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First Evidence of Aerobic Biodegradation of BTEX Compounds by Pure Cultures of *Marinobacter*

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Abstract *Marinobacter vinifirmus* was shown to degrade toluene as sole carbon and energy source under aerobiosis and at NaCl concentrations in the range 30–150 g/L. Maximum toluene consumption rate, total CO_2, and biomass productions were measured in the presence of 60 g/L of NaCl. Under these conditions, 90% of the carbon from toluene was recovered as CO_2 and biomass. Maximum specific toluene consumption rate was about 0.12 mgC toluene mgC biomass^{-1} h^{-1} at NaCl concentrations between 30 and 60 g/L. It decreased to 0.03 mgC toluene mgC biomass^{-1} h^{-1} at 150 g/L. Besides toluene, *M. vinifirmus* degraded benzene, ethylbenzene, and *p*-xylene. Benzene and toluene were utilized to a lesser extent by another *Marinobacter* sp., *Marinobacter hydrocarbonoclasticus*.

Keywords Halophilic bacterium · Toluene biodegradation · Aerobic · *Marinobacter hydrocarbonoclasticus* · *Marinobacter vinifirmus*

Introduction

It is known that polluted hypersaline effluents represent 5% of all treated wastewater [1]. In addition, wastewaters from oil exploration and production or industrial activities can display a wide range of salinities suitable for moderate to extreme halophiles. An exhaustive review of hydrocarbon biodegradation by halophilic microorganisms in saline environments was recently published [2]. It restates previous evidence of hydrocarbon biodegradation by halophilic pure archaeal and bacterial strains in diverse hypersaline environments [3–9]. A biotechnical approach has been taken showing bioremediation of hypersaline effluents on a large scale using a bench-scale sequencing batch reactor (SRB) with moderate and extreme halophilic consortia. For example, two studies conducted in an SRB system showed that 99% phenol in a hypersaline wastewater could be removed by

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bioaugmentation of moderate halophiles [10], and 88% of soluble total organic carbon could also be removed after inoculation of halophilic consortium to treat industrial hypersaline effluent (120 g NaCl/L) [1].

The ability of aerobic halophilic or halotolerant bacteria to degrade petroleum compounds such as polycyclic aromatic hydrocarbons, simple aromatic compounds, and *n*-alkanes has been demonstrated, but little information is available on their ability to degrade light aromatic compounds such as benzene, toluene, ethylbenzene, and xylene (BTEX). To our knowledge, the only study reporting on BTEX biodegradation in hypersaline environments was published by Nicholson and Fathepure [11]. They showed that BTEX compounds were degraded under aerobic conditions by a mixed halophilic bacterial consortium containing *Marinobacter* spp. in 1–2 weeks. Also, [^{14}C] benzene was converted to [$^{14}CO_2$], thus indicating that benzene was completely oxidized at 150 g/L of NaCl. No benzene degradation was observed beyond 150 g/L of NaCl. These results therefore suggest that *Marinobacter* spp. may be an active oxidizer of benzene and potentially of other volatile organic compounds.

In the present study, we report evidence that pure cultures of *Marinobacter* (e.g., *Marinobacter vinifirmus* and *Marinobacter hydrocarbonoclasticus*) aerobically degrade BTEX compounds. We also demonstrate that the recently isolated *M. vinifirmus* can sustain toluene degradation at NaCl concentrations up to 120 g/L.

Materials and Methods

Inocula

M. vinifirmus (DSM 17747^T) was isolated from a hypersaline industrial wastewater (120 g salt/L) from a tartaric acid production plant. This strain grows optimally at 50–60 g NaCl/L and 30°C and uses organic acids (e.g., acetate, lactate, and succinate) but not sugars as carbon and energy sources [12].

M. hydrocarbonoclasticus (ATCC 49840^T) was isolated from Mediterranean seawater near a petroleum refinery. It grows optimally at 35 g NaCl/L and 32°C and degrades a wide variety of aliphatic hydrocarbons [5].

Chemicals

Benzene, toluene, ethylbenzene, and *p*-xylene with a purity of ≥99.5% were obtained from Aldrich-Sigma (France).

Culture Media

Both strains were aerobically cultivated in a synthetic basic medium containing (grams per liter): NH_4Cl, 1; $MgCl_2$ ($6H_2O$), 0.2; $CaCl_2$ ($2H_2O$), 0.2; K_2HPO_4, 0.6; KH_2PO_4, 0.6; and NaCl, 60; supplemented with 1 mL of trace elements [13]. The pH was adjusted to 7.3 with 10 N KOH stock solution. Cultures were sterilized by autoclaving for 20 min at 120°C.

Microcosm Experiments

Penicillin bottles (115 mL), stoppered with Teflon valves (Mininert, Alltech, France), were used in microcosm experiments to evaluate the biodegradation of the BTEX by *M.*

vinifirmus and *M. hydrocarbonoclasticus*. Aliquots of 16 ml of culture medium were placed in the bottles. Microcosms were previously inoculated with 18 mg dry biomass/L. Cultures were incubated at 30°C under aerobic conditions in a rotary shaker (125 rpm), and 2 μL of each BTEX compound, corresponding to 1.60 mgC, was added to the bottles. All the concentrations of BTEX compounds, CO_2, and biomass productions are expressed as the mass of carbon (mgC) per bottle (total volume of bottle: 115 mL).

First, pre-cultures of *M. vinifirmus* at each salt concentration with 1 g yeast extract/L and 1.60 mgC toluene as carbon source were used. Two successive enrichments with toluene as the sole carbon source were then performed at 60 g NaCl/L. Final biodegradation experiments were conducted in replicate with 1.60 mgC toluene. To determine salinity concentration for optimal toluene degradation rate, NaCl was directly weighed in the bottles before adding NaCl-free basic medium. The NaCl concentrations tested were: 0, 30, 60, 90, 120, and 150 g/L.

In the BTEX experiments, all the compounds were tested separately. *M. vinifirmus* and *M. hydrocarbonoclasticus* were initially grown on basic mineral medium supplemented with yeast extract (1 g/L) in the presence of each BTEX compound at a concentration of about 1.60 mgC per bottle. For *M. hydrocarbonoclasticus*, the BTEX compounds were used separately as sole carbon sources, and for *M. vinifirmus*, 0.5 g yeast extract/L was added to the mineral medium. In both cases, 1.60 mgC of benzene or toluene or ethylbenzene or xylene per bottle were added.

Analyses

BTEX concentrations were measured periodically by withdrawing 100 μL of headspace gas from the microcosm using a 250-μL gas-tight syringe, as commonly done in previous work [14]. The gas samples were injected into a gas chromatograph (GC) (Hewlett-Packard 6890, France) equipped with a flame ionization detector and an HP-5 column (ME Siloxane, Hewlett-Packard, USA). The oven, injector, and detector temperatures were 80°C, 180°C, and 250°C, respectively. Nitrogen (N_2) was used as carrier gas with a flow rate of 25 mL min^{-1}. For each compound tested, GC response was calibrated to relate the integrated peak area to the total mass of compound in the bottle, as in previous work [14]. In the case of toluene, increasing NaCl concentrations (0 to 150 g/L) and toluene concentrations (0.75 to 3 mgC/bottle) were used. The ratio of peak area to mass of toluene was estimated as a function of salt concentration by polynomial regression. For the benzene, ethylbenzene, and *p*-xylene, the calibration curve was established with an NaCl concentration of 60 g/L. Calibration assays were conducted over a broad range of conditions (with and without shaking the microcosms, various incubation times), and no variations in measured data were observed. Salt concentration was the main factor modifying gas partitioning as previously demonstrated [15]. We took this effect into account by calibrating the toluene gas concentration in the headspace for each of the different NaCl concentrations in the culture media. In addition, all calibration assays were performed in duplicate, and repeatability was checked by triplicate measurement. We observed no deviation in abiotic controls during the incubation time of the experiments. A steady state could thus be assumed at each salt concentration.

Carbon dioxide production was measured in 100 μL gas samples on a GC (Shimadzu 8A, Japan) equipped with a thermal conductivity detector (TCD) and a concentric column CTR-1 (Alltech, USA). The carrier gas was helium at a flow rate of 65 mL min^{-1}. The column, injector, and detector temperatures were 45°C, 150°C, and 150°C, respectively.

Carbon Balance and Coefficient Yields

The calculation of carbon balance and coefficient yields was used only for the toluene degradation test by *M. vinifirmus* at increasing salt concentration. The carbon balance describes the recovery of carbon initial source (C- toluene, mgC) into incremental carbon biomass (C- biomass, mgC), total carbon dioxide in gas and liquid phase released (C- CO_2, mgC), and carbon trapped in potential metabolite compounds (C- endogenous carbon, mgC). The resulting relation gives:

$$\text{Carbon balance}\,(\%) : [\text{C} - \text{biomass} + \text{C} - \text{CO}_2 + \text{C} - \text{endogenous carbon}/\text{C} - \text{toluene}] \times 100$$

To estimate carbon biomass, the dry cell weight (DCW) per unit volume of culture liquid was calculated from 580 nm absorbance measurements (A_{580}) with a UV-visible spectrophotometer (Ultrospec 3000 Pro Amersham Instrument, France). One A_{580} unit corresponded to 5.14 g DCW/L. Dry biomass concentrations were expressed in carbon mass per unit volume, assuming carbon to represent approximately 50% of the biomass dry weight. Samples and measurements were duplicated. Standard deviation was about 10%.

Carbon dioxide fraction in the gas phase was estimated at the end of experiments by TCD gas chromatography. CO_2 dissolved as carbonates was measured by adding concentrated HCl to lower the pH to 1.0 and measuring the evolved CO_2 by TCD gas chromatography as described above. Quantity of carbon dioxide (mgC) was calculated using the ideal gas law.

Finally, to measure endogenous carbon, at the end of experiments vials were aerated under sterile conditions, re-sealed, and incubated for 1 week. Carbon dioxide was measured in the headspace by TCD gas chromatography. Coefficient yield $Y_{x/S}$ was determined as the ratio of biomass carbon increment (x) and toluene carbon consumed (S). $Y_{p/S}$ was determined as the ratio of carbon dioxide produced to toluene carbon consumed (S). These two coefficient yields were expressed in mgC/mgC.

Modeling and Determination of Toluene Consumption Rate

The Gompertz model was used to determine toluene consumption kinetics [16]. In this model, consumed toluene is a function of time (t) according to the expression: $S=A\exp[-\text{Bexp}^{(-Kt)}]$, where S is the toluene carbon consumed (mgC), K is the toluene consumption rate (h^{-1}), A is the initial mass of toluene (mgC), t is time (h), and B is the parameter related to the initial conditions (dimensionless). The maximum toluene consumption rate (V_{max}) was calculated from the model parameters as $V_{max}=0.368AK$, expressed in mgC h^{-1}. Maximum specific toluene consumption rate ($V_{max,spec}$) was obtained by dividing V_{max} by the carbon mass of dry biomass and was expressed in mgC toluene mgC biomass^{-1} h^{-1}.

Results

Toluene Degradation by *M. vinifirmus* at Different Salt Concentrations

Figure 1 shows the dynamics of toluene consumption with toluene as sole carbon source at five NaCl concentrations (30, 60, 90, 120, and 150 g/L). Toluene was not degraded in the absence of NaCl. A growth test with yeast extract as the sole carbon source showed that *M. vinifirmus* was unable to grow in the absence of NaCl and confirmed that it was a

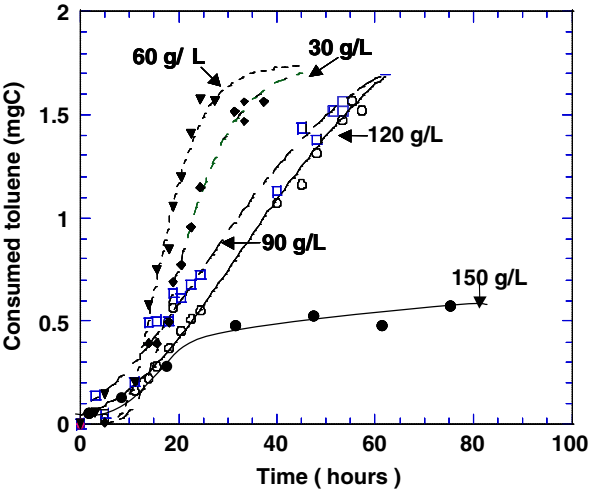

Fig. 1 Toluene consumption by *Marinobacter vinifirmus* strain FB1^T against time with different NaCl concentrations. *Solid and dashed lines* represent data fitted by the Gompertz model. Microcosms were grown with toluene (1.60 mgC/bottle) as sole carbon source. The toluene exhaustion was considered complete when values of total toluene mass consumed were about 1.60 mgC. Autoclaved and non-inoculated controls established for each NaCl concentration tested showed no degradation of toluene (data not shown). The results are means ± 0.05 mgC standard deviations for duplicate microcosms

halophilic bacterium. The fastest toluene degradation was observed at 30 and 60 g NaCl/L. Total toluene degradation was first observed after 35 and 25 h of incubation, in the presence of 30 and 60 g NaCl/L, respectively, and then after 55 h of incubation in the presence of 90 and 120 g l^{-1} NaCl. At 150 g NaCl/L, carbon toluene consumption increased slowly to reach about 1.2 mgC, (about 70% of the initial carbon added) after 24 days. Abiotic control bottles (autoclaved and non-inoculated) showed no evidence of toluene consumption or loss through the Teflon valves (data not shown).

Total CO_2 (TC), carbon biomass (TB) production, and the maximum consumption rate of toluene carbon (V_{max}) were determined for each NaCl concentration (Fig. 2) after substrate exhaustion at 30, 60, 90, and 120 g NaCl/L and after 24 days of incubation at 150 g NaCl/L. In the absence of NaCl, TC, TB, and V_{max} were undetectable. Maximum TC (1 mgC), TB (0.5 mgC) and V_{max} (0.12 mgC h^{-1}) were obtained at 60 g l^{-1} of NaCl. Between 60 and 150 g NaCl/L, TC, TB, and V_{max} followed the same pattern and decreased to 0.4 mgC, 0.15 mgC, and 0.015 mgC h^{-1}, respectively. These results confirm that toluene was a carbon and energy source for *M. vinifirmus* strain FB1^T at 30<[NaCl]<150 g/L. Carbon balance, biomass ($Y_{x/S}$) and carbon dioxide ($Y_{p/S}$) yields, and maximum specific toluene consumption rate ($V_{max,spec}$) were calculated for each NaCl concentration (Table 1). At 60 g NaCl/L, coefficient yields $Y_{x/S}$, $Y_{p/S}$, and $V_{max,spec}$ were approximately 0.21, 0.68, and 0.113 mgC_{tol} mgC_{bio}^{-1} h^{-1}, respectively. Carbon balance was about 90%, showing that almost all the toluene carbon was converted to biomass and CO_2. This result shows that toluene was not only more rapidly degraded at 60 g NaCl/L but was also completely oxidized. At [NaCl]>60 g/L, carbon recovered as biomass and CO_2 was lower by about 50%. In this case, significant amounts of carbon could accumulate in the liquid phase as polymers and/or intermediates of hydrocarbon oxidation, no volatile intermediate of toluene degradation being detected. Also, $V_{max,spec}$ values were about half (0.03 to 0.07 mgC_{tol} mgC_{bio}^{-1} h^{-1}) those of $V_{max,spec}$ at 30 and 60 g NaCl/L. According to these results, toluene mineralization by *M. vinifirmus* seems to be highly dependent on NaCl concentration. No data on $V_{max,spec}$ for halophilic bacteria degrading toluene are available. For comparison, values of $V_{max,spec}$ for *Pseudomonas cepacia* G4 [17], a non-halophilic bacterium, growing on toluene in fed-batch and chemostat at 28°C are between 0.016 and 0.7 mgC toluene mgC^{-1} biomass h^{-1} (assuming 50% carbon in biomass).

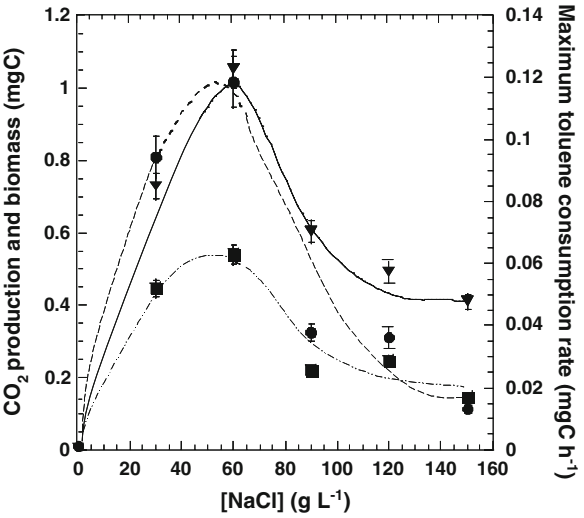

Fig. 2 Maximum toluene consumption rate (*circles*), total CO_2 production (*squares*), and biomass (*inverted triangles*) as a function of NaCl concentration. The rates were obtained by the Gompertz model

BTEX Degradation by *M. vinifirmus* and *M. hydrocarbonoclasticus*

Beside the ability of *M. vinifirmus* to oxidize toluene, we also tested its ability to utilize benzene, ethylbenzene, and *p*-xylene. We also tested BTEX degradation by *M. hydrocarbonoclasticus*, which was reported [5] to degrade only the hydrocarbons tetradecane, hexadecane, phenanthrene, etc. For *M. vinifirmus*, 100% of total benzene and toluene was removed on less than 3 days, while 65% of total ethylbenzene and 20% of total *p*-xylene was removed in 7 days (Fig. 3). In the case of *M. hydrocarbonoclasticus*, over a 7-day period, 10% of benzene, 20% of toluene, 60% of ethylbenzene, and 70% of *p*-xylene were removed. Controls, with autoclaved microcosms (with or without inoculation), showed no significant disappearance of B, T, E, or *p*-X over 7 days.

Discussion

Our results demonstrate for the first time that pure cultures of *Marinobacter* spp. quickly remove toxic volatile compounds such as toluene over a wide range of salt concentrations.

Table 1 Carbon balance, biomass ($Y_{x/S}$), and carbon dioxide ($Y_{p/S}$) yields and maximum specific toluene consumption rate ($V_{max,spec}$) for *Marinobacter vinifirmus* strain FB1^T against NaCl concentration.

NaCl (g/L)	Carbon balance (%)	$Y_{x/S}$ (mgC/mgC)	$Y_{p/S}$ (mgC/mgC)	$V_{max,spec}$ (mgC_{tol} mgC_{bio}^{-1} h^{-1})
30	71.4±7.5	0.23±0.040	0.48±0.073	0.129±0.017
60	89.2±7.6	0.21±0.002	0.68±0.073	0.113±0.02
90	45.3±2.2	0.05±0.002	0.40±0.024	0.063±0.01
120	34.5±7.0	0.07±0.008	0.27±0.078	0.070±0.012
150[a]	35.5±6.9	0.03±0.007	0.33±0.062	0.030±0.008

x biomass, *p* carbon dioxide, *S* toluene

[a] After 24 days

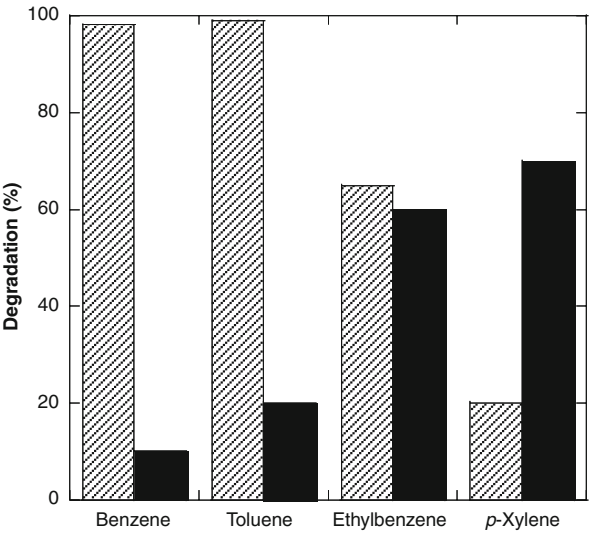

Fig. 3 Percentage of BTEX (benzene, toluene, ethylbenzene, and *p*-xylene) degradation by *Marinobacter vinifirmus* strain FB1^T (*dotted bars*) and *Marinobacter hydrocarbonoclasticus* (*solid bars*) at 60 gL^{-1} NaCl after 7 days. Elimination of benzene and toluene was complete after 3 days for the strain FB1^T. The results are means ±4% standard deviations for duplicate microcosms

Besides, benzene, ethylbenzene, and *p*-xylene were also removed by this bacterium. *M. vinifirmus* removed toluene and benzene faster than *M. hydrocarbonoclasticus*. Removal of ethylbenzene was similar for both strains, while *p*-xylene was preferentially removed by *M. hydrocarbonoclasticus*. The reasons for the differences in the ability of *M. vinifirmus* and *M. hydrocarbonoclasticus* to remove benzene, toluene, ethylbenzene, and *p*-xylene are still unclear.

It is noteworthy that *M. vinifirmus* can sustain toluene biodegradation (1.2 mgC) at 150 g NaCl/L, thus confirming the ecological role played by *Marinobacter* spp. in hydrocarbon oxidation not only in marine but also in hypersaline ecosystems, as previously suggested by Nicholson and Fathepure [18]. In their studies, these authors observed degradation of 1.7 mgC toluene after 6 days incubation at 150 g NaCl/L by a halophilic mixed non-defined microbial culture in which *Marinobacter* spp. were the dominant bacteria, whereas previous studies had shown that an increase in salinity had a detrimental effect on microbial degradation of hydrocarbons [19]. It has been recently reported that *Marinobacter maritimus*, a psychrotolerant microorganism isolated from sea water off the subantarctic Kerguelen Islands, oxidized hydrocarbons and petroleum ethers [20]. However, degradation of light aromatic compounds such as toluene by an axenic culture of a halophilic microorganism had never been reported to date. This has been now demonstrated with *Marinobacter* spp. in this work. Hence, our results, together with those reported recently on *Marinobacter* spp. [11, 20], clearly indicate that members of this genus, possibly metabolically active over a wide range of temperature and salinities, most probably contribute to the overall biodegradation of volatile and non-volatile hydrocarbons in a variety of saline ecosystems, with particular emphasis on marine ones.

Finally, it is clear that besides their ecological significance, *Marinobacter* spp. can be considered potential candidates of industrial interest for decontaminating saline waters containing toxic compounds originating from the petroleum industry.

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