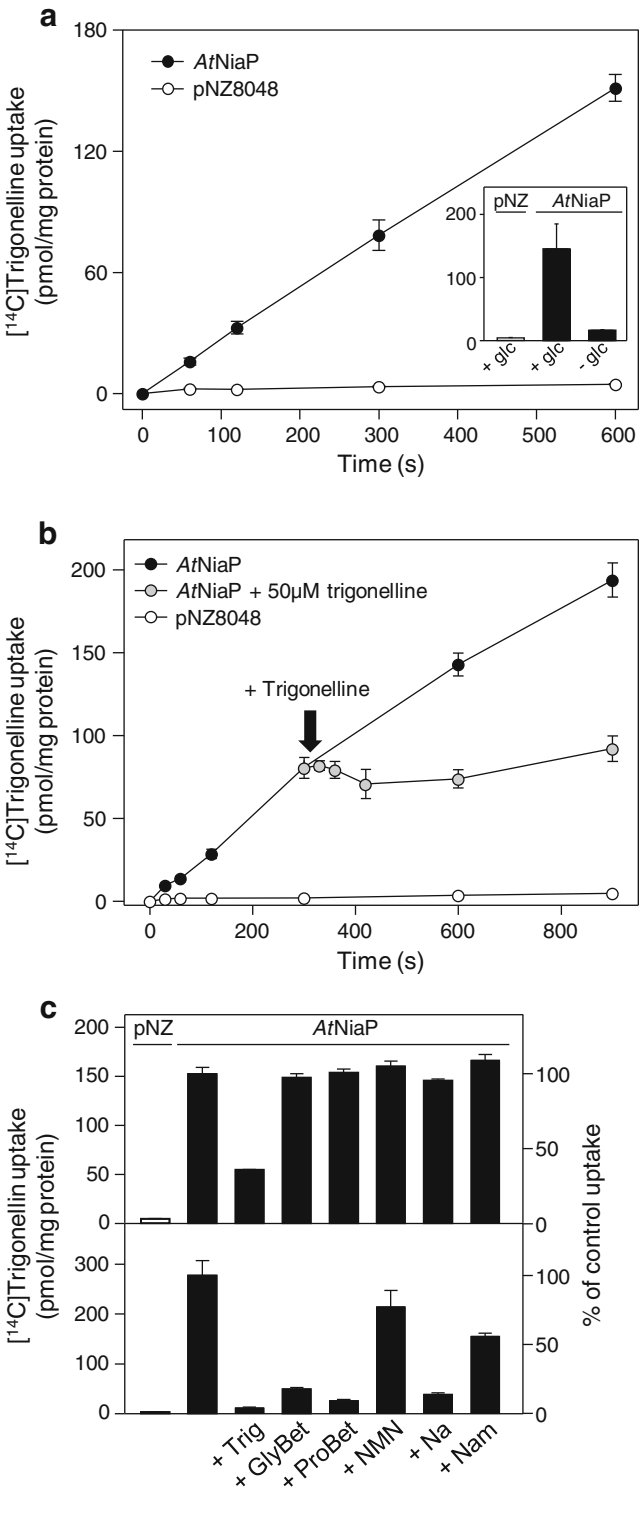

Fig. 5 Transport of [^{14}C]trigonelline by *Arabidopsis* NiaP expressed in *L. lactis*. **a** Uptake of 1.7 µM [^{14}C]trigonelline by cells harboring pNZ8048 alone or expressing *Arabidopsis* NiaP (*At*NiaP); cells were preincubated with 25 mM glucose for 5 min. *Inset* shows uptake at 600 s in cells carrying pNZ8048 alone or expressing *At*NiaP. Cells were preincubated in 25 mM deoxyglucose for 30 min and then without or with 25 mM glucose (*glc*) for 5 min. **b** Effect of adding 50 µM unlabeled trigonelline at 300 s (*arrow*) on the time-course of uptake of 1.7 µM [^{14}C]trigonelline by cells preincubated with 25 mM glucose for 5 min. **c** Initial rate of [^{14}C]trigonelline uptake (measured at 600 s) in presence of 200 µM (*upper panel*) or 2 mM (*lower panel*) of unlabeled compounds: *Trig* trigonelline, *GlyBet* glycine betaine, *ProBet* proline betaine, *NMN* nicotinamide mononucleotide, *Na* nicotinate, *Nam* nicotinamide. Data are means and SE from three replicates

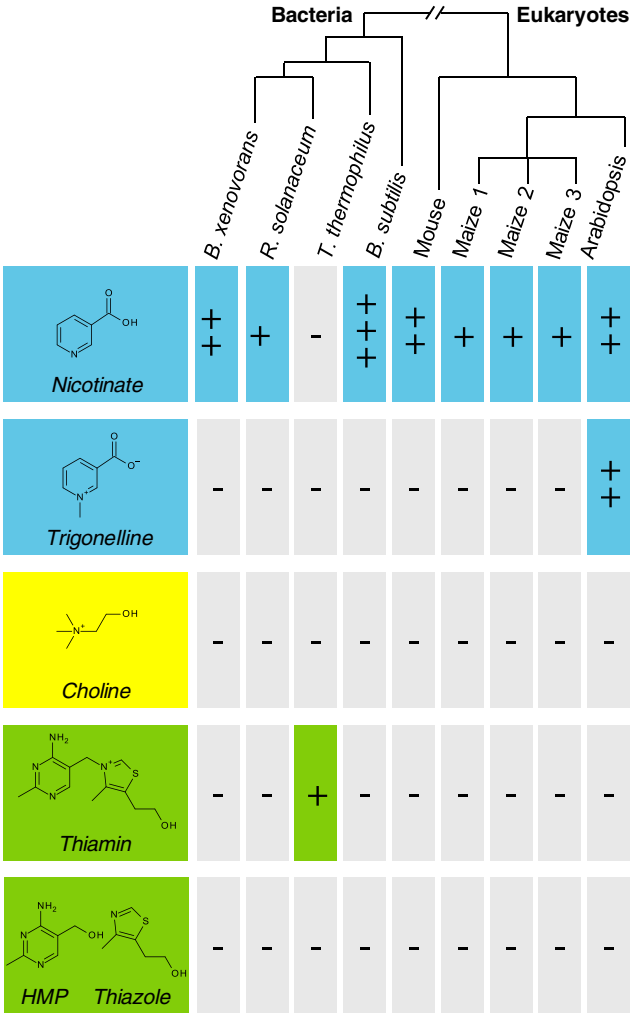

Fig. 6 Summary of the experimental results for NiaP family transporters. Substrate structures are shown on the left. An organismal phylogeny based on Ciccarelli et al. (2006) is shown at the top. The number of *plus symbols* (one to three) indicates the level of transport activity; *minus symbols* denote absence of detectable transport. *Background colors* are as in Fig. 1

facilitator superfamily. Various bacterial, plant, and protistan FBT family members show conserved specificity for unconjugated or conjugated pterins (Klaus et al. 2005), but one FBT protein from the protist *Leishmania* is specific for *S*-adenosylmethionine (Dridi et al. 2010) and certain plant

FBT proteins most probably do not act on pterins (Eudes et al. 2010).

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