Uptake of L-nicotine and of 6-hydroxy-L-nicotine by *Arthrobacter nicotinovorans* and by *Escherichia coli* is mediated by facilitated diffusion and not by passive diffusion or active transport

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The mechanism by which L-nicotine is taken up by bacteria that are able to grow on it is unknown. Nicotine degradation by *Arthrobacter nicotinovorans*, a Gram-positive soil bacterium, is linked to the presence of the catabolic megaplasmid pAO1. L-[¹⁴C]Nicotine uptake assays with *A. nicotinovorans* showed transport of nicotine across the cell membrane to be energy-independent and saturable with a K_m of $6.2 \pm 0.1 \mu\text{M}$ and a $V_{\max}$ of $0.70 \pm 0.08 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. This is in accord with a mechanism of facilitated diffusion, driven by the nicotine concentration gradient. Nicotine uptake was coupled to its intracellular degradation, and an *A. nicotinovorans* strain unable to degrade nicotine (pAO1[−]) showed no nicotine import. However, when the nicotine dehydrogenase genes were expressed in this strain, import of L-[¹⁴C]nicotine took place. *A. nicotinovorans* pAO1[−] and *Escherichia coli* were also unable to import 6-hydroxy-L-nicotine, but expression of the 6-hydroxy-L-nicotine oxidase gene allowed both bacteria to take up this compound. L-Nicotine uptake was inhibited by D-nicotine, 6-hydroxy-L-nicotine and 2-amino-L-nicotine, which may indicate transport of these nicotine derivatives by a common permease. Attempts to correlate nicotine uptake with pAO1 genes possessing similarity to amino acid transporters failed. In contrast to the situation at the blood–brain barrier, nicotine transport across the cell membrane by these bacteria was not by passive diffusion or active transport but by facilitated diffusion.

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INTRODUCTION

L-Nicotine (L-3-pyridyl-*N*-methylpyrrolidine), the main alkaloid synthesized by the tobacco plant, is an *N*-heterocyclic aromatic compound consisting of a pyridine and an *N*-methylated pyrrolidine ring (see left insert of Fig. 1a). The pyrrolidine nitrogen confers the basic character to the molecule, with a pK_a of 11. Nicotine is an amphiphilic molecule soluble in organic media and in water. Nicotine consumption leads to addiction by stimulating nicotinic acetylcholine receptors in the brains of smokers and by increasing dopamine release, which activates the self-reward system.

Nicotine inhaled with the tobacco smoke enters the bloodstream through the lung epithelium and can be located within seconds in the brain. Apparently, it easily crosses the blood–brain barrier without the help of any specific transport system (Crooks *et al.*, 1997; Lockman

et al., 2005). Accordingly, the only mechanism considered for nicotine uptake by mammalian tissues has been passive diffusion of the uncharged nicotine molecules (Schievelbein, 1982). This concept is supported by studies on L-[¹⁴C]nicotine distribution in rat submaxillary gland slices (Putney & Borzeleca, 1971). Nicotine uptake is apparently by passive diffusion only, shows no saturation kinetics and increases with an increase in extracellular pH, which raises the fraction of uncharged nicotine molecules (Putney & Borzeleca, 1971). In contrast, positively charged synthetic quaternary ammonium nicotine analogues, such as *N*-*n*-octylnicotinium iodide, apparently reach the brain by active transport via the blood–brain choline transporter (Allen *et al.*, 2003).

Nicotine of decaying tobacco plants is degraded by soil micro-organisms, which can use this molecule as source of carbon, nitrogen and energy (Andreesen & Fetzner, 2002; Brandsch, 2006). Metabolism of organic compounds by micro-organisms is initiated by their transport across the cell membrane. Aromatic lipophilic compounds may enter the bacterial cell by simple diffusion. In most cases,

Abbreviations: DPl, diphenyleiiodonium; 6HLN, 6-hydroxy-L-nicotine; 6HLNO, 6-hydroxy-L-nicotine oxidase; 6HPON, 6-hydroxy-pseudo-oxynicotine; NDH, nicotine dehydrogenase.

however, micro-organisms have evolved various uptake systems for compounds on which they can thrive. Uptake may be driven directly by ATP hydrolysis, or by secondary active transport, which takes advantage of an ion gradient across the membrane to transport in sym- or anti-port organic molecules into the cell. In this way, uptake can be achieved against the concentration gradient of the compound. Alternatively, uptake of nutrients may be achieved by facilitated diffusion along a concentration gradient with the aid of a permease (Saier, 2000). The transporters for many aromatic compounds have been shown to belong to the secondary active major facilitator superfamily of transporter proteins (Nichols & Harwood, 1997; Prieto & Garcia, 1997; Kahng *et al.*, 2000; Kasai *et al.*, 2001).

There is only limited information available on the uptake of nicotine from the environment by micro-organisms. In contrast to the situation at the blood–brain barrier, it seems not to penetrate the cell membrane of microbes by simple diffusion, and it has been suggested that nicotine uptake by *Arthrobacter nicotinovorans* is inducible. L-[¹⁴C]Nicotine uptake assays show that only bacteria grown with nicotine are able to take up the alkaloid (Igloi & Brandsch, 2003). In *A. nicotinovorans* the ability to use nicotine as a growth substrate has been linked to the presence of the 165 kb catabolic plasmid pAO1. An *A. nicotinovorans* strain without a functional pAO1 copy (pAO1[−]) or *Escherichia coli*, which cannot use nicotine for growth, show no import of nicotine (Igloi & Brandsch, 2003).

In the present work we analysed L-nicotine and 6-hydroxy-L-nicotine (6HLN) uptake by *A. nicotinovorans* and *E. coli* in greater detail, determined its energy requirements, the coupling of nicotine uptake to nicotine breakdown and its dependence on pAO1 genes.

METHODS

Chemicals. All chemicals used were of highest purity available. Diphenylethylideneiodonium (DPI), nigericin, valinomycin, monensin and PMSF were from Sigma-Aldrich. HEPES, MES, EDTA and DTT were from Carl Roth. L-[¹⁴C]Nicotine (4.625×10^7 Bq mmol^{−1}) was a kind gift of Professor Dr Karl Decker, Albert-Ludwigs University, Freiburg.

Bacterial strains, plasmids and growth conditions. *A. nicotinovorans* carrying the catabolic plasmid pAO1 (pAO1⁺) and a derived *A. nicotinovorans* strain unable to degrade nicotine (pAO1[−]) were grown at 30 °C on citrate medium supplemented with mineral salt solution, as described elsewhere (Brühmüller *et al.*, 1972, 1975). *E. coli* XL1-Blue or XL10-Gold, used as host for recombinant plasmids, was grown on LB (lysogeny broth) medium at 37 °C.

Transformation of *A. nicotinovorans* with plasmids by electroporation was performed as described by Gartemann & Eichenlaub (2001). *E. coli* XL1-Blue was made transformation-competent with Roti Transform and transformed as recommended by the supplier (Carl Roth). *E. coli* XL10-Gold ultracompetent cells were purchased from Stratagene.

Cloning of the hypothetical permease genes on pART2. The permease genes (*perm*) were amplified by PCR with the aid of specific primer pairs (1 and 2, 3 and 4, and 5 and 6; Table 1) using whole *A. nicotinovorans* pAO1⁺ cells as template. The amplified DNA of *perm1*, *perm2* and *perm3* was digested with the restriction endonucleases *Xba*I and *Nsi*I, *Kpn*I and *Xba*I, and *Sal*I and *Xba*I, respectively, and the DNA fragments were inserted into plasmid pART2 (Sandu *et al.*, 2005) that had been digested with *Spe*I and *Pst*I (which give cohesive ends compatible with DNA cut with *Xba*I and *Nsi*I), *Kpn*I and *Xba*I, or *Sal*I and *Xba*I, by ligation with the Fast-Link DNA Ligation kit as recommended by the supplier (Epicentre Biotechnologies, Biozym Scientific). The *6hln* gene was amplified with the primer pair 7 and 8 (Table 1), digested with *Xba*I and ligated into pART2*perm1* cut with *Xba*I in such a way as to allow expression of both genes. The ligated plasmid DNA was transformed into *E. coli* XL10-Gold ultracompetent cells. Transformants carrying recombinant DNA were picked, and plasmid DNA isolated according to standard methods (QIAprep Spin Miniprep kit, Qiagen). The isolated pART2 DNA carrying the permease genes 1–3 was then electroporated into *A. nicotinovorans* pAO1[−] and transformants were grown on citrate medium in the presence of 140 µg kanamycin ml^{−1}.

Inactivation of permease genes by insertion of a chloramphenicol-resistance cassette (*cmx*). The *perm1* gene was amplified with the aid of specific primers 9 and 10 (Table 1), and the PCR product was digested with *Bam*HI and *Hind*III and ligated into pET21b cut with the same restriction endonucleases. The *Bam*HI site of the multiple cloning site of pET21b*perm1* was deleted by digestion with *Nhe*I and religation. The *cmx* cassette was isolated from pOKU9, a derivative of pKGT452Cβ (Gartemann & Eichenlaub, 2001), by digestion with *Bam*HI and ligated into the single *Bam*HI site of the *perm1* gene. The *perm2* gene was amplified with primers 11 and 12 (Table 1), cut with the restriction endonucleases *Eco*RI and *Xho*I, and ligated into pMPM-A4 (Mayer, 1995) digested with the same enzymes. The chloramphenicol-resistance cassette of pKGT452Cβ (Gartemann & Eichenlaub, 2001) was amplified with specific primers 13 and 14 (Table 1), and inserted into *perm2* (cut with *Nco*I and blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I), carried on pMPM-A4. *perm3* was amplified with primers 15 and 16 (Table 1), digested with *Hind*III and *Xho*I, and ligated into pH6EX3 (Berthold *et al.*, 1992). The *cmx* cassette isolated from pOKU9 by digestion with *Bam*HI and blunt-ended by treatment with the Klenow fragment of DNA polymerase I was inserted into the *Pml*I site of *perm3*. The plasmids pET21b, pMPM-A4 and pH6EX3 are unable to replicate in *A. nicotinovorans*. The recombinant plasmids were transformed into *E. coli* XL1-Blue competent cells, and recombinant plasmid DNA was isolated from transformants selected on ampicillin plates and introduced by electroporation into *A. nicotinovorans* pAO1⁺. The electroporated cells were spread on plates containing 15 µg chloramphenicol ml^{−1}. Resistant colonies were picked and grown on citrate medium in the presence of chloramphenicol, and the insertion of the *cmx* cassette into the permease gene was verified by PCR with permease-specific primers (27 and 28, 29 and 30, and 31 and 32; Table 1). Colonies with permease genes interrupted by the *cmx* cassette were picked and tested for nicotine uptake.

Construction of a recombinant pART2 plasmid carrying the *ndhMSLcoxFmobA* pAO1 genes. The *coxFmobA* genes were amplified from *A. nicotinovorans* cells as a template (Table 1, primers 17 and 18), and the PCR product was digested with *Sal*I and *Pst*I and ligated into pUCBM20 (Yanisch-Perron *et al.*, 1985) cut with the same enzymes. The *ndhMSL* genes were also amplified with primers 19 and 20 (Table 1) and inserted into the *Eco*RI and *Hinc*II sites of pUCBM20*coxFmobA*. The resulting recombinant plasmid pUCBM20*ndhMSLcoxFmobA* was used as a template for the PCR

Table 1. Oligonucleotide primers used in this study

F, forward; R, reverse.

Primer	Orientation	Sequence (5'–3')	Amplified DNA fragment (fragment size, bp)
Cloning			
1	F	CACCATCTGCTCTAGAAACCTCG	
2	R	CCGAAGTTTCATGCATCTGTGCCGG	<i>perm1</i> (1533)
3	F	CAGAAGGTGGTACCTATGTC	
4	R	GTGCCTCTTGTCTAGAAGCAACTTTAG	<i>perm2</i> (1426)
5	F	CACCTGAAAGGTCGACACCTTTGC	
6	R	CATGCAATGCTCTAGAGGCGGAAATC	<i>perm3</i> (1479)
7	F	AAGCGCCCGATCTAGATAGTTGAAAG	
8	R	AAGTGAGTCTAGAGGTTTCTACG	<i>6hln</i> (1333)
9	F	GCATTGCAGGATCCTTCTTCTG	
10	R	GACGGTTATCAAGCTTCTCCTTATAAAG	<i>perm1</i> (1852)
11	F	CATACAGAAGGAATTCCTATGTCTG	
12	R	CGTCTTTCTTCGCTCGAGACTATTGG	<i>perm2</i> (1460)
13	F	CCTCAACACCGAGCATGCTAAAG	
14	R	CCGGTAGCGGCATGCCCATTTTC	<i>cmx</i> (1845)
15	F	CCGTTAAGCTTCGTGGTC	
16	R	ACCTGCGCCTCGAGTCCTA	<i>perm3</i> (1538)
17	F	CCTAGCAGATCGGGTCGACCATGCGTGACGTTA	
18	R	GCATCGTCCTGCAGGAGGCGATA	<i>coxFmobA</i> (2149)
19	F	GGAAGATGAAGGGAATTCGAGGGAGT	
20	R	TTTTCTTCTTTCAAGTTTCTAGCT	<i>ndhMSL</i> (3885)
21	F	GATGAAACTACCTGCTATCAGATA	
22	R	TCGCATGCCTCTAGAACGCGATACATC	<i>ndhMSLcoxFmobA</i> (5988)
23	F	CTTGAAAGAAGGACGCTTTGTATGAC	
24	R	AAGTGAGTCTAGAGGTTTCTACG	<i>6hln</i> (1315)
25	F	AAGAAGGAAAGCTTTGTATGACGCGA	
26	R	GTCTGCAGGTACCTACGAATGCAG	<i>6hln</i> (1304)
Verification of gene inactivation			
27	F	CGATCGGTTCCGCATACTTCATCG	<i>perm1</i> (532)
28	R	CAAATGAGGAAGTAAGGGATCAC	<i>perm1: cmx</i> (2014)
29	F	GCAGATCGGCCAAATTGTCACA	<i>perm2</i> (230)
30	R	TATCGGTACAAGTGCGTAGACC	<i>perm2: cmx</i> (2075)
31	F	CTCAAGATATAGCCCATGCAGATC	<i>perm3</i> (472)
32	R	GATACGTCCAATCCTCGATAGGCG	<i>perm3: cmx</i> (1954)

amplification with primers 21 and 22 (Table 1) of the *ndhMSLcoxFmobA* DNA, which was cloned, following digestion with *Xba*I, into pART2 cut with *Dra*I and *Xba*I. Expression of the genes was monitored by measuring the nicotine dehydrogenase (NDH) activity in the extracts of *A. nicotinovorans* pAO1[−] transformed with the recombinant plasmid. In order to induce the synthesis of the molybdenum cofactor, the transformed strains were grown in the presence of 150 μ M xanthine.

Construction of recombinant pART2 and pH6EX3 carrying the *6hln* gene of pAO1. The *6hln* gene was amplified from pAO1 by PCR with primer pairs 23 and 24 and 25 and 26 (Table 1), and the amplified DNA was digested with the restriction enzymes *Aat*II and *Xba*I, or *Hind*III and *Kpn*I, and inserted by ligation into the multiple cloning sites of pART2 and pH6EX3, respectively. The ligated recombinant plasmid DNA was transformed into *E. coli* XL10-Gold competent cells. pART2*6hln* plasmid DNA was isolated from *E. coli* XL10-Gold bacteria and introduced by electroporation into *A. nicotinovorans*.

Preparation of *A. nicotinovorans* and *E. coli* membrane vesicles.

Bacterial membrane vesicles were prepared as described in Moser *et al.* (2007), with slight modifications. Briefly, *A. nicotinovorans* was grown in 1 l citrate medium to OD₆₀₀ 0.6–0.7, and the cells were collected by centrifugation, suspended in 50 mM potassium phosphate buffer, pH 6.6, recentrifuged and taken up in 20 ml phosphate buffer. Lysozyme was added (1.5 mg ml^{−1} final concentration, corresponding to 7.6 × 10⁴ U) and the bacterial suspension was incubated for 30 min at 30 °C. Then, Benzonase Nuclease (375 units, Novagen) and PMSF (0.5 mM) were added and the bacteria were broken by two passages in a French pressure cell at 1000 p.s.i. (6900 kPa). The bacterial lysate was centrifuged at 5000 *g* for 25 min, and the resultant supernatant was diluted with 20 ml 50 mM potassium phosphate buffer, pH 6.6, and centrifuged at 150 000 *g* for 2 h in the 45Ti rotor of a Beckman centrifuge. After centrifugation, the membrane pellet was taken up in 50 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. Half volumes of 3 M potassium acetate were added to the membrane suspension and incubated for 15 min on ice.

The suspension was layered on a 750 mM sucrose cushion and centrifuged at 200 000 g for 1 h in a TLA55 Beckman centrifuge rotor. The membrane pellet was suspended in 50 mM HEPES, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF; aliquots of 50 µl were frozen in liquid nitrogen and stored until use.

L-[¹⁴C]Nicotine uptake assays. *A. nicotinovorans* was grown overnight in 10 ml citrate medium to stationary phase (OD₆₀₀ 1.0). Then, 25 µl L-[¹⁴C]nicotine (4.625×10^7 Bq mmol⁻¹) was added to 100 µM. Samples (1.5 ml) were removed at different time points, and the bacteria were pelleted by centrifugation at 18 000 g for 2 min, resuspended in 1 ml double-distilled water and repelleted. The washed bacteria were resuspended in 50 µl 50 mM Tris/HCl (pH 8.0), 10 mM EDTA, 1 mg lysozyme ml⁻¹, treated with 100 µl 200 mM NaOH/1 % SDS (w/v), and neutralized with 75 µl 2 M Tris/HCl (pH 7.0)/0.5 M NaCl. The samples were centrifuged at 18 000 g and the protein concentration in the supernatant was determined with Roti-Quant according to the instructions of the supplier (Roth). A 150 µl volume of the cleared supernatant (18.5 µg protein) was added to 4.5 ml scintillation solution (Roth), and the radioactivity was counted in a Packard liquid scintillation counter for 5 min. The experiments were performed at least three times and the SD was calculated based on these datasets. Scintillation counting revealed that <5 % of the radioactivity was present in the pellets of the lysates.

Alternatively, the bacteria were grown overnight in 10 ml citrate medium in the presence of 1.23 mM unlabelled nicotine. The bacteria were pelleted by centrifugation at 3550 g, washed twice with citrate medium, and resuspended in the same volume of citrate medium; L-[¹⁴C]nicotine (4.625×10^7 Bq mmol⁻¹) was added to 100 µM, and the assays were performed as described above.

Membrane vesicles were tested for nicotine transport in assays containing 50 µg membrane protein in the following buffers: 20 mM MES/KOH, pH 6.0, 100 mM potassium acetate, 10 mM MgSO₄; 20 mM MES/NaOH, pH 6.0, 100 mM sodium acetate, 10 mM MgSO₄; 50 mM HEPES, pH 6.0, 10 mM MgSO₄. L-[¹⁴C]Nicotine was added (12.5 µM), mixed and incubated for 3 min at 30 °C. The assays were centrifuged at 200 000 g for 1 h in a TLA55 rotor, and the membrane pellet was suspended in 100 µl distilled water and added to 4.5 ml scintillation solution, and the radioactivity was counted in a Packard liquid scintillation counter for 5 min.

Uptake of 6HLN by *A. nicotinovorans* pAO1⁻ transformed with pART26hlnO and *E. coli* transformed with pART26hlnO or pH6EX36hlnO. *E. coli* and *A. nicotinovorans* pAO1⁻ carrying pART26hlnO were grown overnight on LB and citrate medium, respectively. Non-transformed *E. coli* XL1-Blue and *A. nicotinovorans* pAO1⁻ strains were employed as controls. The cells were harvested by centrifugation at 5000 g and suspended in 50 mM potassium phosphate buffer, pH 7.1. An assay, dependent on the increase in A₃₃₄ on conversion by 6-hydroxy-L-nicotine oxidase (6HLNO) of 6HLN to 6-hydroxy-pseudooxynicotine (6HPON) ($\epsilon = 20.7$ mM⁻¹ cm⁻¹ at alkaline pH) was performed in a 1 ml volume. A 10 µl volume of cell suspension of *A. nicotinovorans* pAO1⁻ carrying pART26hlnO, 50 µl of cell suspension of *E. coli* pART26hlnO or 5 µl of cell suspension of *E. coli* pH6EX36hlnO was added to 50 mM potassium phosphate buffer, pH 7.1, containing 500 µM 6HLN. The assays were incubated for 10 min at 30 °C, the cells were removed by centrifugation, the supernatant was transferred into 1 ml cuvettes and the absorption at 334 nm was measured in a UV/visible spectrophotometer (Amersham). The pH was adjusted to alkaline by adding 6 µl 5 M NaOH solution and the difference in readings was recorded. Calculation of enzyme activity and product formation was standardized to OD₆₀₀ 1 in the assays.

To determine the effect of ionophores on 6HLN uptake into the cells, 5 µM monensin, 10 µM valinomycin or 2 µM nigericin was added to

a 1 ml assay containing 50 mM potassium phosphate buffer, pH 7.1, and a cell suspension of different bacterial strains. The assay was incubated for 5 min at room temperature before 500 µM 6HLN was added. After incubation for 10 min at 30 °C the sample was processed following the protocol described above.

RESULTS

Kinetics of nicotine uptake

The time-course for L-[¹⁴C]nicotine uptake (Fig. 1a, right insert) by *A. nicotinovorans* grown in citrate medium resulted in a bell-shaped curve (Fig. 1a). Nicotine uptake starts, as shown previously (Igloi & Brandsch, 2003), with a lag-phase of approximately 10 min, which coincided with the appearance of NDH and 6HLNO activity in the bacterial extracts. The bell-shaped curve resulted from the fact that methylamine produced from the pyrrolidine ring carrying the labelled methyl group (Chiribau *et al.*, 2006) was extruded from the cells by the NepAB export pump (Ganas *et al.*, 2007) (Fig. 1a, right insert TLC). After 2 h the culture medium started to turn blue, due to the secretion of nicotine blue, an end-product of nicotine catabolism (Brandsch, 2006). The *A. nicotinovorans* strain lacking a functional copy of pAO1 (*A. nicotinovorans* pAO1⁻) as well as *E. coli* showed no nicotine uptake (Fig. 1a). *Nicotiana tabacum* produces small amounts of the stereoisomer D-nicotine (Armstrong *et al.*, 1999). D-Nicotine inhibited L-[¹⁴C]nicotine uptake, suggesting that the stereoisomers are transported by the same route (Fig. 1a).

When *A. nicotinovorans* grown in citrate medium in the presence of unlabelled nicotine was tested, uptake of L-[¹⁴C]nicotine started immediately, providing evidence for a nicotine-inducible transport mechanism (Fig. 1b).

To investigate the concentration-dependent uptake of L-[¹⁴C]nicotine by *A. nicotinovorans*, pAO1⁺ cells grown in citrate medium overnight were incubated for 60 min with L-[¹⁴C]nicotine. As shown in Fig. 1(c), the nicotine uptake by these cells was saturable. A K_m of $6.2 \mu\text{M} \pm 0.1$, in good agreement with previous results (Igloi & Brandsch, 2003), and a V_{max} of $0.70 \pm 0.075 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ were determined. In contrast, *A. nicotinovorans* pAO1⁻ showed no nicotine uptake even at high nicotine concentrations (Fig. 1c). The slight increase in radioactivity in the samples can be explained by non-specific contamination at these high nicotine concentrations. Similarly, *E. coli* showed no nicotine uptake (results not shown). These results suggested that nicotine uptake by *A. nicotinovorans* is not by passive diffusion.

Coupling of nicotine uptake and nicotine degradation

Nicotine degradation starts with the hydroxylation of nicotine at C6 of the pyridine ring which is performed by the molybdenum cofactor (MoCo)-dependent NDH. Replacement of molybdenum in the growth medium with

Fig. 1. Time-dependent and concentration-dependent nicotine uptake by bacterial strains. (a) *A. nicotinovorans* (■), *A. nicotinovorans* pAO1⁻ (▲) and *E. coli* XL1-Blue (◆) bacteria grown overnight in citrate medium or LB medium in the absence of nicotine were incubated with 100 μM L-[¹⁴C]nicotine, and time-dependent nicotine uptake was assayed as described in Methods. (□) L-[¹⁴C]Nicotine uptake by *A. nicotinovorans* in the presence of 1.5 mM D-nicotine. Inserts: left, L-[¹⁴C]nicotine carrying the radioactive label at the C atom of the methyl group (indicated by an asterisk); right, TLC displaying the disappearance with time of L-[¹⁴C]nicotine (N) from, and the appearance of [¹⁴C]methylamine (MA) in, the growth medium. (b) Time-dependent uptake of L-[¹⁴C]nicotine by *A. nicotinovorans* grown overnight in citrate medium in the presence of unlabelled nicotine. (c) Concentration-dependent kinetics of nicotine uptake by *A. nicotinovorans* (■) and *A. nicotinovorans* pAO1⁻ (▲) grown overnight in citrate medium and then incubated with different concentrations of L-[¹⁴C]nicotine for 1 h.

tungsten leads to the formation of an inactive cofactor and inhibition of NDH activity (Grether-Beck *et al.*, 1994; Baitsch *et al.*, 2001). When L-[¹⁴C]nicotine uptake by bacteria grown in the presence of molybdenum was compared with uptake by bacteria grown in the presence of tungsten, it became apparent that uptake of nicotine was blocked in bacteria grown with tungsten (Fig. 2a). Control measurements of NDH activity in extracts of tungsten-grown

bacteria confirmed the absence of enzyme activity (results not shown). However, when tungsten was added to L-[¹⁴C]nicotine uptake assays performed with bacteria grown in the presence of molybdenum and unlabelled nicotine, tungsten did not inhibit L-[¹⁴C]nicotine uptake (Fig. 2b). Apparently, active MoCo-containing NDH was present in these bacteria and tungsten no longer inhibited NDH activity and thus nicotine uptake.

Fig. 2. Dependence of nicotine uptake on nicotine turnover. (a) *A. nicotinovorans* was grown overnight in citrate medium in the presence of molybdenum (■) or tungsten (▲), and time-dependent L-[¹⁴C]nicotine uptake assays were performed with the bacteria as described in Methods. (b) *A. nicotinovorans* was grown in citrate medium overnight in the presence of nicotine and molybdenum, and time-dependent L-[¹⁴C]nicotine uptake assays were performed with the washed bacteria in the absence (■) and presence (▲) of added tungsten. (c) *A. nicotinovorans* pAO1⁻ was transformed with the expression plasmid pART2 carrying the *ndhMSLcoxFmobA* genes and grown for 24 h at 30 °C in 100 ml citrate medium supplemented with 150 μM xanthine. The bacterial cells were centrifuged and resuspended in 1.5 ml 100 mM potassium phosphate buffer, pH 7.0, in the presence of 200 μM L-[¹⁴C]nicotine, 200 μl samples were taken from the assays at the time points indicated, centrifuged, and 7 μl of the supernatant was applied to TLC plates (as described in Ganas *et al.*, 2007). Control assays were performed with the untransformed *A. nicotinovorans* pAO1⁻ strain. The TLC plates were subjected to autoradiography and the films developed after 7 days of exposure.

When *A. nicotinovorans* pAO1⁻, which is unable to take up nicotine, was transformed with pART2 carrying the *ndhMSL* and the *coxFmobA* genes, which are required for synthesis of molybdopterin cytosine dinucleotide (MCD) specific for NDH (Sachelaru *et al.*, 2006), L-[¹⁴C]nicotine uptake became detectable by analysis of the growth medium (Fig. 2c). L-[¹⁴C]Nicotine taken up by the bacteria was converted into [¹⁴C]6HLN by NDH, and in the absence

of additional nicotine-degrading enzymes, [¹⁴C]6HLN was released into the medium, where it was detected by TLC.

Uptake of 6HLN by *A. nicotinovorans* pAO1⁻ and *E. coli* expressing a functional *6hln* gene

Taken together, the results presented above indicated that nicotine breakdown was a prerequisite for nicotine uptake.

Since 6HLN and nicotine were apparently taken up by *A. nicotinovorans* by the same mechanism, we addressed the question of whether bacterial cells can be made competent for 6HLN uptake by expressing the *6hln* gene. The *6hln* gene was expressed in *A. nicotinovorans* and *E. coli* from the expression plasmids pART2 and pH6EX3. 6HLN was added to bacterial suspensions and the formation of 6HPON was assayed as described in Methods. As 6HPON is no longer degraded by the cells and is released into the culture medium, its presence can be measured by

the increase in A_{334} following a shift to $\geq$ pH 9.5. In the absence of 6HLN uptake, its concentration in the assay should not change and no 6HPON should be formed. It was found that the expression of *6hln* conferred upon the bacterial strains the ability to take up 6HLN, as shown by the formation of 6HPON (Fig. 3a). The level of 6HLNO produced from the *6hln* gene under the control of the IPTG-inducible *tac* promoter of pH6EX3 in *E. coli* (1673 mU mg^{-1}) was higher than the level of 6HLNO produced from the constitutively expressed *6hln* gene

Fig. 3. Uptake of 6HLN by bacterial strains. 6HLN uptake assays were performed as described in Methods with *A. nicotinovorans* pAO1⁻ transformed with pART26hln, or with untransformed controls and *E. coli* transformed with pART26hln or pH6EX36hln, or with untransformed controls (a), in the presence of the additions indicated in the figure. (b) Saturation kinetics of 6HLN uptake by *A. nicotinovorans* pAO1⁻ carrying pART26hln (■) and by *E. coli* pART26hln (▲). (c) Saturation kinetics of 6HLN uptake by *E. coli* pH6EX36hln.

under the control of the *6hdno* promoter of pART2 in *A. nicotinovorans* pAO1[−] (31.29 mU mg^{−1}) and in *E. coli* (3.36 mU mg^{−1}). Addition of DPI, an inhibitor of flavoenzymes which penetrates bacterial cells (Brandsch & Bichler, 1991), to the assays reduced the level of 6HPON in a concentration-dependent manner, an indication that 6HLNO activity was required for the production of 6HPON from externally added 6HLN (Fig. 3a). Uptake of 6HLN by *E. coli* and *A. nicotinovorans* pAO1[−] was saturable, consistent with transport of the molecule by facilitated diffusion (Fig. 3b, c).

As found for nicotine uptake (Ganas *et al.*, 2007), dissipation of the Na⁺, K⁺ and H⁺ gradients by monensin, valinomycin and nigericin, respectively, did not significantly affect 6HLN uptake (results not shown).

L-[¹⁴C]Nicotine uptake assays with membrane vesicles

If a permease for nicotine was expressed by *A. nicotinovorans* in response to nicotine in the growth medium, one might expect a higher number of permease molecules in the membranes of nicotine-grown bacteria than in membranes from bacteria grown in the absence of nicotine. When membrane preparations were incubated with L-[¹⁴C]nicotine, no difference was found in nicotine content between membrane vesicles from bacteria grown in the presence and those grown in the absence of nicotine. The application of a Na⁺ or K⁺ gradient, or the absence of an ion gradient had no effect on the results. Membrane vesicles prepared from *E. coli* XL10-Gold showed the same level of radioactivity, which was considered to be background binding of L-[¹⁴C]nicotine to membranes (results not shown). Apparently, no nicotine permease was present in the membrane preparations, or the absence of nicotine-degrading enzymes prevented nicotine uptake by the membrane vesicles.

Specificity of nicotine uptake

Assays were performed with *A. nicotinovorans* pAO1⁺ in the presence of compounds structurally related to nicotine, which might be expected to inhibit the uptake of L-[¹⁴C]nicotine if the transport involved a permease. Fig. 4 shows that the strongest inhibition of L-[¹⁴C]nicotine uptake was observed with 2-amino-L-nicotine, followed by 6HLN, while 2,6-dihydroxypyridine had a slight effect, and 2,6-

Fig. 4. Inhibition of L-[¹⁴C]nicotine uptake by structurally related compounds. *A. nicotinovorans* was grown overnight in citrate medium in the presence of unlabelled nicotine, and the washed bacteria were used in nicotine uptake assays in the presence of various compounds which were added simultaneously with L-[¹⁴C]nicotine. L-[¹⁴C]Nicotine uptake assay without additions (■) and in the presence of 1 mM of the following compounds: (▲) 6HLN, (◆) 2-amino-L-nicotine, (□) 2,6-dihydroxynicotinic acid, (△) pyridine, (◇) 2,6-dihydroxypyridine.

dihydroxynicotinamide and pyridine had no effect. Since positively charged synthetic quaternary ammonium nicotine analogues are actively transported across the blood–brain barrier by a choline transporter (Allen *et al.*, 2003), we added choline or ethanolamine to the assays but observed no inhibition of nicotine uptake (results not shown).

Attempts at identification of the nicotine permease(s)

Within the *nic*- gene cluster of pAO1 there are three hypothetical genes that exhibit similarity to genes of amino acid transporters (Fig. 5) (Igloi & Brandsch, 2003). Since

Fig. 5. pAO1 gene cluster with putative permease genes. Schematic representation of the position of the genes on pAO1 and inactivation of the permease genes by the insertion of a chloramphenicol-resistance cassette (*cmx*). Indicated are the PCR primers (see Table 1) used for verification of the insertion events.

nicotine uptake has been shown to be influenced by the presence of amino acids in the medium (Igloi & Brandsch, 2003), these predicted permeases were considered to be candidates for nicotine transporters. Expression of the

Fig. 6. L-[¹⁴C]Nicotine uptake assays performed with *A. nicotinovorans* strains. Assays were performed with *A. nicotinovorans* (■) and *A. nicotinovorans* carrying *cmx*-inactivated *perm1* (□), *perm2* (△) and *perm3* (◇) genes, grown overnight in the absence (a) or presence (b) of unlabelled nicotine. (c) L-[¹⁴C]Nicotine uptake assays performed with *A. nicotinovorans* pAO1⁻ (■), and *A. nicotinovorans* pAO1⁻ transformed with pART2*perm1* (□), pART2*perm2* (△) or pART2*perm3* (◇) grown overnight in citrate medium.

putative pAO1 permease genes has been shown previously to be dependent on the presence of nicotine in the growth medium (Sandu *et al.*, 2003; Chiribau *et al.*, 2004). If one of these genes encoded a nicotine permease, its inactivation should affect nicotine uptake by *A. nicotinovorans*. Therefore, inactivation of the individual permease genes was performed with the aid of a chloramphenicol-resistance cassette (*cmx*) that interrupted the respective ORF (Fig. 5). Insertion of the *cmx* cassette was verified by amplification with specific primers for the permease genes, as described in Methods, and the effect on nicotine uptake was tested. L-[¹⁴C]nicotine uptake by these strains was not significantly affected, irrespective of whether they were grown in the absence (Fig. 6a) or presence of unlabelled nicotine (Fig. 6b). The expression of the permease genes in *A. nicotinovorans* pAO1⁻ when introduced on pART2 did not confer the ability to take up L-[¹⁴C]nicotine by these cells (Fig. 6c). Because nicotine transport is coupled to its breakdown, we transformed *E. coli* with pART2 carrying in addition to a permease gene the *6hlnO* gene (pART2*perm16hlnO*). The transformed bacteria exhibited 6HLNO activity, but there was no increased 6HLN uptake detectable when compared with the strain expressing the *6hlnO* gene alone (results not shown).

DISCUSSION

What drives nicotine uptake into bacterial cells: is it by concentration-dependent facilitated diffusion or is it by primary or secondary active transport? It was known from previous studies that a proton-gradient-dependent small multidrug resistance pump, NepAB, was involved in the export of labelled breakdown products of nicotine (Ganas *et al.*, 2007). Compounds that block ATP synthesis by dissipating the proton gradient [carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP)] and compounds that dissipate the K⁺ (valinomycin), the Na⁺ (monensin) or the K⁺ and proton gradient (nigericin) across the cell membrane resulted in the inhibition of the NepAB export pump. In this situation, uptake of nicotine by the cells continued, leading to an increase in the level of intracellular radioactivity (Ganas *et al.*, 2007). This conclusion was supported by the experiments presented in this work with regard to the energy-independent 6HLN uptake by *A. nicotinovorans* and *E. coli* expressing 6HLNO. These findings indicated that nicotine and 6HLN uptake was not driven by a primary or secondary active transport mechanism. Since nicotine

uptake by *A. nicotinovorans* seemed to be neither by passive diffusion nor by energy-dependent transport, a mechanism of facilitated diffusion across the bacterial membrane appears to be active.

The delay in L-[¹⁴C]nicotine uptake by *A. nicotinovorans* has been attributed to the need to induce the expression of nicotine transport and degradation genes (Igloi & Brandsch, 2003). Since methylamine carrying the [¹⁴C] label is not further metabolized by *A. nicotinovorans* but secreted into the medium (Chiribau *et al.*, 2006; Ganas *et al.*, 2007) the time-dependent uptake of nicotine showed a bell shaped curve. L-[¹⁴C]nicotine could be taken up without delay by bacteria pre-grown in the presence of unlabelled nicotine, supporting a nicotine-inducible transport mechanism. The saturation kinetics for nicotine uptake pointed to a mechanism of facilitated diffusion by a permease, contrary to the suggested mechanism of passive diffusion of nicotine across the blood–brain barrier and rat submaxillary gland cells (Putney & Borzeleca, 1971; Schievelbein, 1982; Lockman *et al.*, 2005).

The presence of ORFs encoding putative amino acid permeases (Burkovski & Krämer, 2002) within the *nic* gene cluster of pAO1 suggested a possible involvement of these gene products in nicotine transport. Such a gene arrangement is common in the organization of bacterial catabolic pathways (Nichols & Harwood, 1997; Kahng *et al.*, 2000; Rojas *et al.*, 2001; Kasai *et al.*, 2001; Hearn *et al.*, 2003). Expression of these putative permease genes in the presence of nicotine in the growth medium pointed to their possible involvement in nicotine catabolism by *A. nicotinovorans*. However, all efforts to functionally link these permease genes to nicotine catabolism failed. The gene-inactivation experiments and the inability to induce nicotine uptake in *A. nicotinovorans* pAO1[−] and in *E. coli* by expression of the *perm* genes, either alone or in combination with genes of nicotine-degrading enzymes, showed that these putative permeases were not the nicotine transporters. Additional support for the assumption that these permeases are not nicotine transporters is the fact that *Nocardioideis* sp. JS614 harbours a nicotine catabolic pathway almost identical to that of *A. nicotinovorans* (Ganas *et al.*, 2008), but lacks the permease genes found on pAO1. The physiological function of the hypothetical amino acid permeases in nicotine catabolism, if any, remains unclear.

Alternatively, a constitutively synthesized permease may fortuitously mediate nicotine transport. Since facilitated diffusion across the cell membrane by a permease is along the concentration gradient of the transported solute, a modification inside the cell of the imported molecule maintains the outside–inside gradient. Usually this is accomplished by the action of an enzyme that metabolizes the imported molecule. Indeed, nicotine transport was coupled to its intracellular breakdown. This was demonstrated by nicotine uptake following transformation and expression of the *ndhMSL* genes in *A. nicotinovorans* pAO1[−], a strain previously unable to do so. The coupling

of nicotine uptake and nicotine breakdown may maintain the outside–inside nicotine gradient and thus facilitate its transport, similar to the mechanism of glucose uptake by the well-studied glucose transporters of mammalian cells (Mueckler, 1994). Also, 6HLN transport required the intracellular presence of 6HLNO. As with nicotine, uptake of 6HLN was saturable and energy-independent in both *A. nicotinovorans* pAO1[−] and *E. coli*, in accord with a transport mechanism by facilitated diffusion. Higher 6HLNO activity correlated with increased 6HLN uptake, and inhibition of 6HLNO activity by the flavoenzyme inhibitor DPI (Brandsch & Bichler, 1991) decreased 6HLN uptake. In the absence of 6HLNO from the bacteria, the 6HLN concentration in the assays did not change. Only in the presence of the enzyme did 6HPON appear in the cell-free supernatant of the import assays. These findings demonstrated that 6HLN entered the cells and was transformed by 6HLNO into 6HPON, which, not being further metabolized, was released into the medium.

The incompatibility of the *E. coli* cofactor molybdopterin guanosine dinucleotide (MGD) with NDH, which requires the molybdopterin cytosine dinucleotide (MCD) cofactor, prevented L-[¹⁴C]nicotine import assays with *E. coli*. Previous work (Roduit *et al.*, 1997; Chiribau *et al.*, 2006; Ganas *et al.*, 2007) as well as this study show that when 6HLN accumulates inside bacterial cells during nicotine breakdown, it is released into the medium. The same occurred with 6HPON, which accumulated in *E. coli* and *A. nicotinovorans* in the presence of 6HLNO.

6HLN accumulated inside the bacteria and was exported, presumably because of overloading of the degradation capacity of the pathway. Export of 6HLN from and import of 6HLN into the bacterial cells may take place along the concentration gradient of 6HLN by the same permease. From the experiments showing inhibition of nicotine uptake by structurally related compounds one may speculate that L-nicotine, D-nicotine, 6HLN and 2-amino-L-nicotine share the same permease. Both 6HLN and D-nicotine also induce expression of the *nic* genes of pAO1 (Gloger & Decker, 1969; Schenk *et al.*, 1998). The absence of the pyrrolidine ring in pyridine and hydroxypyridine renders these compounds inactive with respect to nicotine uptake. Apparently, the pyrrolidine ring of nicotine is one of the structural determinants recognized by the permease that facilitates nicotine transport. The nature of this permease, however, remains to be established.

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