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Urea uptake by the marine bacterium *Deleya venusta* HG1

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The uptake (transport and metabolism) of urea was studied in a strain of the marine bacterium *Deleya venusta*, measuring the uptake of [^{14}C]urea *in vivo* and the urease reaction *in vitro*. Urea uptake *in vivo* was sodium-dependent and exhibited a K_m value of 1·4 μM for urea, a broad pH optimum between pH 6·0 and 8·5, a distinct temperature optimum at 35 °C and a requirement for energy. Urease activity *in vitro* exhibited a K_m value of 0·86 mM for urea and showed maximum activities at pH 8·5 and 60 °C; the enzyme was neither dependent on the presence of sodium, nor inhibited by metabolic inhibitors. Synthesis of the urea uptake system was subject to nitrogen control; ammonium resulted in a repression of the system, whereas high uptake rates were observed after growth with nitrate or incubation of the cells in the absence of a nitrogen source. The uptake reaction *in vivo*, but not the urease activity *in vitro*, was decreased greatly in the presence of ammonium. This inhibition was relieved by methionine sulphoximine (MSX), a potent inhibitor of glutamine synthetase; in mutant strains impaired in this enzyme no inhibition of urea uptake by ammonium was observed. These results suggest that glutamine formed from ammonium rather than ammonium itself regulates urea uptake activity in *D. venusta*.

Introduction

The importance of urea as a nitrogen source in the marine environment has been pointed out in numerous publications during the last 20 years (Remsen, 1971; Herbland, 1976; Kristiansen, 1983; Lund & Blackburn, 1989; Paerl, 1991). It is therefore surprising that whilst several studies describe urea utilization by estuarine and marine micro-organisms (ZoBell & Feltham, 1935; Taga, 1970; Savidge & Johnston, 1987), there are no reports on the transport of this compound in marine prokaryotes; this may partly be due to the common assumption that there is no need for a specific bacterial permease system for such a small, uncharged molecule. However, the permeability coefficients of bacterial membranes for urea are low (between 2·7 and 4·6 × 10^{-8} cm s^{-1}; Jahns *et al.*, 1988), and ambient urea concentrations in, for example, marine environments rarely exceed the micromolar range (Remsen *et al.*, 1974). Energy-dependent uptake systems for urea have been reported in freshwater and marine algae (Syrett & Bekheet, 1977; Williams & Hodson, 1977; Rees *et al.*, 1980), yeasts and other fungi (Cooper & Sumrada, 1975; Pateman *et al.*, 1982) and several non-marine bacteria (Jahns *et al.*, 1988). In this paper, some properties of the urea uptake system in a strain of a newly isolated marine bacterium and its regulation, both of synthesis and activity, are described.

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Abbreviations: MSX, methionine sulphoximine; GS, glutamine synthetase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide.

Methods

Media and growth conditions. Deleya venusta strain HG1 was grown at 26 °C and pH 7·5 in a mineral medium (Schlegel *et al.*, 1961) containing 500 mM-NaCl, 0·5% (w/v) glucose and the nitrogen sources indicated in the experiments. Unless otherwise indicated, cells were harvested at the end of exponential growth, washed twice in 50 mM-phosphate buffer pH 7·5 (31 mM-KH_2PO_4, 19 mM-Na_2HPO_4) containing 500 mM-NaCl, resuspended in the same buffer containing 0·5% glucose and stored on ice for up to 4 h before the uptake experiments were performed. The optical density of the cell suspensions was measured at 436 nm.

Isolation of mutants. Mutants lacking glutamine synthetase (GS; EC 6.3.1.2) activity were isolated on the basis of their inability to grow with low concentrations of urea as the nitrogen source. A mutagenesis procedure using ethylmethanesulphonic acid was employed as previously described (Jannsen *et al.*, 1981), with the following modifications: carbenicillin was replaced by ampicillin (50 μg ml^{-1}); in the counterselection step, 0·5 mM-urea was used instead of glutamate and ammonium sulphate, and surviving cells were grown in a mineral medium supplemented with 1 mM-glutamine and 5 mM-ammonium chloride.

Uptake experiments and enzyme activities. Cells suspensions adjusted to OD_{436} values between 0·3 and 1·0 were incubated at 30 °C for 5 min prior to addition of [^{14}C]urea; uptake was measured as previously described (Jahns & Kaltwasser, 1989) by following the decrease of the [^{14}C]urea concentration in the suspending medium. Disintegration of the cells was achieved by ultrasonic treatment with a Branson B12 sonifier at a maximum output of 70 W. Enzyme activities were determined in the cell-free supernatant, obtained by centrifugation of the sonified cell suspensions at 4 °C and 50000 *g* for 15 min. Unless otherwise indicated, urease activity (EC 3.5.1.5) was determined in a buffer containing 100 mM-diethylbarbituric acid and 20 mM-urea at pH 8·5 by the indophenol method according to Zawada & Sutcliffe (1981), measuring the formation of ammonium from hydrolytic breakdown of urea; for determination of GS activity, the magnesium-dependent synthetic assay or the manganese-dependent transferase assay, both at pH 8·2, were employed as described by Bender *et al.*, (1977), except that the CTAB solution was replaced by distilled water. Urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}; enzyme activities are given in nmol ammonium (urease) or nmol γ-glutamylhydroxamate (GS) formed (mg protein)$^{-1}$ min^{-1}.

Determination of protein, urea and ammonium. Ammonium was determined by the indophenol method (Zawada & Sutcliffe, 1981); urea was measured by the same method after its hydrolysis to ammonia by the action of jack bean urease. Protein was determined in cell suspensions according to Schmidt *et al.* (1963), with the modification that only 10% of the indicated amount of potassium iodide was used, and in cell-free extracts according to the Lowry method; bovine serum albumin was used as a standard.

Reproducibility of results. All results presented are mean values from at least two independent experiments; SD values not exceeding ±20% of the mean values are not indicated. The Tables and Figures show representative results from single experiments, which were repeated at least once. Experiments for the determination of the K_m value of urea uptake and urease were repeated at least three times; linear correlations were fitted by regression analysis, yielding values for r of between 0·985 and 0·995.

Results and Discussion

Isolation and characterization of strain HG1

The strain under study, designated HG1, was isolated in September 1990 from surface waters of the North Sea in Helgoland, FRG, at a distance of about 500 m from the coast. The following properties of this bacterium indicate that it belongs to the genus *Deleya* (Baumann *et al.*, 1983). Growth of the strictly aerobic, Gram-negative rod occurred only in the presence of NaCl concentrations of 200 mM or more (up to 3000 mM, the highest concentration tested, with an optimum at 700 mM); NaCl could not be replaced by other cations such as KCl, CsCl or LiCl. Electron microscopy revealed that this motile bacterium had peritrichous flagella; poly-β-hydroxybutyrate [extracted from the cells and identified by the methods of Law & Slepecky (1961) and Williamson & Wilkinson (1958)] was accumulated as a storage compound; D-glucose, D-gluconate and glycerol were readily utilized by this bacterium; these traits differentiate the isolated organism from other aerobic marine species such as *Pseudomonas* or *Alteromonas*.

Cell-free extracts from strain HG1 were analysed by SDS-PAGE and compared with those of *D. venusta* strain A84 (ATCC 35137), *D. cupida* strain A79 (ATCC 27124) and *D. aesta* strain A134 (ATCC 27128) (kindly provided by E. F. DeLong, Woods Hole Oceanographic Institution, MA, USA); practically identical protein patterns for *D. venusta* A84 (ATCC 35137) and strain HG1 were observed (results not shown). The utilization of maltose and the inability to metabolize D-mannose were further characteristics distinguishing strain HG1 from *Deleya* species other than *D. venusta* (Valderrama *et al.*, 1991). However, strain HG1 was susceptible to 50 μg ampicillin ml^{-1}, in contrast to *D. venusta* strain NCMB 1979 (Valderrama *et al.*, 1991). Interestingly, both strains HG1 and *D. venusta* strain A84, but not the other species of *Deleya* investigated, exhibited a strong odour characteristic of fresh peas during growth. The strain has been deposited in the German collection of Microorganisms and Cell Cultures (DSM), Braunschweig, FRG as *Delaya venusta* HG1 DSM 7049.

Kinetics of urea uptake

When grown with 10 mM-nitrate as the nitrogen source, *D. venusta* strain HG1 rapidly transported urea. Uptake was a saturable process showing typical Michaelis–Menten kinetics, with a K_m value of 1·4 ± 0·7 μM for urea and a V_{max} of 7·8 ± 3·0 nmol urea (mg protein)$^{-1}$ min^{-1} ($n = 4$). An affinity about one order of magnitude lower has been described for the urea uptake systems of non-marine bacteria (Jahns *et al.*, 1988) and fungi (Pateman *et al.*, 1982; Cooper & Sumrada, 1975). The affinity of the urea uptake system of *D. venusta* strain HG1 is in the range of that described for several marine algae (McCarthy, 1972), for which K_m values between 0·14 and 0·85 μM for urea have been measured. This high affinity may facilitate the utilization of urea at the low concentrations usually occurring in marine systems (Remsen *et al.*, 1974).

The urease of strain HG1 exhibited a K_m value of 0·86 ± 0·23 mM for urea and a V_{max} of 497 ± 142 nmol ammonium (mg protein)$^{-1}$ min^{-1} ($n = 5$) in nitrate-grown cells. Maximum uptake activities were observed between pH 6·0 and 8·5 and at 35 °C; urease activity, in contrast, was highest at pH 8·5 and 60 °C (Fig. 1). Based on temperature-dependence determinations, apparent activation energies of 40·3 kJ mol^{-1} and 86·3 kJ mol^{-1} were calculated from Arrhenius plots for urease and urea uptake, respectively.

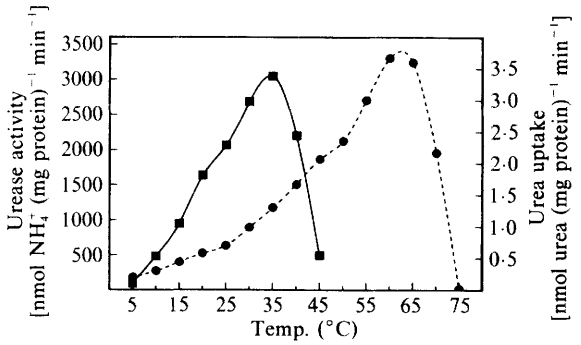

Fig. 1. Temperature-dependence of urease and urea uptake in *D. venusta* strain HG1. Cells grown with 10 mM-nitrate as the nitrogen source were washed twice and incubated at the temperature indicated for 10 min before uptake was started by addition of 25 μM-[^{14}C]urea (6×10^5 Bq mmol^{-1}; ■). Similarly, cell-free extracts were incubated for 10 min at the temperatures indicated prior to the determination of urease activity (●).

Table 1. *Effect of inhibitors and the lack of an energy source on urea uptake in D. venusta strain HG1*

Cells were grown with 10 mM-nitrate. Inhibitors were added 5 min prior to the addition of 25 μM-[^{14}C]urea (6×10^5 Bq mmol^{-1}). CCCP and DCCD were dissolved in ethanol (final ethanol concentration 1%, v/v); ethanol alone had no effect. Urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}.

Inhibitor	Concn (mM)	Urea uptake
Control	–	3·99
Without glucose	–	0·87
CCCP	0·02	0·55
DCCD	0·02	3·75
Cyanide	1·0	0·16
NaN_3	10	0·51

Table 2. *Effect of monovalent and divalent cations on urea uptake in D. venusta strain HG1*

Cells were grown with 10 mM-nitrate as the nitrogen source. For the uptake assay, cells were suspended in 100 mM-Tris/HCl, pH 7·5, containing 0·5% glucose and the compounds indicated; uptake was started by the addition of 35 μM-[^{14}C]urea (6×10^5 Bq mmol^{-1}). Urease activity is given in nmol ammonium formed (mg protein)$^{-1}$ min^{-1}; urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}.

Compound added	Concn (mM)	Urea uptake	Urease activity
None	–	0·29	422
NaCl	250	3·59	462
	500	4·85	444
	1000	3·06	418
KCl	500	0·34	425
LiCl	500	1·50	463
CsCl	500	0·29	443
$MgCl_2$	500	0·33	454

Effect of inhibitors on urea uptake

Urea uptake in *D. venusta* strain HG1 was an energy-dependent process, as indicated by the effect of various metabolic inhibitors or by the absence of glucose (Table 1). Azide, cyanide and the protonophore CCCP almost completely inhibited uptake, while the ATPase inhibitor DCCD exerted only a minor effect. These results implied that ATP was not the direct driving force for urea uptake, but that an electrochemical ion gradient provided the energy necessary for the uptake reaction.

Inhibition by CCCP was strongly dependent on the pH of the assay; at pH 7·3, a 95% inhibition of urea uptake was observed, whereas at pH 8·0 and pH 8·6, uptake was inhibited by about 70% and 10%, respectively (results not shown). Similar observations have been described for the inhibitory action of CCCP on amino acid transport in three marine bacteria, in one moderate halophile, and to a lesser extent in *Escherichia coli* (MacLeod *et al.*, 1988), and have been attributed to the acidic p*K* value, about 6·1, of this protonophore. With increasing pH, the proportion of the deprotonated, anionic form of CCCP increases; the anion, however, can cross the membrane at only 1% of the rate of the protonated, electrically neutral form (Kasianowicz *et al.*, 1984).

None of the inhibitors described exhibited an inhibitory effect on the urease reaction *in vitro* (data not shown). If passive diffusion were the only mechanism of translocation of urea into the cell, these differences between *in vitro* urease and *in vivo* urea uptake, and those between the kinetic parameters as well as the dependence on temperature and pH, would not have been observed. The findings are evidence for the existence of an energy-dependent translocation of urea via a permease system exhibiting a high affinity towards urea, and that the subsequent hydrolysis via urease is a distinct process in *D. venusta* strain HG1.

Sodium dependence of urea uptake

The *in vivo* uptake of urea in *D. venusta* strain HG1 depended on the presence of sodium in the incubation medium; maximum uptake rates were observed at 500 mM-NaCl, the ambient sodium concentration of seawater. Only negligible amounts of urea were taken up in the absence of sodium, or when it was replaced by other cations (Table 2); only lithium replaced sodium to an appreciable extent. Such a stimulatory influence of

sodium was not observed for the urease reaction *in vitro* (Table 2), providing further evidence for the existence of a urea permease in *D. venusta* strain HG1. A dependence of urea uptake on sodium has also been described for a marine diatom, *Phaeodactylum tricornutum* (Rees *et al.*, 1980). More experiments would be required to decide whether sodium is directly involved in the uptake of urea via a sodium-ion/substrate symport mechanism in *D. venusta* strain HG1, as shown for the uptake of α-aminoisobutyric acid in *Alteromonas haloplanktis* (Niven & MacLeod, 1980), or whether urea uptake indirectly depends on the presence of sodium, which may be necessary for transport and/or oxidation of glucose and thus generation of metabolic energy, required for the urea uptake reaction. Such interdependences between sodium-dependent membrane transport and sodium-dependent oxidation of an energy source have recently been described for another species of *Deleya*, *D. aesta* strain 134, by Berthelet & MacLeod (1991).

Regulation of urea uptake activity

Urea uptake was strongly inhibited by ammonium (Table 3), which delayed the onset of the uptake reaction; this delay was proportional to the amount of added ammonium (results not shown). The possibility that urea and ammonium were taken up by a common transport system exhibiting a higher affinity towards ammonium was ruled out by experiments with methionine sulphoximine (MSX). This compound is known to be a strong inhibitor of GS (Meister, 1974). In *D. venusta* strain HG1, MSX markedly reduced GS activity *in vitro* (97% inhibition at a concentration of 5 mM-MSX; results not shown). MSX relieved the inhibitory effect of ammonium on the uptake of urea (Table 3); in the absence of added ammonium, urea uptake was stimulated more than 2-fold by MSX (Table 3). These results indicate that a product formed intracellularly from ammonium in a GS-dependent reaction inhibited the urea uptake reaction. The finding that added glutamine but not glutamate strongly interfered with the uptake reaction (Table 3) indicated that glutamine was this inhibitor.

In order to confirm this hypothesis, attempts were made to isolate mutant strains of *D. venusta* strain HG1 lacking GS or with impaired enzyme activity. Eight independent mutant strains that depended on the presence of glutamine in the growth medium were isolated after mutagenesis with ethylmethanesulphonic acid. Two of these mutants, *D. venusta* strains HG1-II and HG1-V, exhibited less than 1% of the GS activity measured in the wild-type. An inhibition of urea uptake by ammonium was no longer observed in these strains

Table 3. *Effect of ammonium and MSX on urea uptake and urease activity in D. venusta strain HG1*

Cells were grown with 10 mM-nitrate as the nitrogen source. Ammonium was added 10 s prior to the start of the uptake and enzyme assays, while in the experiments with MSX, cells and cell-free extracts were pre-incubated for 30 min with this inhibitor; uptake was started by addition of 200 μM-[^{14}C]urea (6×10^5 Bq mmol^{-1}). Urease activity is given in nmol ammonium formed (mg protein)$^{-1}$ min^{-1}; urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}.

Inhibitor	Concn (mM)	Urea uptake	Urease activity
None	–	3·59	472
MSX	5	7·72	459
Ammonium	0·1	0·47	480
Glutamine	0·1	0·33	467
Glutamate	0·1	3·58	478
MSX + ammonium	5 + 0·1	7·01	463

Table 4. *GS, urease and urea uptake in D. venusta strain HG1 and the mutant strains HG1-II and HG1-V*

Cells grown in mineral medium supplemented with 1 mM-glutamine and 5 mM-ammonium chloride were washed twice and incubated for 6 h in the absence of an added nitrogen source. Urea uptake activity was measured in the presence or absence of 0·1 mM-ammonium. GS activity was determined by the magnesium-dependent synthetic assay. Enzyme activities are in nmol γ-glutamylhydroxamate (GS) and nmol ammonium (urease) formed (mg protein)$^{-1}$ min^{-1}; urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}.

D. venusta strain	GS activity	Urease activity	Urea uptake	
			$-NH_4^+$	$+NH_4^+$
HG1	417	646	6·72	0·64
HG1-II	2	739	4·25	4·50
HG1-V	2	609	5·47	5·22

(Table 4). These results confirm that it was not the added ammonium which interfered with the uptake of urea in *D. venusta* strain HG1, but that the glutamine formed via GS was probably responsible for the observed inhibition of the uptake reaction. A comparable regulatory effect of intracellular glutamine has been discussed for the uptake of ammonium/methylammonium in *Klebsiella pneumoniae* (Kleiner & Castorph, 1982), *Azotobacter vinelandii* (Jayakumar & Barnes, 1984) and *Escherichia coli* (Jayakumar *et al.*, 1987).

Regulation of synthesis of the urea uptake system

When *D. venusta* strain HG1 was grown with 5 mM-urea as sole nitrogen source, ammonium was excreted into the

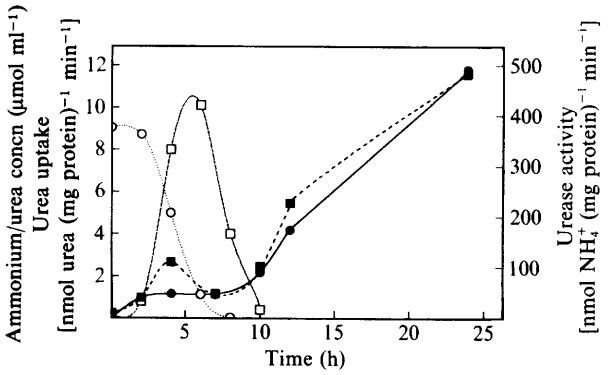

Fig. 2. Urease and uptake of urea in *D. venusta* strain HG1. Cells grown with 20 mM-ammonium as the nitrogen source were washed twice and resuspended in growth medium containing 10 mM-urea as the nitrogen source. □, Ammonium; ○, urea; ●, urease; ■, uptake of 25 μM-[^{14}C]urea (6×10^5 Bq mmol^{-1}).

Table 5. *Influence of the nitrogen source used for growth on glutamine synthetase, urease and urea uptake activity in D. venusta strain HG1*

Cells pregrown with 20 mM-ammonium as the nitrogen source were harvested during exponential growth, incubated in growth medium with the nitrogen sources indicated and harvested during exponential growth (cells incubated without a nitrogen source were harvested after 12 h) GS activity was determined by the manganese-dependent transferase assay. Enzyme activities are in nmol γ-glutamylhydroxamate (GS) and nmol ammonium (urease) formed (mg protein)$^{-1}$ min^{-1}; urea uptake activity is given in nmol urea taken up (mg protein)$^{-1}$ min^{-1}.

Nitrogen source	GS activity	Urease activity	Urea uptake
Nutrient broth	16	18	0·0
NH_4Cl (10 mM)	3	8	0·2
KNO_3 (10 mM)	244	354	14·0
Urea (5 mM)	6	213	1·0
Glutamine (5 mM)	3	99	1·0
Without a fixed nitrogen source	214	26	16·2

medium, and low rates of urea uptake were observed. The activity of the uptake system increased after both urea and ammonia were exhausted (Fig. 2). Cells grown with nitrate and subsequently incubated in growth media containing 20 mM-ammonium showed a rapid decline of urea uptake activity. In contrast, the transfer of ammonium-grown cells to a growth medium without an added nitrogen source resulted in a rapid increase of the urea uptake rate (data not shown). These observations suggest that, as has been reported for several other bacteria studied (Jahns *et al.*, 1988), urea uptake is subject to nitrogen control. The formation of the uptake system is repressed by ammonium, and derepression occurs in the absence of reduced nitrogen compounds.

GS and urease also turned out to be regulated by the nitrogen source supplied for growth. The regulation, however, was not strongly parallel; while urea uptake was derepressed in the absence of a fixed nitrogen source, urease exhibited only low activities (Table 5).

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