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NOTE

Urea production by the marine bacteria *Delaya venusta* and *Pseudomonas stutzeri* grown in a minimal medium

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ABSTRACT: The present experiment showed that the marine bacteria *Delaya venusta* and *Pseudomonas stutzeri* produced urea when grown in a minimal medium without an external supply of organic nitrogen. The urea production rate depended on the bacterial state of growth, and the highest urea accumulation rates in the medium were found in the growth deceleration phase and in the beginning of the stationary phase. Urea did not accumulate in *D. venusta* cells, whereas the intracellular accumulation of urea in *P. stutzeri* cells exceeded urea accumulation in the medium during exponential growth. Further, *D. venusta* could, in contrast to *P. stutzeri*, hydrolyse urea. We suggest that intracellular purines and pyrimidines (in particular RNA) were potential sources for the observed urea production.

KEY WORDS: Marine bacteria · Urea · Production · Turnover · Growth phases

Urea is an important organic nitrogen compound in the marine environment (e.g. Remsen 1971, McCarthy et al. 1977, Sörensson & Sahlsten 1987, Price & Harrison 1988, Lomstein et al. 1989, Cochlan & Harrison 1991, Therkildsen & Lomstein 1994). Urea production has been related to the presence and input of readily degradable organic material (Satoh 1980, Lomstein et al. 1989, Therkildsen & Lomstein 1994) and the highest concentrations of urea have been found in the sediment surface, which is characterized by a low C/N ratio. A low C/N ratio has been used as an indicator of high quality organic matter (Blackburn 1986, Lomstein et al. 1989, Therkildsen & Lomstein 1994). Bacteria can play a significant role in the overall production of urea in marine sediments (Pedersen et al. 1993a, Therkildsen & Lomstein 1994). Purines and pyrimidines are potential precursors for bacterial urea production (e.g. Vogels & Van der Drift 1976, Busse et al. 1984, Gottschalk 1986, Kaspari & Busse 1986). A substrate addition experiment with anoxic, defaunated marine sediment showed a considerable urea production, when the sediment was supplemented with adenosine 5'-monophosphate (AMP), cytidine 5'-monophosphate (CMP), and 16S ribosomal RNA (Therkildsen et al. 1996). However, bacterial urea production from purines and pyrimidines is even more favourable under oxic conditions (Vogels & Van der Drift 1976). Bovine serum albumin did not stimulate sediment urea production, which indicated that urea was not an important intermediate in protein degradation (Therkildsen et al. 1996). Culture experiments with *Thiosphaera pantotropha* showed that the bacteria produced urea during exponential growth when cultured under oxic and anoxic conditions in a minimal medium (Pedersen et al. 1993b).

The present experiment was designed to investigate urea production by the marine bacteria *Delaya venusta* and *Pseudomonas stutzeri* grown without any supplements of organic nitrogen under oxic conditions. Urea production was followed during the different growth phases of the cultures. *D. venusta* is an aerobic bacterium belonging to the family Halomonadaceae. The uptake, transport and turnover of urea by *D. venusta* has been studied by Jahns (1992). *P. stutzeri* is a facultative, aerobic, nonfluorescent, denitrifying pseudomonad commonly encountered in diverse habitats, including marine environments, soil, and sewage (Ward & Cockcroft 1993).

Methods. *Delaya venusta* was grown at 30°C, at pH 7.3, in a minimal medium and under oxic conditions. *Pseudomonas stutzeri* was grown at 18°C, at pH 7.0, in a similar minimal medium and under oxic conditions. The minimal medium contained the following in g l^{-1}: Na_2HPO_4, 2.44; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.05; NaCl, 29.22;

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and 5 ml of the trace element solution described in Finster et al. (1992). Glucose was added to a final concentration of 10 mM.

Samples were withdrawn from the cultures at regular time intervals to follow the change in the urea concentration in the medium, the total concentration of urea (medium+bacteria) and bacterial density. The concentration of urea in the medium was measured on 0.2 µm Nucleopore filtered samples by the diacetylmonoxime method (Mulvenna & Savidge 1992). The total concentration of urea (medium+bacteria) was measured by addition of the acid reagent from the diacetylmonoxime method directly to unfiltered samples. The diacetylmonoxime-sample mixture was thoroughly mixed and reacted for 10 min before centrifugation at 11 000 × *g* for 10 min. The remaining reagents in the Mulvenna & Savidge (1992) urea method were added to the supernatant, and the concentration of urea was measured. Bacterial density was measured as optical density on a Milton Roy, Spectronic 601 spectrophotometer at 600 nm. Additional samples were withdrawn from the cultures in the beginning and at the end of the experiment to measure the NH_4^+ concentration. NH_4^+ was measured by the salicylate-hypochlorite method (Bower & Holm-Hansen 1980).

Urea turnover was measured twice in *Delaya venusta* and *Pseudomonas stutzeri* cultures by the ^{14}C-urea isotope dilution technique (Lund & Blackburn 1989). Eight subsamples (2 ml culture) were collected and transferred to 10 ml sterile glass containers (Exetainer, Labco) in each turnover measurement. Half of the samples were used to follow the change in the concentration of urea during incubation and the remaining samples were injected with ^{14}C-urea. The ^{14}C-incubation was modified slightly from Lund & Blackburn (1989): (1) the incubation was performed as a 4 point time course (~0, 1.0, 1.5, 3.5 h); (2) the activity of the injected 10 µl of tracer was 9.6 nCi µl^{-1} (56 mCi mmol^{-1}; Amersham Radiochemical Center); (3) the incubation was terminated by addition of 2 ml 2.5% NaOH; (4) the scintillation fluid was pH-adjusted OPTIFLUOR™ from Packard (1:10 v/v 0.1 M NaOH:OPTIFLUOR™). Urea turnover rates were calculated by the nonsteady-state model I, described in Lund & Blackburn (1989).

The purity of the *Delaya venusta* cultures were confirmed microscopically before and after the experiment and during the experiment by the characteristic pea (*Pisum* sp.) odour of the cultures. Purity of the *Pseudomonas stutzeri* cultures were confirmed microscopically and by the micromethod API 20 NE for identification of nonenteric Gram-negative rods (bioMérieux) before and after the experiment. *D. venusta* and *P. stutzeri* cultures were purchased from DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

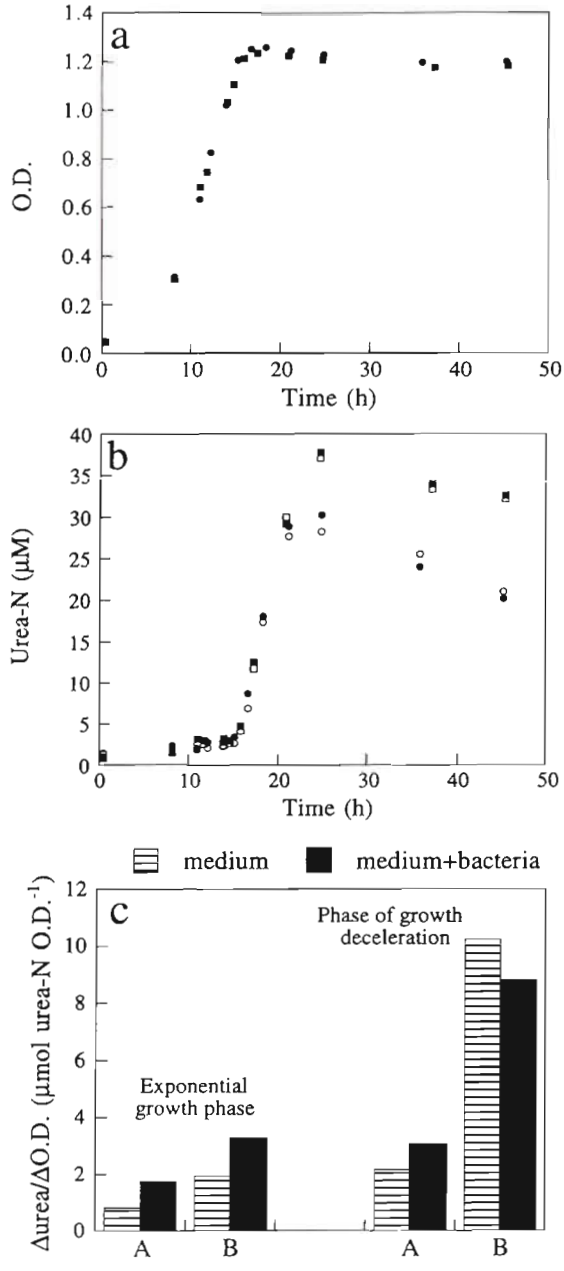

Fig. 1. *Delaya venusta*. (a) Optical density (O.D.) vs time for 2 cultures. Exponential growth phase: from t = 0.0 h to t = 12.5 h; growth deceleration phase: from t = 12.5 h to t = 16.0 h; stationary phase: from t = 16.0 h to t = 50.0 h. (b) Urea concentrations in medium (open symbols) and medium+bacteria (solid symbols) vs time for 2 cultures. (c) Individual Δurea/ΔOD rates during exponential growth and in the growth deceleration phase for 2 cultures (A, B)

Results and discussion. ***Urea production:*** *Delaya venusta* and *Pseudomonas stutzeri* produced urea during the exponential growth phase, in the phase of growth deceleration, and in the stationary phase, when they were grown in a minimal medium under oxic con-

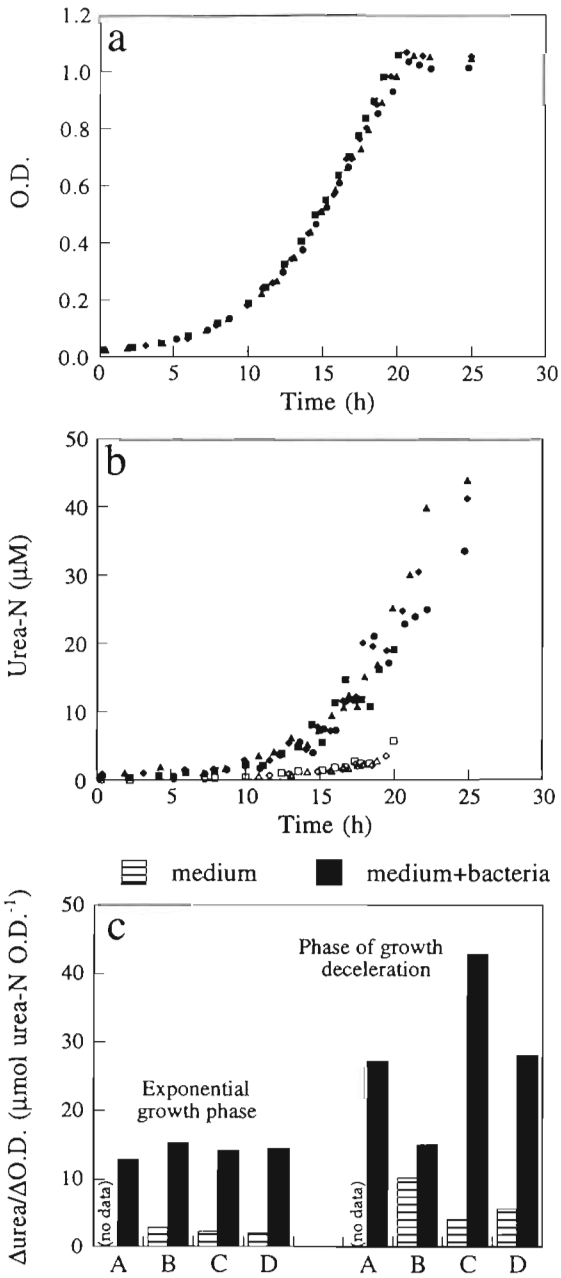

Fig. 2. *Pseudomonas stutzeri*. (a) Optical density (O.D.) vs time for 4 cultures. Exponential growth phase: from t = 1.0 h to t = 16.5 h; growth deceleration phase: from t = 16.5 h to t = 20.0 h; stationary phase: from t = 20.0 h to t = 30.0 h. (b) Urea concentrations in medium (open symbols) and medium+bacteria (solid symbols) vs time for 4 cultures. (c) Individual Δurea/ΔOD rates during exponential growth and in the growth deceleration phase for 4 cultures (A, B, C, D)

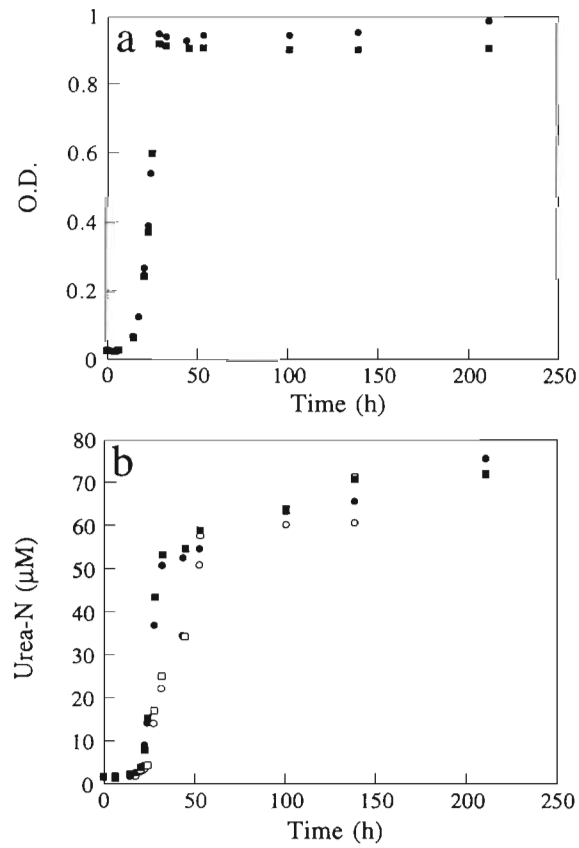

Fig. 3. *Pseudomonas stutzeri*. (a) Optical density (O.D.) vs time for 2 cultures. Exponential growth phase: from t = 10 h to t = 25 h; growth deceleration phase: from t = 25 h to t = 27 h; stationary phase: from t = 27 h to t = 210 h. (b) Urea concentrations in medium (open symbols) and medium+bacteria (solid symbols) vs time for 2 cultures

ditions (Figs. 1 to 3). The different growth phases were as defined by Buchanan (1918).

The change in optical density (OD), the urea concentration in the medium and the summed medium+bacteria urea concentration with time in 2 *Delaya venusta* cultures are shown in Fig. 1a and b, respectively. The mean generation time was 2.84 ± 0.01 h. The urea concentration in the medium and medium+bacteria extract were similar, which indicated that there was no urea accumulation within *D. venusta* cells. The increase in the urea concentration in the medium was proportional to bacterial density (Δurea/ΔOD) during exponential growth (1.9 ± 1.0 µmol urea-N OD^{-1}) and in the subsequent phase of growth deceleration (6.1 ± 4.0 µmol urea-N OD^{-1}). The rates were means of rates from individual cultures. The Δurea/ΔOD for the individual cultures are shown in Fig. 1c. The increase in urea concentration was also linearly related to time (Δurea/Δtime) in the phase of growth deceleration and was 0.7 ± 0.3 µmol urea-N l^{-1} h^{-1}. The ^{14}C-urea turnover incubation took place from t = 14.8 h to t = 18.1 h, and covered the phase of growth deceleration and the beginning of the stationary phase. Urea turnover was ~0.6 µmol urea-N l^{-1} h^{-1}. The highest rate of urea accumulation in the medium was found in the beginning of the stationary phase (from t = 16 h to $t \approx$

22 h), where urea accumulated at a rate of 4.8 ± 0.3 µmol urea-N l^{-1} h^{-1}, despite bacterial density remaining constant. However, after some time (t > 24 h), the urea concentration in the medium declined (Δurea/Δtime = −0.3 ± 0.1 µmol urea-N l^{-1} h^{-1}) due to reduced urea production and a substantial urea turnover (1.8 µmol urea-N l^{-1} h^{-1} from t = 36.0 h to t = 39.8 h). It is likely that *D. venusta* hydrolysed urea during most of the experiment even though NH_4^+ was present in the medium in concentrations >2 mM. Continuous measurements of urea turnover in *D. venusta* cultures, grown under similar conditions, showed that urea turnover took place during all 3 growth phases (authors' unpubl. data). Contrary to these findings, Jahns (1992) found urea uptake by *D. venusta* to be strongly inhibited by an NH_4^+ concentration of 0.1 mM.

Four *Pseudomonas stutzeri* cultures were used to study the exponential and deceleration phases (Fig. 2a, b), while 2 remaining cultures were used to study the stationary phase (Fig. 3a, b). The mean generation time in the *P. stutzeri* cultures was 3.1 ± 0.1 h. Urea accumulation in the medium and in the medium+bacteria were linearly related to bacterial density during exponential growth. The intracellular accumulation of urea exceeded urea accumulation in the medium, as the urea accumulation rates in the medium and medium+bacteria were 2.4 ± 0.4 (n = 3) and 14.1 ± 1.0 µmol urea-N OD^{-1} (n = 4), respectively. The Δurea/ΔOD for the individual cultures are shown in Fig. 2c. A similar difference between the urea accumulation rate in the medium and in the medium+bacteria was found in the growth deceleration phase, where the Δurea/ΔOD increased to 6.7 ± 3.2 and 28.2 ± 11.4 µmol urea-N OD^{-1}, respectively. After the bacteria entered the stationary phase ($t \geq 25$ h), there was an accumulation of urea in the medium and the Δurea/Δtime was ≥1.5 µmol urea-N l^{-1} h^{-1} in the medium and ≥0.7 µmol urea-N l^{-1} h^{-1} in medium+bacteria (Fig. 3b). After ~25 h in the stationary phase the urea concentration in the medium and in the medium+bacteria were approximately the same, and urea continued to accumulate in the medium at a rate of 0.11 ± 0.03 mol urea-N l^{-1} h^{-1} during the rest of the experiment (to t = 210 h). It is not known why *P. stutzeri* cells accumulated urea during exponential growth and in the phase of growth deceleration. There was no urea turnover in the *P. stutzeri* cultures during any of the growth phases and this result was in agreement with results of the API 20 NE micromethod, which showed that there was no urease activity. Bacteria may have an energetic advantage by excretion of urea compared to NH_4^+ in marine sediments, as the sediment concentration of NH_4^+ often is much higher than the concentration of urea (Rosenfeld 1981, Lomstein et al. 1989). The concentration of NH_4^+ in natural surface sediments can vary between <100 and 10000 µM (Blackburn & Henriksen 1981, Rosenfeld 1981, Lomstein et al. 1989). The NH_4^+ concentration in the medium decreased from 7.56 mM at the beginning of the experiment to 2.32 mM at the end of the experiment.

Potential urea precursors: There is a considerable synthesis and break down of internal RNA during bacterial growth (e.g. Norris & Koch 1972, Kjelleberg et al. 1987, Mason & Egli 1993). Some of the RNA is converted to smaller building blocks which is used in the synthesis of new RNA, while part of the RNA is further degraded to urea and NH_4^+. The mRNA degradation, in particular, is high during exponential growth (e.g. Norris & Koch 1972) and may have been a potential source for the observed urea production in the present experiments. The degradation of RNA increases immediately after carbon exhaustion (Mason & Egli 1993) and will ultimately result in a decrease in bacterial RNA content (e.g. Kjelleberg et al. 1987). Accordingly, we found the highest urea production rates in the phase of growth deceleration and when the cultures entered the stationary phase.

Conclusion: The present experiment showed that bacterial urea production can take place without an externally supplied organic nitrogen source and that the urea production rate depended on the bacterial state of growth. We suggest that intracellular purines and pyrimidines, in particular RNA, were potential sources for the observed urea production.

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