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ORIGINAL ARTICLE

Influence of ε-caprolactam on growth and physiology of environmental bacteria

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Abstract ε-Caprolactam was found to have an effect on ecologically important soil bacteria. It inhibited the growth of several *Bacillus* sp. and *Rhizobium* sp. but cells of *Arthrobacter* sp. were able to grow in the presence of caprolactam. *Sphingomonas* sp. lost its inherent capacity to produce extracellular polymer (EPS) if grown in medium containing caprolactam. In the case of raw domestic sewage, the diversity of native bacteria was diminished in presence of caprolactam. Polluted sea water yielded predominantly one type of caprolactam-degrading bacteria of the genus *Achromobacter*. These cells efficiently utilized up to 10 g caprolactam/L as the sole source of carbon and nitrogen in synthetic medium even in the presence of 20 g NaCl/L. Compared to cells of *Arthrobacter* sp., cells of *Achromobacter* sp. accumulated high amount of 6-aminocaproic acid due to degradation of caprolactam. When using caprolactam as sole source of carbon and nitrogen, *Achromobacter* cells showed unique physiological ability to produce EPS upon prolonged incubation in solid medium and in broth with low phosphate (C:N:P ratio 100:20:0.05). Hydrolyzed cell-free EPS had glucose as its major component though the only substrate provided in the medium for growth was caprolactam.

Keywords *Achromobacter* · *Arthrobacter* · Bacteria · Caprolactam · Extracellular polymeric substance · Sewage · Seawater · Soil

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Introduction

Microbial activity in soil has numerous functions contributing to soil fertility. It is involved in organic nutrient cycles, the release of minerals and fixation of nutrients from the air, rendering nutrients more accessible and easily transportable to plant roots, prevention of aggressive plant pathogens taking hold, improving the ability of plants to withstand disease effects, the decrease of inorganic fertilizer loss through erosion and leaching, short-term immobilization, and decrease of persistence of pesticides in soils. However, the soil biological ecosystem (manifested both by microbial and enzymatic activities) can be significantly impaired by the presence of a toxic or xenobiotic compound, in either a reversible or irreversible mode depending on the chemical concentration, persistence (biodegradability), inherent toxicity, bioavailability and the mode of inhibition (Gianfreda and Rao 2008). One such xenobiotic compound is caprolactam. Caprolactam ($C_6 H_{11}NO$) is a man-made chemical used almost exclusively to produce polycaprolactam, commonly known as nylon-6. It has been reported to be toxic to plants and animals (Shama and Wase 1981). Derivatives of caprolactam have been proposed to have antibacterial activity (Bogatcheva et al. 2011). Caprolactam-containing wastewater or solid waste thus poses a disposal problem.

Only some microorganisms that have the ability to utilize caprolactam either as sole growth substrate or in presence of other carbon sources, especially in controlled waste treatment plants or in laboratory studies, have been reported (Litvinenko et al. 1993; Kulkarni and Kanekar 1998; Wang and Lee 2007). There are no reports of the effect of caprolactam on various individual environmental bacteria. The present study shows the inhibitory effect of caprolactam

on some typical bacteria and alteration in the physiology of bacteria that otherwise play an important role in the environment from the perspective of microbial ecology and agriculture. The present study also describes for the first time the ability of an *Achromobacter* sp. obtained from polluted seawater to utilization high concentrations of caprolactam as a sole substrate for growth. A unique physiological feature was the production of an exopolysaccharide (EPS) composed of glucose, although the only carbon source supplied in the medium was caprolactam—a toxic and xenobiotic compound.

Materials and methods

Bacteria

Soil isolates *Bacillus*, *Rhizobium* and *Sphingomonas* and *Arthrobacter* were procured from the culture collection of the Department of Microbiology, MS University of Baroda, India. Locally procured raw domestic sewage was used as the source of sewage bacteria. *Achromobacter* sp. was isolated from polluted seawater and identified at the Microbial Type Culture Collection (MTCC) and Gene Bank, IMTECH, Chandigarh, India (Baxi and Shah 2007).

Media

The growth media used for the growth of each respective bacterial isolate contained the appropriate carbon, nitrogen sources and other nutrients. For example, for the growth of *Sphingomonas paucimobilis* and *Rhizobium* sp., the basal synthetic medium was supplemented with either 40 g sucrose/L and 1 g potassium nitrate/L or 10 g mannitol/L and 1 g sodium glutamate/L, respectively. The synthetic basal medium used contained (in g/L) KH_2PO_4 0.2, K_2HPO_4 0.6, NaCl 0.3, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot H_2O$ 0.1, $FeCl_3$ 0.1. The pH was adjusted to 7.2±0.2. *Achromobacter* sp. was isolated and subsequently grown in the basal synthetic medium supplemented with 10 g caprolactam/L as sole source of carbon and nitrogen. The concentration of sodium chloride and phosphate was varied as mentioned in the text. All the media were autoclaved at 115 °C for 20 min. For study of accumulation of 6-ACA during caprolactam utilization, or for study of 6-ACA utilization, equal quantity of biomass of each culture priorly grown on caprolactam (10 g/L) was centrifuged at 10,000 *g* (Eltek centrifuge, http://www.eltekindia.com) for 20 min, washed and resuspended into either fresh caprolactam medium or 6-ACA medium (at 250 mg/L cell dry weight) and incubated on a rotary shaker (180 rpm) at 30(±2) °C for 4 h and 6-ACA in the culture supernatant was detected by paper chromatography. Culture supernatants obtained after removal of biomass were spotted on Whatman filter paper (number 3) and the chromatograms were developed using a solvent system composed of *n*-propanol: ethyl acetate: ammonia: water (6:1:1:3). 6-ACA was detected using ninhydrin reagent (0.2 % ninhydrin in acetone).

Analytical methods

Growth of bacteria was monitored by measuring the optical density of the broth spectrophotometrically at 600 nm. For EPS-producing bacteria, growth was monitored prior to EPS formation (up to 24 h). The caprolactam content of broth was estimated spectrophotometrically (Bergmann 1952) after removal of cells by centrifugation. Viscosity of the broth was measured using Brookfield DV II+viscometer using a small sample adapter at a spindle speed of 1 rpm. Polymer was extracted from the culture supernatant of broth after removal of cells from the broth by high speed centrifugation. Protein was detected using the standard methods described by Bradford (1976) and Lowry (1951). The total carbohydrate content of cell free polymer was estimated spectrophotometrically (Dubois et al. 1956). Uronic acid was detected

Table 1 Effect of caprolactam on the growth ability of soil bacteria. Bacterial isolates were inoculated into 50 mL different media in 250 mL Erlenmeyer flasks and incubated on a rotary shaker (180 rpm) at 30(±2) °C for 24 h–7 days. −No growth; + growth (turbidity OD 600_{nm}=0.1–0.6); P+exopolysaccharide (EPS) formation

Culture	Property of culture	Growth medium[a]	Growth medium with caprolactam[b]
Bacillus sphaericus	Insecticidal	+	−
Bacillus brevis	Anti-fungal	+	−
Bacillus sp.	Anti-fungal	+	−
Bacillus sp.	Biosurfactant producer	+	−
Rhizobium sp.	Nitrogen fixation	+	−
Sphingomonas paucimobilis	Exopolysaccharide production	+, P +	+
Arthrobacter citreus	Degradation of amides	+	+

[a] Growth medium used for the routine growth of each respective isolate contained the appropriate carbon, nitrogen sources and other nutrients

[b] 10 g caprolactam/ L was added to the medium

Table 2 Effect of caprolactam on growth ability of domestic sewage water microflora. Raw sewage (50 mL) was incubated in 250 mL Erlenmeyer flasks on a rotary shaker in either the absence (−) or presence (+) of caprolactam (5 g/L). The viable count and bacterial diversity were observed using rich medium (Luria agar)

Colony forming units /mL						Different morphological types of colonies					
− Caprolactam			+ Caprolactam			− Caprolactam			+ Caprolactam		
0 h	3 h	18 h	0 h	3 h	18 h	0 h	3 h	18 h	0 h	3 h	18 h
2.5×10^5	2.3×10^6	4×10^6	3×10^5	1.4×10^7	2.6×10^7	4	4	4	4	1	1

spectrophotometrically (Dische 1962). The EPS was hydrolyzed using 2 M sulfuric acid at 100 °C for 0.5 to 2 h. The hydrolysate was neutralized and the products were analyzed by chromatography. The presence of glucose in the hydrolysate was confirmed using the glucose oxidase method according to details provided in the commercial kit (Qualigens, Mumbai, India) based on the original method of Huggett and Nixon (1957). Detection of 6-aminocaproic acid was done on chromatograms using ninhydrin reagent. All experiments and analyses were done twice or in triplicate sets.

Results and discussion

The viable count of bacteria (colony forming units of bacteria / g soil) decreased from 2×10^{11} to 1×10^7 when fertile garden soil was incubated for 30 days with solid waste of a nylon-6 plant. The solubilized solid waste contained caprolactam as a major constituent (Baxi and Shah 2007). The viable count remained the same in soil incubated in the absence of waste. Thus the effect of caprolactam on individual terrestrial bacteria was studied. The model bacteria chosen were either dominant culturable soil bacteria or important heterotrophic bacteria (Maier and Pepper 2000). Some laboratory maintained soil bacterial isolates of selected genera used were of the genera *Bacillus*, *Rhizobium*, *Sphingomonas* and *Arthrobacter*. Each of the bacterium used had a potential for a beneficial ecological role or application. In the absence of caprolactam, each of these soil bacteria grew profusely in their respective optimized medium containing the optimized sources of carbon (sucrose, glucose, or mannitol) and nitrogen (ammonium, nitrate or peptone) and other inorganic nutrients and growth factors. However, bacteria of the genera *Bacillus* and *Rhizobium* were not able to grow in their respective optimized growth medium if caprolactam (10 g/L) was added as an additional ingredient to the optimized medium (Table 1). This result indicated a general toxic effect of caprolactam on survival of bacteria. *Sphingomonas paucimobilis* GS1 was able to grow in presence of caprolactam but it was unable to utilize caprolactam as sole source of carbon. However, the conspicuous property of formation of EPS was lost. *Arthrobacter citreus* is an amide-utilizing soil isolate and was thus able to grow in the presence of caprolactam—a cyclic amide.

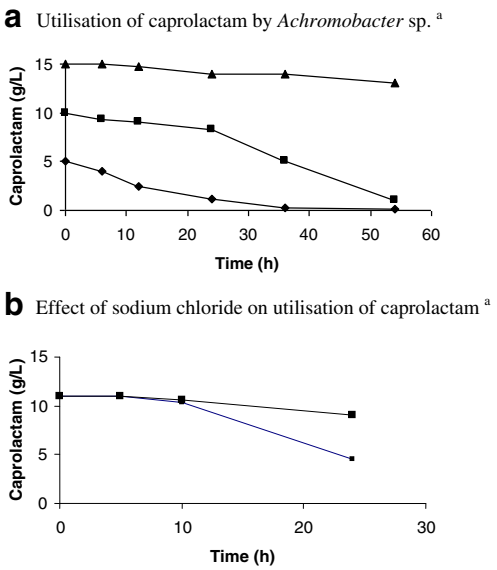

Fig. 1 **a** Utilization of caprolactam by *Achromobacter* sp. **b** Effect of sodium chloride on utilization of caprolactam. Bacterial cells were grown in 50 mL basal synthetic medium containing 5–15 g caprolactam/L as the sole source of carbon and nitrogen, in 250 mL Erlenmeyer flasks on a rotary shaker (180 rpm) at 30(±2) °C. **a** ♦, ■, ▲5, 10, 15 g caprolactam/L, respectively. **b** ■, ▪=0.3, 20 g NaCl /L, respectively with 10 g caprolactam/L

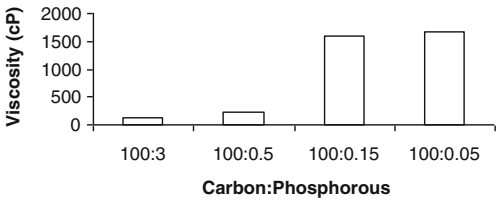

Fig. 2 Effect of phosphate on the viscosity of culture broth of *Achromobacter* sp. The bacterial cells were grown as described in Fig. 1. The medium contained 10 g caprolactam/L. Varying concentrations of phosphates were used to attain different C:P ratios (100:3 to 100:0.05). Medium with 0.6 g K_2HPO_4 and 0.2 g KH_2PO_4 /L gave a C:P ratio of 100:3. The viscosity (centipoise) of the broth was measured using Brookfield DV II+viscometer using a small sample adapter at a spindle speed of 1.0 rpm

Subsequently, several diverse samples of soil / sediment from different locations such as garden, rhizosphere, garbage dump site, riverbed, sea coast were screened for isolation of caprolactam-degrading bacteria. The failure to obtain potent caprolactam-degrading organisms from locations that have not been exposed to caprolactam or other pollutants could be due to the absence of such organisms or due to the toxic effect of caprolactam on soil microflora.

When raw domestic sewage was incubated in the presence of caprolactam, after 3 h of incubation it was found that the diversity of native bacteria present in raw domestic sewage was diminished (Table 2). The types of colonies of different, morphologically distinct bacteria initially present was clearly decreased and only one typical bacterial species eventually survived in the raw sewage. Diversity of bacteria in sewage is a property that is very important for self purification and stabilization of sewage and, if adversely affected, the sewage will not undergo sufficient remediation. Although the total number of sewage bacteria increased 10-fold upon incubation in the absence of caprolactam as compared to 40-fold in the presence of caprolactam, the latter increase was due to only one single morphological type of bacteria. The morphology of the isolate was rod shaped (Gram negative). As the characteristics of sewage may change on a daily basis, the isolate was not further characterized.

The polluted seawater sample also yielded only one prominent Gram negative bacterial isolate, *Achromobacter* sp., which could also utilize caprolactam as the sole source of carbon and nitrogen. Caprolactam (10 g/L) was consumed efficiently by the cells in broth (Fig. 1a). The supplementation of 20 g/L sodium chloride did not inhibit the utilization of caprolactam (Fig. 1b) probably because the isolate was obtained from seawater. Though *Achromobacter* sp. has been reported to be able to act upon aminocaprolactam and oligomers of nylon-6 (Kinoshita et al. 1975), this is the first report of an *Achromobacter* sp. degrading caprolactam, whose structure and stability differ from those of the former two substrates. Caprolactam contains an epsilon lactam (cyclic/internal amide) bond, which is much more stable (with respect to physicochemical and enzymatic hydrolysis) than the linear amides found in acetamide, or the linear oligomers found in nylon or in smaller lactam rings (beta lactam). Similarly, aminocaprolactam is labile to high temperature (degrades to lysine) but caprolactam is stable even to autoclaving (121 °C).

The broth culture of *Achromobacter* exhibited an increase in viscosity after 24 h of incubation, indicating accumulation of EPS. The appearance of highly mucoid colonies on medium containing caprolactam as sole source of carbon and nitrogen after prolonged incubation for 6–7 days at 30(±2) °C and subsequent storage at 4–8 °C was another unique physiological feature. This is the first report of synthesis of EPS by a bacterium whilst using only caprolactam—a xenobiotic and

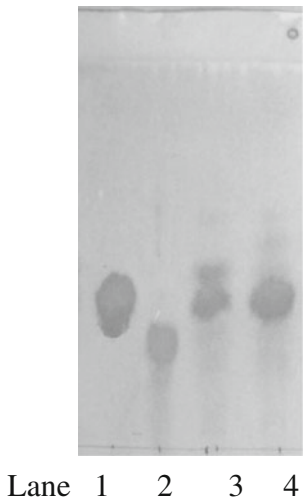

Fig. 3 Chromatogram of hydrolysed extracellular polymeric substance (EPS). The chromatogram was developed using *n*-butanol: acetic acid: diethyl ether: water (9: 6: 3: 3.5). Components were detected using silver nitrate reagent specified for carbohydrates. Lanes: *1* Glucose; *2* maltose; *3*, *4* EPS hydrolyzed for 1.5 h or 2 h, respectively, using 2 M sulfuric acid at 100 °C

Table 3 Functional groups present in the extracellular polymeric substance (EPS) as assessed by infrared (IR) spectroscopy analysis using the KBr pellet method

Frequency (cm^{-1})	Likely linkage / functional groups
3,437.36	-OH -H bonded / CHO unsubstituted
2,930.27	-CH stretching
1,736.94	-COOR asymmetric
1,590.99	C=O | O
1,455.27	-CH bending
1,414.94	C = O stretching asymmetric
1,376.07	-CH deformation
1,255.30	C-O-C stretching / acetyl glycosidic
1,041.97	OH deformation
889.98	C-O-S

Table 4 Accumulation and utilization of 6-aminocaproic acid (6-ACA) by bacterial cultures

Bacterial culture	Presence of 6-ACA (at 4 h of incubation)	
	In caprolactam medium[a]	In 6-ACA medium[a]
Achromobacter sp.	++	+++
Arthrobacter citreus	+	+

[a] Medium contained inorganic salts and either caprolactam or 6-amino-caproic acid as sole substrate (10 g/l) . +, ++, +++ indicate relative amount of 6ACA formed or residual remaining after utilization

toxic compound—as the sole source of carbon and nitrogen. The production of EPS by xenobiotic-utilizing bacteria of genera *Pseudomonas* or *Sphingomonas* (Stolz 2009) is reported so far only in one case of *Pseudomonas mendocina* P2d (Royan et al. 1999), which produced EPS when the substrate was sodium benzoate. Exponentially growing cells of *Pseudomonas mendocina* P2d were incapable of producing EPS. Hence the authors proposed that the EPS was formed as a protective measure against the toxic effects of benzoate.

EPS formation has several important functions in microbial ecology in specific environmental niches (Harrah et al. 2006; Weiner 1997; Bhaskar and Bhosle 2005). Microbial EPS have also been produced in vitro using rich or synthetic media, and media with a high substrate ration of carbon to other nutrients (Sutherland 1996). In the case of medium containing caprolactam as the sole source of carbon and nitrogen, the C:N ratio due to caprolactam ($C_6H_{11}NO$) was always 100:20. But a decrease in the level of phosphorous (P) enhanced the production of EPS and caused a marked increase in broth viscosity (Fig. 2). Maximum viscosity was obtained when C:P was 100:0.15 or 100:0.05, but if the concentration of P was decreased further, the growth of bacterial cells ceased. Probably the level of P had a role in triggering the formation of EPS, and depletion of P led to biosynthesis of EPS during growth on caprolactam. This finding is important because, although wastewaters are usually deficient in phosphate, cells of *Achromobacter* will be able to grow, degrade caprolactam and produce EPS/ biofilm also in low phosphate conditions.

The EPS of *Achromobacter* was harvested from the viscous broth containing caprolactam and a low level of phosphate (C:P=100:0.15). Protein and uronic acids were not detected in the EPS. The EPS contained 60–65 % carbohydrate as estimated by the total carbohydrate estimation method. The acid-hydrolyzed EPS contained, glucose (Rf=0.37; Fig. 3). A progressive time course hydrolysis of the polymer was carried out for 0.5 h to 6 h; intensity of spots was maximal at 2 h after which spot intensity decreased. Amino sugars were concluded to be absent as no ninhydrin-positive compound was detected on TLC. The presence of glucose in the hydrolysate was confirmed using the glucose-specific glucose oxidase method. The glucose concentration estimated by this method was 15 %, and appeared to be less than the amount of glucose evident from chromatography analysis. It was possible that some component present in the acid-hydrolyzed EPS had an inhibitory effect on the activity of glucose-detection enzymes. The glucan type of polymer was analysed by infrared (IR) spectroscopy and the results (Table 3) indicated the absence of acid and peptide linkages.

The formation of this glucan-type EPS from caprolactam was not a feature of all caprolactam-degrading bacteria because the other caprolactam-degrading bacterial isolate obtained from soil and *Arthrobacter citreus* did not produce EPS even in medium containing low phosphate (C:P=100:0.15 or 100:0.05). Another striking physiological difference was that the culture supernatant of *Achromobacter* sp. showed accumulation of 6-aminocaproic acid (6-ACA—the product of hydrolysis of caprolactam) whereas the same was not true for *Arthrobacter citreus* (Table 4). The further metabolism of 6-ACA has been shown to be the "bottleneck" of caprolactam degradation, and ACA-accumulating strains grow poorly on caprolactam (Litvinenko et al. 1993). The two different caprolactam-utilizing bacteria differed in their metabolism of caprolactam. *Achromobacter* (but not *Arthrobacter*) accumulated 6-ACA while degrading caprolactam and if 6ACA (instead of caprolactam) was provided directly as a substrate, utilization of this 6ACA by *Achromobacter* was slower than that by *Arthrobacter*. This indicated that *Arthrobacter* could utilize caprolactam at a faster rate than *Achromobacter*, which instead accumulated ACA or EPS.

Concluding remarks

The growth of several bacteria of soil and sewage origin were inhibited in the presence of caprolactam and its presence led to alterations in the physiology of *Sphingomonas* sp. *Achromobacter* sp. utilized caprolactam as the sole source of carbon and nitrogen. *Achromobacter* sp. had different physiology as compared to *Arthrobacter* sp. with respect to 6ACA accumulation and production of EPS using caprolactam as sole nutrient. Such EPS was composed of glucose, although the only source of carbon supplied was caprolactam. The EPS-based biofilm of *Achromobacter* thus has great potential for use in the bioremediation of water/ wastewater containing caprolactam.

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