# Preliminary characterization of an estuarine, benzoate-utilizing *Vibrio* sp. isolated from Durban Harbour, South Africa

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High production volume chemicals (HPVCs) are those that are produced in quantities greater than 1000 tons per year in at least one OECD member country. About 65% of these chemicals contain aromatic rings. Due to their important role in modern society, aromatic hydrocarbons tend to be important constituents of wastewaters and, as such, the quantity of these chemicals present in aquatic environments has increased. Nevertheless, many HPVCs are a potential source of carbon and energy and can therefore be exploited by bacteria as growth substrates. During the course of our screening for aerobic, HPVC-utilizing bacteria, an estuarine strain with the ability to utilize benzoic acid was isolated from Durban Harbour. The isolate was tentatively identified as a member of the genus *Vibrio* based on a host of taxonomically-relevant morphological and biochemical characteristics, along with the analysis of the 16S rRNA gene sequence. Growth experiments along with oxygen uptake measurements indicated that the isolate catabolizes benzoic acid via the *ortho*-pathway. With only limited information as to the capability of *Vibrio* spp. to mineralize aromatic compounds, this discovery adds useful information highlighting the metabolic versatility of the genus *Vibrio*.

**Keywords** Biodegradation; high production volume chemicals; benzoic acid, *Vibrio* spp.; *ortho*-pathway

## 1. Introduction

Only limited data are available concerning the environmental fate of anthropogenic chemicals being fed into the marine environment by river discharges or other inputs [1]. High production volume chemicals – an important group of such organic pollutants - are defined as those compounds that are produced in quantities exceeding 1000 tons per year in at least one OECD country [2]. About 65% of those chemicals identified as HPVCs contain aromatic rings [3]. Aromatic hydrocarbons are known to play an important role in modern society and tend to be relevant constituents of domestic and industrial wastewaters. Consequently, the overall concentration of these chemicals in aquatic environments has increased.

Benzoic acid is one such example of an HPVC with an estimated global production capacity of more than 600 000 tons per year [4]. Anthropogenic releases of benzoic acid into the environment largely involve emissions into water and soil from its use as a preservative. Risk assessments involving toxicity tests have concluded that benzoic acid has the potential to cause harm to aquatic life by causing cell multiplication inhibition and developmental defects [4,5].

Contaminated environments are known to harbour a range of pollutant-degrading bacteria that play a crucial role in tackling certain aromatic contaminants. Hence, numerous efforts have been made to characterize bacterial communities and their responses to pollutants. Although many investigations have undertaken to explore the ability of bacteria in freshwater environments to catabolize aromatic pollutants, the catabolic activities of marine and estuarine bacteria have received somewhat limited attention. Indeed, only a limited number of references point toward the capability of *Vibrio* species to utilize aromatic compounds [6].

In the early 1930s a phenol-oxidizing bacterium was isolated from polluted wastewater which, based on certain morphological and biochemical characteristics, was designated *Vibrio* O1 [7]. The organism was found to mineralize a number of simple aromatic compounds (including benzoic acid) and played a pivotal role in the elucidation of the pathways involved in aromatic hydrocarbon catabolism. However, it was later revealed that this original strain had been lost and that many subsequent studies had been conducted with a completely different organism [8].

Kiyohara and Nagao [9] isolated two bacterial strains (designated $s176_P1$ and $s183_P1$) displaying the ability to utilize phenanthrene as a sole source of carbon and energy. These isolates were tentatively identified as *Vibrio* spp. although comprehensive details concerning the morphological and physiological characteristics of these microorganisms were not documented. Isolate $s176_P1$ could utilize naphthalene, salicylic and benzoic acid. Similarly, $s183_P1$ utilized salicylic acid and benzoic acid but was unable to use naphthalene.

A more recent study reports the isolation and detailed characterization of an aromatic hydrocarbon-degrading strain of *Vibrio cyclotrophicus* (now *V. cyclitrophicus*) from marine sediments contaminated with creosote [10]. Growth experiments showed that the isolate utilized several two- and three-ring aromatic hydrocarbons, including phenanthrene and naphthalene as a sole source of carbon and energy.

There is only limited information available concerning the capability of members of the genus *Vibrio* to mineralize aromatic compounds. The discovery of a new estuarine *Vibrio* isolate displaying the ability to utilize benzoic acid aerobically as a sole source of carbon and energy is therefore highlighting the metabolic versatility of the genus *Vibrio*.

In addition, it indicates that members of this genus might exhibit an important function in the elimination of aromatic pollutants in estuarine environments.

## 2. Materials and methods

### 2.1 Isolation and identification of the organism

*Vibrio* sp. strain KM1 was isolated from enrichment cultures inoculated with water samples collected from Durban Harbour (Kwa-Zulu Natal, South Africa) by using benzoic acid (1.5 mM) as the sole source of carbon and energy and initially using an artificial seawater medium [11]. The morphological and physiological characterization of the isolate was performed according to standard procedures [12,13]. In addition two commercially-available biochemical test systems (the VITEK system and the API 20NE) were employed. Appropriate type-strains were used as controls for each physiological characteristic tested.

### 2.2 Media and culture conditions

As it was established that the isolate did not require artificial seawater medium for optimum growth, it was routinely grown using a mineral salts medium containing per liter 3.5 g $Na_2HPO_4 \cdot 2H_2O$, 1 g $KH_2PO_4$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2 \cdot 6H_2O$, 50 mg $Ca(NO_3)_2 \cdot 4H_2O$ and 12g NaCl, and 0.5 ml of a trace elements solution containing per liter 5 g EDTA, 3 g $Fe(II)SO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 50 mg $Co(II)Cl_2 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 20 mg $NiCl_2 \cdot 6H_2O$, 30 mg $Na_2MoO_2 \cdot 2H_2O$, 50 mg $ZnSO_4 \cdot 7H_2O$ and 20 mg $H_3BO_3$ [14]. Benzoic acid was added to a final concentration of 5 mM. Cells were routinely grown in 100 ml Erlenmeyer flasks containing 20 ml of the mineral salts medium, and incubated at 25°C on a bench-top orbital shaker at 200 rpm in the dark. Biomass formation over time was monitored by measuring optical density at 600 nm and by analysis of protein following alkaline lysis of harvested cells (see section 2.7) using the method of Bradford [15]. Total cell counts were obtained by using a bacterial counting chamber (Thoma-Neu, dimensions: 0.0025 $mm^2$ x 0.01 mm). The decrease in benzoic acid concentration over time was analyzed using UV spectroscopy at 275 nm and the disappearance of the aromatic substrate was verified by scanning culture supernatants in a range from 200-400 nm. Cultures incubated in the absence of benzoic acid as the utilizable carbon source served to demonstrate that biomass formation depended on the utilization of this aromatic compound, while incubations without active cells present served as abiotic controls.

### 2.3 DNA extraction, PCR and sequence analysis

DNA from a single colony of the isolate was extracted using a freeze-thaw method. Five freeze-thaw cycles were employed with freezing cells in liquid nitrogen and subsequent heating at 85°C for 3 minutes per cycle. The 16S rRNA gene sequence was amplified using the forward primer fD1 (AGAGTTTGATCCTGGCTCAG) and reverse primer rP2 (ACGGCTACCTTGTTACGACTT) [16]. The parameters for PCR-based amplification were as follows: An initial denaturing cycle at 95°C for 4 minutes, followed by 25 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 3 minutes. A final extension cycle was carried out at 72°C for 7 minutes. The size of the amplification product was verified by gel electrophoresis (2% agarose, TBE buffer pH 8) and the isolated DNA sequenced (Inqaba Biotechnologies, Pretoria, South Africa). The sequence obtained (about 1100 bp) was aligned using the Basic Local Alignment Search Tool (BLAST) (BLAST, www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and compared with small-subunit (SSU) sequences deposited with NCBI GenBank (www.ncbi.nlm.nih.gov) and the RDP database (www.rdp.cme.msu.edu). A phylogenetic tree employing *Vibrio* spp. type strain sequences obtained from RDP was constructed using *MEGA* version 4 [17] based on the neighbour joining method [18] with resampling for 1000 replicates. The 16S rRNA gene sequence of *E. coli* was used as an out-group.

### 2.4 Microscopy

Cells were routinely examined and counted by phase contrast microscopy (Motic, BA310). Cell morphology and flagellation was verified by TEM (Philips, CM120 Biotwin) using formvar-coated copper grids and staining of an overnight culture with 2% phosphotungstic acid.

### 2.5 Utilization of additional aromatic compounds

The ability of the organism to utilize other carbon sources structurally related to benzoic acid was tested. The organism was grown in mineral salts medium supplemented separately with phenol, *p*-cresol, salicylate, catechol, 3,4-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid at a concentration of 1.5 mM. Cultures were incubated as stated previously. The turbidity of each culture was observed every 24 hours for 72 hours by measuring the optical density at 600 nm.

 

### 2.6 Specific oxygen uptake

Cells grown to the late exponential phase in mineral salts medium supplemented with either 5 mM benzoic acid or succinic acid were harvested by centrifugation (10000 x g, 4°C), washed three times with phosphate buffer (20 mM, pH 7.4, containing 200 mM NaCl) and resuspended in the same buffer to an optical density at 600 nm of 0.8. The ability of resting cells to oxidize selected aromatic compounds was verified by measuring specific oxygen uptake rates at 25°C using a Hansatech Oxytherm Electrode Unit. The final concentration of test compounds was 1 mM unless otherwise stated. Endogenous respiration rates were measured prior to adding the test compound and accounted for.

### 2.7 Protein determination

The lysis of cells prior to protein determination was obtained by heating cells for 10 minutes at 95°C in the presence of 0.15 N NaOH. Bradford's protein assay [15] was used to determine soluble protein using BSA (bovine serum albumin) as a standard.

## 3. Results and discussion

The strain (designated KM1) isolated from water samples obtained from Durban Harbour (Kwa-Zulu Natal, South Africa) is a Gram-negative, asporogenous, motile and slightly curved rod (about 2.0 x 0.8 μm) with a single, polar flagellum. The bacterium is both oxidase and catalase positive and utilized a wide variety of simple and complex carbohydrates. This mesophilic (optimum growth range 20 – 37°C) and facultatively anaerobic isolate tolerates high NaCl concentrations (8% w/v) but is sensitive to the vibriostatic agent O/129. Strain KM1 was able to hydrolyze starch, a feature that has previously been considered a presumptive positive test for *Vibrio* spp. [19]. Based on these and other relevant results obtained by employing a number of recommended tests [12,13], the organism was tentatively identified as *V. pelagius* (basonym, now *Listonella pelagia*) using a set of biochemical keys specific for the Genus *Vibrio* [20]. Although these keys provide a convenient system for the rapid identification of suspected *Vibrio* isolates, the reliability of this physiological scheme is uncertain as working conditions such as temperature, incubation period and the composition of test media have not yet been standardized [21]. Therefore, it is believed that these keys might be misleading for species identification within the genus *Vibrio* [22].

Two commercial methods (API 20NE and VITEK) were also employed for the elucidation of the biochemical characteristics of the organism. Based on the API 20NE profile, the isolate was identified as *Vibrio fluvialis*; while the VITEK system identified the bacterial isolate as *Vibrio alginolyticus*. These commercial systems have been developed for use in clinical microbiology laboratories for the routine identification of bacteria of medical importance and, as such, the applicability of these systems to the identification of environmental species is uncertain. Indeed, these commercial databases may not encompass enough biodiversity to accurately provide a definitive identification of such isolates [23].

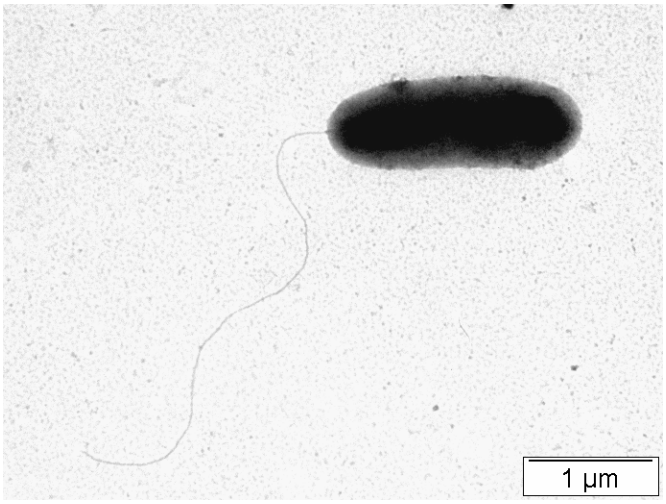


**Fig. 1** TEM image of Vibrio sp. strain KM1 showing a curved, rod-shaped cell with a single polar flagellum. Samples were viewed under high vacuum at a magnification of 18 000 x.

A very important diagnostic feature for the identification of *Vibrio* species has always been the presence of flagella and thus motility. Flagella staining using the method of Blenden and Goldberg [24] and subsequent light microscopy as well as TEM (transmission electron microscopy) revealed the presence of a single, polarly-inserted flagellum (Fig 1) which is keeping with the conventional features described for *Vibrio* species [12].

Sequence analysis of the amplified 16S rRNA gene revealed that strain KM1 exhibited the highest sequence similarity (98%) with *Vibrio natriegens* (GenBank accession number FJ171336.1, V. *natriegens strain* WT01) and an environmental isolate (accession number EU077544.1, *Vibrio* sp. HN011). 16S rRNA gene sequences for *Vibrio* spp. type strains were obtained from the Ribosomal Database Project and a phylogenetic tree (Fig 2) was constructed using MEGA4 [17]. Based on the morphological, physiological and 16S rRNA gene sequence properties of the isolated strain KM1, the organism was assigned to the genus *Vibrio*. However, further genomic profiling including, for example, DNA-DNA hybridization with type strain DNA is required before the isolate KM1 can be assigned to a species.

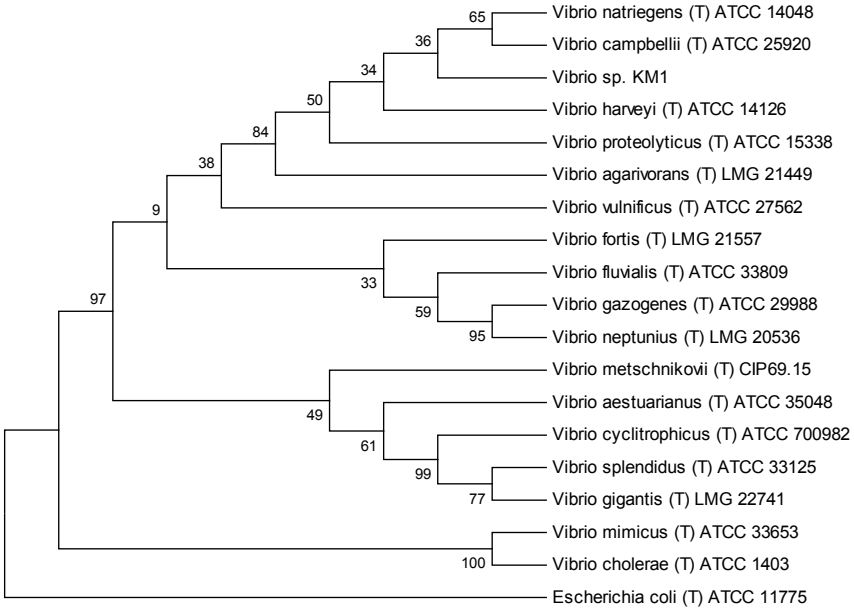


**Fig. 2** Neighbour-joining tree and phylogenetic affiliation of Vibrio sp. KM1 based on selected 16S rRNA gene sequences for type strains obtained from the Ribosomal Database Project. The 16S rRNA gene sequence of E. coli was used as an out-group.

The ability of *Vibrio* sp. KM1 to grow with benzoic acid as the sole source of energy and carbon is demonstrated in Figure 3. Growth of the bacterium expressed as the increase in optical density at 600 nm (data for protein and total cell numbers not shown), was tightly correlated to the removal of benzoic acid from liquid cultures. Cell-free cultures showed no decrease in benzoic acid concentration over time thus verifying that the loss of benzoic acid was not due to abiotic processes.

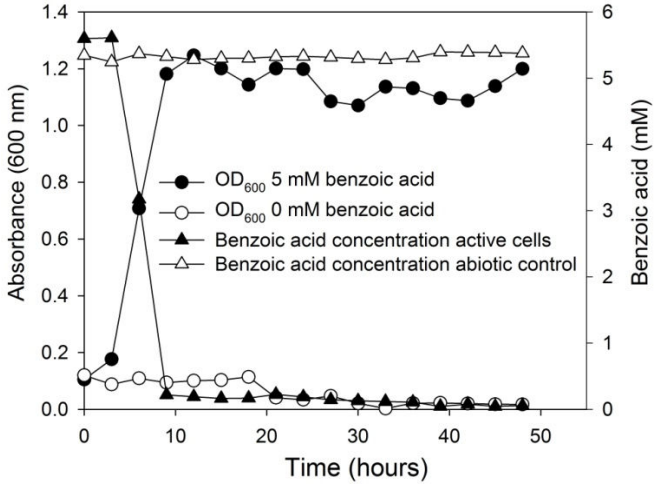


**Fig. 3** Growth of Vibrio sp. KM1 measured as a function of optical density at 600 nm over 48 hours of incubation at 25°C and 200 rpm in the presence of benzoic acid as sole source of carbon and energy.

This is not unexpected on physicochemical grounds as the half life for benzoic acid in the aquatic environment is expected to be rather long due to the limited tendency for volatilization [4]. So far, *Vibrio* sp. KM1 was not able to grow

 

at the expense of phenol, *p*-cresol, salicylic acid, 3,4- or 2,5-dihydroxybenzoic acid, although biomass formation was observed at the expense of catechol. The utilization of other potential substrates is currently under investigation.

The rapid growth observed for *Vibrio* sp. KM1 (doubling time about 1.6 hours) with benzoic acid as a sole carbon and energy source is of particular interest. *V. natriegens* is a marine bacterial species known to exhibit a generation time of 9.8 minutes under optimum conditions and with all nutrients provided [25,26]. Isolate KM1 shows a doubling time of about 24 minutes under similar conditions. This seems to indicate that similar to *V. natriegens* the isolated strain *Vibrio* sp. KM1 is remarkable in exhibiting rather quick growth as an ecophysiological property.

Results obtained from oxygen uptake measurements (Table 1) indicate that the catabolic sequence employed by *Vibrio sp.* KM1 for the oxidation of benzoic acid is induced by growth on the aromatic HPVC benzoic acid. A highly increased specific oxygen uptake rate was detected in the presence of catechol for cells grown with benzoic acid, making benzoic acid metabolism via this intermediate – which was utilized as carbon source by strain KM1 - very likely. Succinate-grown cells were not induced for the oxidation of benzoic acid and catechol.

**Table 1** Substrate specific oxygen uptake rates determined for resting cells of *Vibrio* sp. KM1.

| Substrate | Specific activity* after growth with | |
|---|---|---|
| | Succinate | Benzoic Acid |
| Succinate | 166 | 30 |
| Benzoic Acid | 19 | 242 |
| Catechol | 14 | 199 |

*Specific oxygen uptake rates are given in nmol $O_2$ consumed per minute per mg of protein and are corrected for endogenous respiration. The data shown represent the mean of 4 independently performed experiments.

In order to elucidate the catabolic pathway involved in the productive utilization of benzoic acid by *Vibrio* sp. KM1 as well as to gain insight into the regulation of the catabolic sequence, experimental work is now underway to verify the specific enzymatic steps and key intermediates involved in the mineralization of benzoic acid.

## 4. Concluding remarks

The taxonomic profile obtained for *Vibrio* sp. KM1 confirms that this South African isolate is indeed a member of the genus *Vibrio*. Although there are now about 74 validly described species within the genus, the ecophysiological versatility and biodiversity of these heterotrophic bacteria has not been fully elucidated yet as new species isolated from the marine environment are frequently reported [22]. Although the sequence analysis performed demonstrated that the isolated strain KM1 is closely related to *Vibrio natriegens*, it is quite likely that *Vibrio* sp. KM1 might represent a novel species. The fact that this *Vibrio* isolate can mineralize the high production volume chemical benzoic acid aerobically demonstrates that members of this genus might contribute towards the elimination of aromatic pollutants from estuarine environments.

**Acknowledgements** Financial support provided in the form of a DAAD/NRF postgraduate bursary for Karis Moxley is gratefully acknowledged.

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