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New Findings on Aromatic Compounds' Degradation and Their Metabolic Pathways, the Biosurfactant Production and Motility of the Halophilic Bacterium *Halomonas* sp. KHS3

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Abstract

The study of the aromatic compounds' degrading ability by halophilic bacteria became an interesting research topic, because of the increasing use of halophiles in bioremediation of saline habitats and effluents. In this work, we focused on the study of aromatic compounds' degradation potential of *Halomonas* sp. KHS3, a moderately halophilic bacterium isolated from hydrocarbon-contaminated seawater of the Mar del Plata harbour. We demonstrated that *H*. sp. KHS3 is able to grow using different monoaromatic (salicylic acid, benzoic acid, 4-hydroxybenzoic acid, phthalate) and polyaromatic (naphthalene, fluorene, and phenanthrene) substrates. The ability to degrade benzoic acid and 4-hydroxybenzoic acid was analytically corroborated, and Monod kinetic parameters and yield coefficients for degradation were estimated. Strategies that may enhance substrate bioavailability such as surfactant production and chemotactic responses toward aromatic compounds were confirmed. Genomic sequence analysis of this strain allowed us to identify several genes putatively related to the metabolism of aromatic compounds, being the catechol and protocatechuate branches of β-ketoadipate pathway completely represented. These features suggest that the broad-spectrum xenobiotic degrader *H*. sp. KHS3 could be employed as a useful biotechnological tool for the cleanup of aromatic compounds-polluted saline habitats or effluents.

Introduction

Aromatic compounds are organic molecules containing one or more aromatic rings, specifically benzene rings. Among the major environmental pollutants, these compounds are the most persistent due to the high thermodynamic stability of the benzene moiety.

Contamination of saline and hypersaline environments with aromatic compounds as a result of industrial activities and urban water effluents is frequent, posing a serious environmental problem. Furthermore, over the last few years, oil refineries have generated a large amount of aromatic compounds-contaminated highly saline wastewater during crude oil extraction [1–3], that need to be adequately treated to reduce the contaminant load.

Many of the standard physicochemical treatment processes used to decontaminate wastewater have limited (if any) application in the presence of salt, are prohibitively expensive, or may be only partially effective [4]. In most cases, biodegradation constitutes the more adequate mechanism for pollutants removal [5, 6]. Therefore, the use of salt-tolerant microorganisms (as halophilic archaea and moderately halophilic bacteria) that are able to degrade aromatic compounds is essential in a biodegradation program of saline wastewaters [7–9]. In addition, the study of pollutants biodegradation by native microorganisms is becoming of extreme ecological importance [10], and the indigenous microorganisms based technology is strongly applied for the waste management [11].

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Many reports have been published describing pollutants-degrading bacteria from different genera [12–14] but much less information is available on halophilic degraders [15–19]. Furthermore, studies of genetics, biochemistry, and pathways of aromatic compounds degradation in halophiles and halotolerants are limited [20].

Several studies indicate that bacteria of the genus *Halomonas* are an interesting target to look for potential aromatic compounds degraders of saline contaminated waters [7, 9, 17, 19, 21, 22]. However, there are few studies [23] that go beyond the degradative ability of any of the studied species. In this study, we analyzed (1) the biodegradation of the aromatic compounds such as benzoic acid and 4-hydroxybenzoic acid by *H*. sp. KHS3, a moderately halophilic bacteria recently isolated, (2) the biosurfactant production and chemotaxis ability, and (3) the genetic pathways of aromatic compounds degradation, in order to know the potential of this microorganism for future biodegradation technologies of saline contaminated waters. *Halomonas* sp. KHS3 was isolated from hydrocarbon(HC)-contaminated seawater of the Mar del Plata harbour and is able to grow using gasoil as the sole carbon and energy source [24]. Its draft genomic sequence has recently been published [25].

Materials and Methods

Biological Material

The *H*. sp. KHS3 strain used in this study was previously isolated [24] from HC-contaminated seawater of Mar del Plata harbour based on its ability to grow in gasoil (a complex mixture of aromatic and aliphatic HC) as the sole carbon and energy source.

Screening of the Catabolic Potential of *H*. sp. KHS3

To study the aromatic compound and different HC utilization by *H*. sp. KHS3, the microorganisms were incubated in 20 ml of H1 minimal medium (composition in g l^{-1}: K_2HPO_4, 11.2; KH_2PO_4, 4.8; $(NH_4)_2SO_4$, 2; $MgSO_4$, 0.132; $FeSO_4$, 5×10^{-4}, pH 7) with 3% NaCl (w/v) and a pure compound (Table 1) as the sole carbon and energy source. The concentrations of the substrates were selected according to literature [7, 14, 17, 26–29]. A 3% NaCl (w/v) was chosen for analyses because it is the salt concentration of the seawater within the harbor from where this strain was isolated. A washed cell suspension of a culture pre-grown in kerosene (0.2%; v/v) was used as inoculum. The initial cell concentration was 0.1 (OD_{600nm}). Cultures were incubated at 25 °C in dark at 150 rpm and cell growth was determined periodically by turbidity (OD_{600nm}) using an UV/VIS spectrometer (T80, PG Instruments). Cultures were maintained until bacterial growth reached the stationary phase (from 48 to 120 h depending on the growth substrate employed). Controls without inoculums or without substrate were monitored during 25 days under the same conditions.

A linear correlation between cells number (cells counted by optic microscopy with a Neubauer chamber) as a function of $OD600_{nm}$ was done $Y = 38.73 \times Abs600_{nm} + 11.31$; $R^2 = 0.871$ (Y = cells $\times 10^{-6}$).

Degradation of Aromatic Compounds

In order to analyse the degradation ability of the strain, benzoic acid (BA) and 4-hydroxybenzoic acid (4-HBA) were used as model aromatic compounds since their metabolic pathways have been previously described in detail for other microorganisms [30, 31]. *H*. sp. KHS3 cells were previously acclimated in BA or 4-HBA (50 mg l^{-1}) and then transferred to 20 ml of H1 medium with an initial concentration of 0.1 (OD_{600nm}), in a 500 ml flask. Different concentrations of BA (10, 50, 100, 200, 400 mg l^{-1}) and 4-HBA (10, 25, 50, 100 mg l^{-1}) were tested as the only carbon source. Cultures were incubated at 25 °C in dark at 150 rpm and cell growth was determined periodically by turbidity (OD_{600nm}). The results showed are representative of the three independent experiments.

Benzoic acid (10 mg l^{-1}) and 4-HBA (50 mg l^{-1}) degradation was determined by HPLC-DAD at the time of inoculation (T_0) and 2 days after the stationary growth phase was reached (T_f). Negative controls without bacterial inoculums were incubated in the same conditions as mentioned above, and measured at T_f to test for a possible abiotic loss.

One-millilitre culture samples were centrifuged in order to remove the cells, filter (0.22 µm pore size acetate cellulose membranes), and store at −20 °C until HPLC analysis was carried out.

For analysis, an Agilent 1260 HPLC was used with in-line degasser, quaternary pump (400 bar), variable automatic injector, column heater, and diode array detector (DAD). The mobile phase consisted of phosphoric acid (0.1%; v/v)/ methanol (9/1) with isocratic elution at a flow rate of 1 ml min^{-1}. The column used was a Zorbax Eclipse C18 of 75 mm × 4.6 mm i.d., 3 µm particle side. An injection volume of 50 µl was chosen. The analysis was performed at 230 nm (BA) and 244 (4-HBA). The compounds were quantified by measuring its area against a standard curve.

Kinetic Parameter Determination

Monod kinetic parameters and yield coefficients for BA and 4-HBA were estimated [14, 32]. Monod's model adequately describes cell growth kinetics and can be used to describe complex degrading systems of different strains. Thus, we calculated the generation time, td (h), of the microorganisms

(time period in which cell population is doubled); the maximum specific cell growth rate (μ_{max} (h^{-1}), an important parameter in modeling microbial growth); the duration of the lag phase (tL (h), period when the bacteria are adjusting to the environment); the global yield coefficient biomass-substrate (*Yx/s*, ($Abs600_{nm}$/mg l^{-1}); apparent yield of bacterial cells on substrate consumption); and the residual substrate concentration (*S*).

Biosurfactant Production Assays

To test the biosurfactant production by *H*. sp. KHS3, cells were first cultured in the presence of fluorene (100 mg l^{-1}) or naphthalene (30 mg l^{-1}) as the sole carbon and energy source. At the exponential growth phase, a cell free supernatant was obtained by centrifugation of the culture broth at 10,000×*g* for 20 min. The presence of biosurfactants was determined by the drop-collapsing test [33] and the emulsification assay [34]. The drop-collapse assay was performed spotting 20 µl of the supernatant on a solid surface and assayed for bead formation. The evidence of emulsification was then confirmed by the shape of the droplet. If the drop remained beaded, the result was scored as negative. If the drop spread and collapsed, the result was scored as positive for the presence of biosurfactant. In the emulsification assay, 2 ml of the cell free culture broth and 2 ml of hexadecane were vortexed for 1 min and incubated at 25 °C for 24 h. A turbid stable emulsification in the flask indicated a decrease in the surface tension generated by surfactant compounds present in the medium.

Chemotaxis Assays

Swimming plate assays were carried out as described by Lanfranconi *et al*. [35]. *H*. sp. KHS3 was grown in H1-3% NaCl (w/v) with naphthalene (30 mg l^{-1}) as the only source of carbon and energy. Two microliters of the culture at exponential growth phase (OD_{600nm} of 1.0) were spotted into the centre of the semi-solid agar plates (H1 medium with the addition of 3% (w/v) NaCl, 0.25% (w/v) agar) containing phenanthrene (10 mg l^{-1}), fluorene (5 mg l^{-1}), naphthalene (6 mg l^{-1}), phthalate (1 mg l^{-1}), salicylic acid (12 mg l^{-1}), BA (1 mg l^{-1}), or 4-HBA (1 mg l^{-1}) and incubated at 25 °C for 3 days. A plate without any carbon source in the agar was used as negative control. Plates were checked periodically. Chemotaxis was observed qualitatively as the formation of expanding rings of growth.

Genetic Pathway Identification

The draft genome sequence of *H*. sp. KHS3 has been recently published [25]. The strain was taken from glycerol stock and plated in nutrient medium (H1 medium supplemented with 2% (w/v) NaCl, 0.5% (w/v) yeast extract, 1% (w/v) agar–agar) and incubated 36 h at 30 °C. Once grown, a colony was picked up and inoculated in a liquid culture medium with the following composition: H1 medium with 2% (w/v), NaCl, 0.5% (w/v) yeast extract. Cells were cultivated at 30 °C until stationary phase. Samples were taken and DNA extraction was performed. As is described in Gasperotti et al. (2015), genomic DNA was sequenced on an Illumina HiSeq 1500 instrument using 2 × 100-bp reads, resulting in 400-fold genome coverage. Trimming and correction, contig assembly, crude scaffolding, misassembly correction, and final scaffolding were done using A5-miseq pipeline. The NCBI Prokaryotic Genomes Annotation Pipeline was used for genome annotation and the RAST annotation server was used for subsystem classification and functional annotation. A total of 4,493 coding sequences (CDSs) and 68 structural RNAs (60 tRNAs, 8 rRNA) were predicted. Traces of potential extrachromosomal elements were not detected. The whole-genome shotgun project is deposited at DDBJ/EMBL/GenBank under the accession no. JWHY00000000 [25].

In order to identify the genetic pathways of aromatic compounds degradation a screening of the subsystem classification and functional annotation of the genome was done with RAST server (Rapid Annotation Using Subsystem Technology Version 2.0, http://rast.nmpdr.org/). Positional gene order analyses were done in RAST and Integrated Microbial Genomes & Microbiomes (IMG-M ER).

Results

Screening of the Catabolic Potential of *H*. sp. KHS3

A preliminary screening aromatic compounds and different HC catabolic potential of *H*. sp. KHS3 was carried out in order to select some compounds to deepen the study of its catabolism. Therefore, *H*. sp. KHS3 strain was tested for its ability to grow on pure aromatic compounds and n-alkanes HC as the only carbon sources. *H*. sp. KHS3 exhibited a selective substrate profile (Table 1), being able to grow in the polyaromatic HC naphthalene, fluorene and phenanthrene reaching high cell densities (OD_{600nm} ranging between three and six) and also in single aromatic compounds as benzoic acid, 4-hydroxybenoic acid, salicylic acid, and phthalate. No growth was evidenced on linear HC hexane, undecane, cyclohexane, and hexadecane at the concentrations used.

For substrates that did not support *H*. sp. KHS3 growth, cells were withdrawn from tested cultures after a week of incubation and plated on LB-agar in order to check their substrate tolerance. Bacterial growth was observed in all cases except for xylene and cyclohexane, indicating tolerance of *H*. sp. KHS3 to most tested substrates.

Table 1 Substrate profile of *H*. sp. KHS3

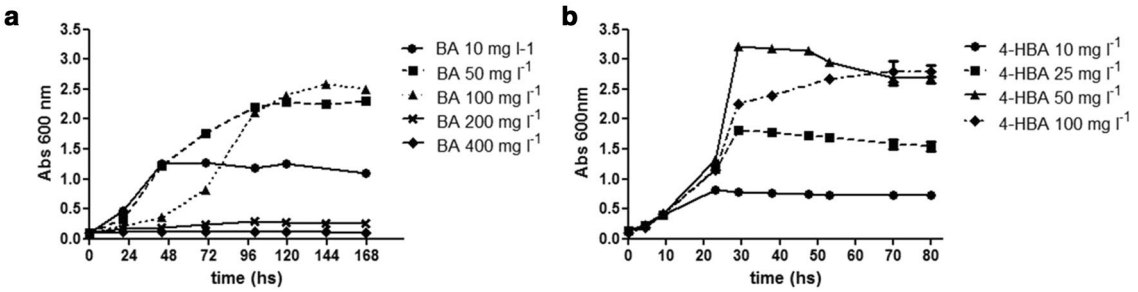
Substrate	Concentration	Growth
Naphthalene	30 mg l^{-1}	++
Phenanthrene	200 mg l^{-1}	+++
Fluorene	100 mg l^{-1}	+++
Anthracene	250 mg l^{-1}	−
Benzoic acid	10 mg l^{-1}	+
Salicylic acid	50 mg l^{-1}	+
Phthalate	25 mg l^{-1}	+
4-hydroxybenzoic acid	50 mg l^{-1}	++
Biphenyl	25 mg l^{-1}	−
α-Naphthol	150 mg l^{-1}	−
β-Naphthol	150 mg l^{-1}	−
Phenylbutazone	5 mg l^{-1}	−
Phenol	100 mg l^{-1}	−
Xylene	100 mg l^{-1}	−
Toluene	100 mg l^{-1}	−
Bencene	100 mg l^{-1}	−
Hexane	0.5% (v/v)	−
Hexadecane	0.5% (v/v)	−
Undecane	0.5% (v/v)	−
Cyclohexane	0.2% (v/v)	−

Cells were cultured in H1 minimal medium with the corresponding compounds as the only carbon source as described in section "Materials and methods". Growth was considered: (+++), if the maximum OD_{600nm} value of the culture (stationary phase) was superior to six; (++) if the maximum OD_{600nm} value of the culture was between three and six; (+) if the maximum OD_{600nm} value of the culture was three; (-) No growth was observed after 25 days. In cases where no growth was observed, concentrations of substrates smaller than those expressed in the table were also tested in order to exclude an inhibitory effect of the substrate concentration. Linear HC are in italic letter

Benzoic Acid and 4-Hydroxybenzoic Acid Degradation

In order to analyse the aromatic degradation ability of the strain, benzoic acid (BA) and 4-hydroxybenzoic acid (4-HBA) were used as model aromatic compounds since their metabolic pathways have been previously described in detail for other microorganisms [30, 31]. Figure 1 shows growth curves of *H*. sp. KHS3 in BA (Fig. 1a) and 4-HBA (Fig. 1b) as the only carbon and energy source in batch reactors. The cell density increased with the concentration of BA using 10, 50, and 100 mg l^{-1}; however, it did not change at higher concentrations of the substrate (200 and 400 mg l^{-1}). In the 4-HBA assays, an increase in cell growth was observed for all the concentration tested. Kinetic parameters from the growth curves in BA and 4-HBA were obtained for the initial points at the exponential growth. Cells grown in BA or 4-HBA presented the maximum $\mu_{máx}$ at 50 mg l^{-1} (Fig. 1c). The generation time (t_d, h) of the organisms in 4-HBA was around 6 h from 10 to 100 mg l^{-1} of initial substrate concentration. Also, the td in the presence of BA ranged between 12 and 25 h for 10 to 200 mg l^{-1} of substrate, and 99 h for 400 mg l^{-1}, respectively. Cells growth did not present lag phase ($t_{lag} = 0$ h) for all the BA and 4-HBA concentrations employed, possibly due to previous acclimation with these compounds (see "Materials and methods").

The effect of initial substrate concentration on the maximum recorded biomass concentration [36] indicated toxicity in presence of 100 mg/l of 4-HBA (Fig. S1). When using BA the first symptoms of toxicity by initial substrate concentration was detected between 50 and 100 mg l^{-1}, being very strong from 200 mg l^{-1} (Fig. S1). Therefore, to



c

	BA(mg l^{-1})					4-HBA (mg l^{-1})			
	10	*50*	*100*	*200*	*400*	*10*	*25*	*50*	*100*
μ máx (h^{-1})	1.156	0.056	0.03	0.027	0.007	0.116	0.117	0.122	0.120

Fig. 1 Growth of *H*. sp. KHS3 in benzoic acid (BA) and 4-hydroxybenzoic acid (4-HBA). Cells were grown in H1 minimal medium with different concentrations of **a** BA or **b** 4-HBA as the only carbon source, at 25 °C and 150 rpm in dark. Cell growth was measured periodically by turbidity (OD_{600nm}). The showed results are representative of three independent experiments. **c** Maximum specific cell growth rate, μ_{max} (h^{-1}) was obtained from the growth curves at the exponential phase. $OD_{600nm} = 1$ corresponds to 50.04×10^{-6} cells

analyze the 4-HBA and BA degradation ability of *H*. sp. KHS3, we selected a non-toxic initial concentration of 4-HBA (50 mg l^{-1}) and BA (10 mg l^{-1}). HPLC analyses confirm degradation of 4-HBA and BA (Table 2). After 96 h of growth in approximately 91.6% and 51.85% of biodegradation was observed, respectively.

Comparing BA (10 mg l^{-1}) and 4-HBA (50 mg l^{-1}) growth parameters, the maximum $\mu_{máx}$ was achieved in the presence of 4-HBA, although the maximum cell yield (0.216 Abs/mg l^{-1}) was obtained with BA (Table 2; Fig. 1c).

Biosurfactant Production and Chemotaxis

H. sp. KHS3 strain was tested for its ability to produce biosurfactants. The droplet collapsing test and the emulsification assays were used. The results of both assays indicated the presence of extra-cellular biosurfactants after growing *H*. sp. KHS3 in H1 medium with fluorene (Fig. 2a, b) and naphthalene (data not shown for simplicity) as the only carbon sources. However, the CTAB-agar plate test of Siegmund and Wagner [53] indicated the presence of extracellular rhamnolipids production (data not shown).

With respect to chemotactic responses, when *H*. sp. KHS3 was inoculated into semi-solid agar plates containing phenanthrene (10 mg l^{-1}), fluorene (5 mg l^{-1}), naphthalene (6 mg l^{-1}), phthalate (1 mg l^{-1}), salicylic acid (12 mg l^{-1}), BA (1 mg l^{-1}), and 4-HBA (1 mg l^{-1}) as the only carbon source, a positive chemotactic response was observed (Fig. 2c).

Table 2 Biodegradation of benzoic acid (BA) and 4-hydroxybenzoic acid (4-HBA). *H*. sp. KHS3 was grown in H1 minimal medium with BA (10 mg l^{-1}) or 4-HBA (50 mg l^{-1}) as the only carbon source

	Initial concentration (mg l^{-1})	Final concentration (mg l^{-1})	Abiotic loos (%)[a]	Biodegradation (%)[b]	μ (h^{-1})	*Yx/s* (A600/mg l^{-1})
BA	10.24 ± 0.43	0	48.15	51.85	0.026	0.216
4-HBA	50 ± 0.4	4.2	0	91.6	0.122	0.069

Concentration of both substrates in the culture supernatant was estimated by HPLC at initial time and after 96 h of the start of cultures (T_f). A negative control with no cells was included at final time to analyze the abiotic loss. The results are the average and SD of three independent analyses. For maximum specific cell growth rate μ_{max} (h^{-1}) and global yield coefficient biomass-substrate *Yx/s* (Abs_{600}/mg l^{-1}) calculation see "Materials and Methods"

[a]Difference between the initial concentration (taken as 100%) and final concentration (T_f) in the negative control (abiotic loos)

[b]Difference between the initial concentration (taken as 100%) and final concentration (T_f) in flasks inoculated with bacteria, subtracting abiotic loos

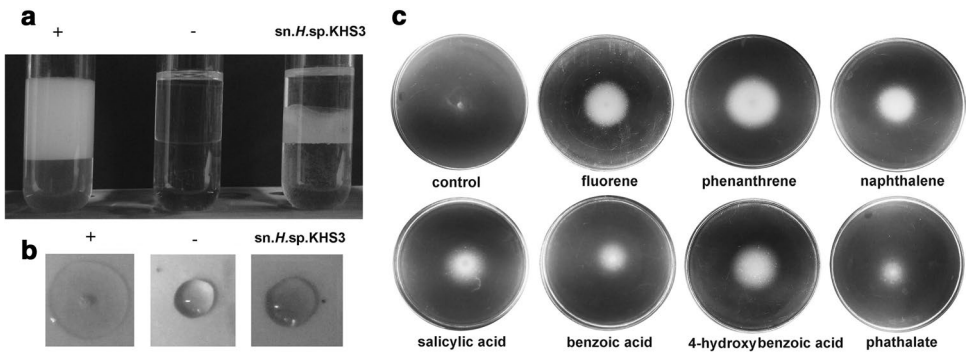


Fig. 2 Biosurfactant production and chemotactic responses of *H*. sp. KHS3 towards aromatic compounds. **a** Emulsification Assay. *H*. sp. KHS3 was cultured 48 h with fluorene. Then, 2 ml of hexadecane were mixed with 2 ml of cell-free culture supernatant, vortexed and left to stand 24 h (sn *H*. sp. KHS3). **b** Droplet collapse assay. A drop of 20 µl of the cell-free supernatant coloured with methylene blue were placed on a solid surface, left to stand 5 min and then photographed. H1 medium without addition (−) or with 1% Tween 20 (+) were used as negative and positive controls in both assays, respectively. **c** Swarm assay. 2 µl of *H*. sp. KHS3 culture at the exponential growth phase was placed in the centre of soft agar plates containing phenanthrene (10 mg l^{-1}), fluorene (5 mg l^{-1}), naphthalene (6 mg l^{-1}), phthalate (1 mg l^{-1}), salicylic acid (12 mg l^{-1}), BA (1 mg l^{-1}), or 4-HBA (1 mg l^{-1}). Negative control had no carbon source. Plates were photographed after 72 h of incubation at 25 °C. The results are representative of at least three independent experiments

In silico Analysis of the Aromatic Catabolic Pathways of *H.* sp. KHS3

The draft genome sequence of *H.* sp. KHS3 has recently been published [25]. A detailed analysis of the genomic sequence was carried out in order to identify putative genes involved in aromatic compounds catabolic pathways. RAST (http://rast.nmpdr.org) annotation showed the presence of 61 genes related to the aromatic compounds metabolism (Table S1) including genes of β-ketoadipate, homogentisate and gentisate pathways, benzoate, biphenyl, p-hydroxybenzoate, salicylate, and some involved in n-heterocyclic aromatic compound degradation. No alkane monooxygenase genes were detected, this being consistent with the inability of *H.* sp. KHS3 to growth in the presence of alkanes as the only carbon source (described above, "Biological Material" section).

Among the 61 genes putatively related to aromatic compounds catabolism of *H.* sp. KHS3, it was possible to identify the whole central catabolic pathway of β-ketoadipate described in diverse species [37], including catechol (*cat* genes) and protocatechuate (*pca* genes) branches (Fig. 3). Besides, genes of peripheral pathways leading benzoate and 4-hydroxybenzoate to the β-ketoadipate central pathway are also present (*ben* and *pob* genes respectively), being consistent with the ability of the strain to degrade BA and 4-HBA. As shown in Fig. 3, *pcaL* gene of *H.* sp. KHS3 encodes a bifunctional protein. This is the result of a *pcaC* (encoding 4-carboxymuconolactone decarboxylase) and *pcaD* (genes β-ketoadipate enol-lactone hydrolase) gene fusion as has been reported in other species [38].

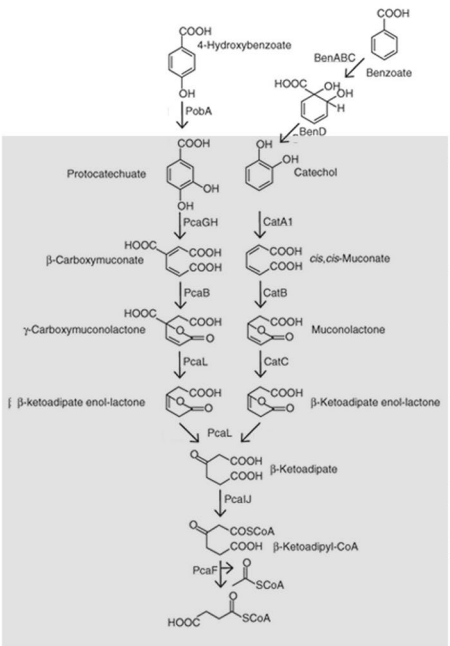
Concerning to the β-ketoadipate pathway regulation, the previously described transcriptional regulators *catR* and *pcaR* [37, 39] as well as *pobR*, a positive regulator of *pobA* in the 4-HBA degradation, were also identified in the genome of *H.* sp. KHS3 (Fig. 3).

Ortho cleavage of the β-ketoadipate pathway observed at genomic level is also consistent with a purple colour development in Rothera test (indicative of β-ketoadipate formation; [40]) observed for *H.* sp. KHS3 (data not shown).

Organization of *pca*, *cat*, and *ben* Genes of *H.* sp. KHS3 and Comparison with Equivalent Catabolic Genes From Other Degrading Bacteria

In *H.* sp. KHS3 genome, *pca* genes are arranged in a single cluster (*pcaBIJFLHG*) (Fig. 4a) between positions 295,589–302,558 base pairs (bp), and *cat* genes are grouped with the *ben* genes in the same cluster (cluster *catBCAbenABCDE*) (Fig. 4b) between positions 226,767–231,186 bp.

The arrangement and organization of genes of *pca* and *cat-ben* clusters observed in *H.* sp. KHS3 were compared



Name	Subsystem	Role	n° CDS
benA	Benzoate degradation	Benzoate 1,2-dioxygenase α-subunit (EC 1.14.12.10)	1
benB		Benzoate 1,2-dioxygenase β-subunit (EC 1.14.12.10)	1
benC		benzoate dioxygenase, ferredoxin reductase component	1
benD		1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase (EC 1.3.1.25)	1
benE		Benzoate transport protein	2
catA1	Catechol branch of β-ketoadipate pathway	Catechol 1,2-dioxygenase (EC 1.13.11.1)	2
catB		Muconate cycloisomerase (EC 5.5.1.1)	3
catC		Muconolactone isomerase (EC 5.3.3.4)	1
pcaL	Catechol and Protocatechuate branch of β-ketoadipate pathway	3-oxoadipate enol-lactone hydrolase/ 4-carboxymuconolactone decarboxylase [EC:3.1.1.24, EC:4.1.1.44]	1
pcaI		3-oxoadipate CoA-transferase subunit A (EC 2.8.3.6)	1
pcaJ		3-oxoadipate CoA-transferase subunit B (EC 2.8.3.6)	1
pcaF	Protocatechuate branch of β-ketoadipate pathway	Beta-ketoadipyl CoA thiolase (EC 2.3.1.-)	2
pcaG		Protocatechuate 3,4-dioxygenase α-chain (EC 1.13.11.3)	1
pcaH		Protocatechuate 3,4-dioxygenase β-chain (EC 1.13.11.3)	1
pcaB		3-carboxy-cis,cis-muconate cycloisomerase (EC 5.5.1.2)	1
pcaR		Pca regulon regulatory protein PcaR	1
pobA	p-Hydroxybenzoate degradation	P-hydroxybenzoate hydroxylase (EC 1.14.13.2)	1
catR	DNA-binding regulatory protein	Aromatic HC utilization transcriptional regulator CatR (LysR family)	1
pobR	None	Transcriptional regulator PobR, AraC family	1

Fig. 3 The β-ketoadipate pathway, genes, and peripheral reactions in *H.* sp. KHS3. The figure shows the catechol and protocatechuate branch of β-ketoadipate pathway deduced from the genome of *H.* sp. KHS3 (highlighted in gray). All genes of this pathway (named next to the arrows) are present in the genome of this strain, including the benABCD and pobA genes that funnel benzoate and 4-hydroxybenzoate to the β-ketoadipate pathway. In the table are listed these genes (Subsystem, role and number of coding DNA sequences (CDS) of these RAST annotated genes) and the transcription factors possibly involved in the regulation of the β- ketoadipate pathway in *H.* sp. KHS3

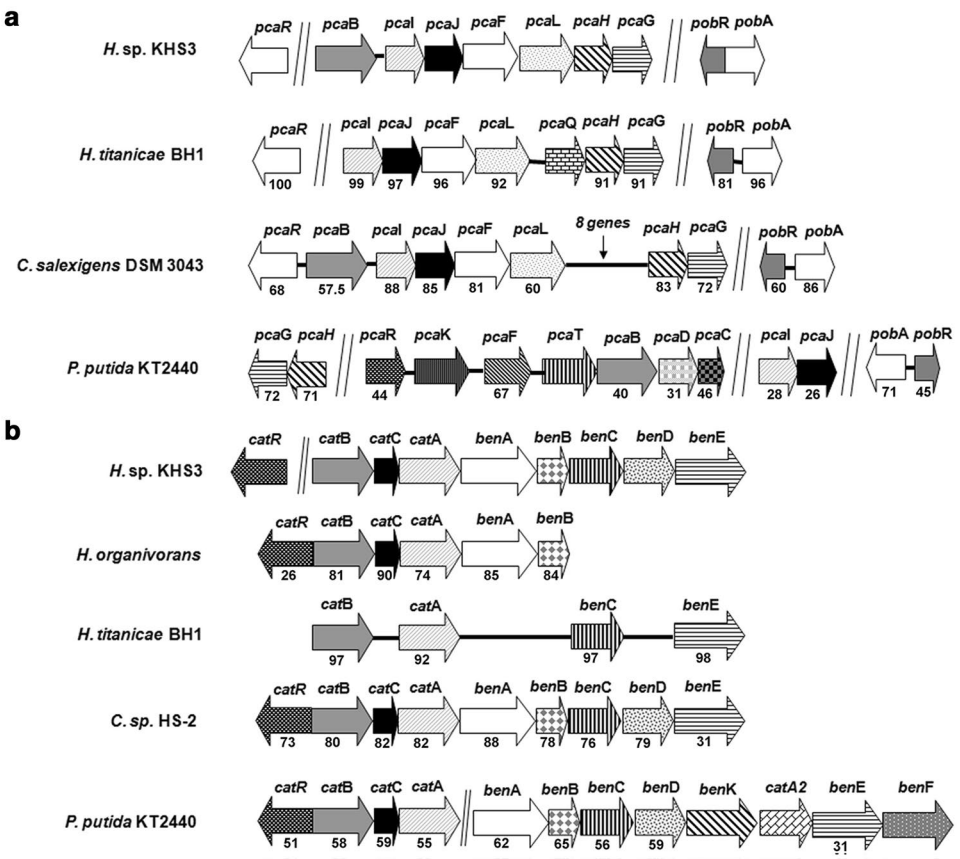


Fig. 4 Organization of **a** *pca* and **b** *cat-ben* gene clusters of *H*. sp. KHS3 and comparison with equivalent clusters from other aromatic compounds-degrading bacteria and *H. titanicae* BH1. Genes (listed in Fig. 3.) are represented by arrows. Two vertical lines indicate that the genes are not adjacent in the genome. Numbers beneath the arrows indicate the percentage of amino acid sequence identity between the encoded gene product and the equivalent product from *H*. sp. KHS3. *pcaQ*: transcriptional regulator-LysR family; *pcaT*: alpha-ketoglutarate permease; *pcaD*: 3-oxoadipate enol-lactone hydrolase; *pcaC*: 4-carboxymuconolactone decarboxylase; *benK*: benzoate transporter; *benF*: benzoate-specific porin. 8 genes: eight genes not related to aromatic compounds degradation

with those of other equivalent clusters of aromatic compounds degrading bacteria and with *Halomonas titanicae* BH1 (Fig. 4). Evidences previously obtained indicate that *H*. sp. KHS3 is a strain of *H. titanicae* (personal communication). As can be seen in the table in Fig. 3, there are more than one *benE, catA, catB*, and *pcaF* coding DNA sequences (CDS). Only genes clustered with other degrading genes were taken into consideration for comparisons. A similar arrangement of all *pca* genes in a single cluster can be seen in *H. titanicae* BH1 and *C. salexigens* DSM3043 (Fig. 4a). However, the gene order in *H*. sp. KHS3 cluster (*pcaBIJFLHG*) is different of those *pca* clusters. In *P. putida* KT2440, the *pca* genes are scattered in the genome (Fig. 4a).

The *pobA* and *pobR* genes that encodes p-hydroxybenozate hydroxylase and its transcriptional activator in different bacteria [37] are located at positions 267,937–270,170 bp in *H*. sp. KHS3 genome, and are divergently transcribed (Fig. 4a). A *pcaR* (a regulatory protein of the protocatechuate branch of the β-ketoadipate pathway from *P. putida* PRS2000 [39]) homologue gene is located at position 327,990–328,769 in *H*. sp. KHS3.

The arrangement of *cat-ben* genes in a single cluster is similar among members of *Halomonadaceae* family (Fig. 4). The *H*. sp. KHS3 gene order (*catBCAbenAB-CDE*) is similar to that of *Chromohalobacter* sp. HS-2 and to the *catBCAbenAB* cluster described in *H. organivorans* (Fig. 4b). In *P. putida* KT2440 (Fig. 4b), *cat*, and *ben* genes are in different clusters. The transcriptional regulator *catR* gene is located separately from *cat-ben* cluster in *H*. sp. KHS3 (position: 205,305–206,207 bp), whereas in other species it precedes the *cat* genes (Fig. 4b).

Discussion

H. sp. KHS3 is a moderately halophilic bacteria isolated from HC-contaminated seawater of the Mar del Plata harbor [24]. In this work, we demonstrated that *H*. KHS3 can grow using different monoaromatic and polyaromatic compounds as the only carbon and energy source in a saline environment. Several *Halomonas* strains have been described as efficient aromatic compounds degraders [7, 15, 17, 18, 22, 26, 41]. For example, *H. organivorans* is characterized by its ability to use a wide range of organic compounds as the only carbon source including BA, 4-HBA and salicylic acid [7], which are also substrates for *H*. sp. KHS3 as was determined in this work. To our knowledge, most of the studies on *Halomonas* sp. focused on degradation of monoaromatic compounds rather than polycyclic and heterocyclic compounds. We have demonstrated that *H*. sp. KHS3 has the ability to

grow using the polyaromatics phenanthrene, fluorene, and naphthalene as the only carbon source. Similar ability has been demonstrated by Dastgheib et al. [22], in a mixed culture (Qphe-SubIV) composed of one *Halomonas* strain and one *Marinobacter* strain. However, the degradation of phenanthrene by Qphe-SubIV was mainly due to the *Marinobacter* strain and the *Halomonas* strain playing an auxiliary role in the degradation by utilizing phenanthrene metabolites, whose accumulation in the media could be toxic [22]. Furthermore, Dziewit et al. [42] revealed that *Halomonas* sp. ZM3 uses phenanthrene as the sole source of carbon and energy. On the other hand, Yang et al. [43] showed fluorene degradation by *Halomonas* sp. 19-A. It would be interesting to expand the study of the PAHs degradative ability of *H*. sp. KHS3 strain, to know its potential to be used as an integral tool of the biodegradation programs design for saline waters contaminated with these compounds.

In this work, we analyzed and demonstrated for the first time the BA and 4-HBA degradation ability of *H*. sp. KHS3 by employing batch reactors (laboratory scale). We used these compounds as a model of aromatic compounds since their metabolic pathways have been previously described in detail for other microorganisms [30, 31]. Furthermore, 4-HBA is present as a contaminant in certain highly saline industrial effluents [44]. 4-HBA is also a common intermediate in the biodegradation of lignin and is, therefore, an important compound in the short-term cycling of environmental carbon [44]. BA occurs naturally in many plants and it serves as an intermediate in the biosynthesis of many secondary metabolites. Salts of BA are used as food preservatives, and BA is an important precursor for the industrial synthesis of many organic substances [45].

After 96 h of growth in BA (10 mg l^{-1}) or 4-HBA (50 mg l^{-1}) as the only carbon source, we observed approximately 52 and 91.6% of biodegradation, respectively. It is important to note the analysis of the abiotic loss of these compounds, because we found that BA decreased by 58% due to abiotic factors after 96 h. This fact must be taken into account for biodegradation analyses, since most of the time it remains unnoticed.

A kinetic study of the reactions including an accurate estimation of the growth parameters could be an important contribution since they are necessary to establish reaction mechanisms and to provide a rational basis for reactors design. Information on kinetic parameters is scarce or non-existent in the literature concerning BA and 4-HBA by alkalophiles or halophiles. To our knowledge, only Oie et al. (2017) [17] analyzed kinetic parameters of *H. campisalis* grown in BA; however, they employed different units of cell density and cell yield than those employed by our research group. When we analyzed the results presented in other publications with the same kinetic parameter determination method used in our work, we observed that, interestingly, *H*. sp. KHS3 had higher cell yield based on substrate utilization ($Y_{x/s}$: 0.069 Abs/mg l^{-1} in 4HBA, and 0.22 Abs/mg l^{-1} in BA) than other reports ($Y_{x/s}$: from 0.00065 to 0.014 Abs/mg l^{-1}) [44, 46, 47].

Many aromatic compounds degrading microorganisms possess special physiological mechanisms to increase the availability of low aqueous solubility compounds [48–52]. Biosurfactants production may enhance the bioavailability of these compounds by either increasing it apparent solubility in the aqueous phase, or by expanding the contact surface area due to emulsification [49]. In this work, drop collapse assay, emulsification tests, and a colorimetric method [53] confirmed the production of surfactant by *H*. sp. KHS3, including extracellular rhamnolipid production. These findings open new doors for future research in this field.

In addition, there are previous descriptions of chemotaxis as an advantage for degradation of hydrophobic compounds [5, 50, 54, 55]. It was previously described that *Halomonas* sp. KHS3 showed chemotactic responses towards gasoil [24], which is a mixture of aliphatic and aromatic HC. In this work, our results show that *H*. sp. KHS3 actually shows chemotactic responses towards all the tested pure aromatic compounds that it can employ as carbon source. To date, information on chemotactic responses in Halomonadaceae family is rather scarce, and our results represent the first report of this behavior in a Halomonas strain. The biosurfactants production and chemotaxis responses of *H*. sp. KHS3 could confer to this microorganism a competitive advantage over other degraders.

Little is known about the aromatic compounds degradation pathways in halophilic organisms, however, a few recent evidences [15, 17, 23, 26] have shown that the degradation occurs using enzymes described for many non-halophiles. In this work, we explored the recently sequenced genome of *H*. sp. KHS3 and found that the central carbon catabolism via, known as β-ketoadipate pathway (*ortho* cleavage pathway), is completely represented, including catechol (*cat* genes) and protocatechuate (*pca* genes) branches. This is the first report where the complete catechol and protocatechuate branches of β-ketoadipate are described for a *Halomonas* strain. Genes encoding enzymes that lead BA (*BenABCD genes*) and 4-HBA (*PobA*) towards the β-ketoadipate pathway are also present in *H*. sp. KHS3. Therefore, our results support the hypothesis that the degradation of BA and 4-HBA would be through β-ketoadipate pathway encoded in its genome. García *et al*. [26] arrived to a similar conclusion for *Halomonas organivorans*, evaluating the presence of genes and enzymatic activity of catechol-1,2-dioxygenase and protocatechuate-3,4-dioxygenases. Additional evidences of β-ketoadipate pathway as the main way for aromatic metabolism in *Halomonas* sp. have been also reported [15, 17, 56]. Recently, the gene cluster *catRBCAbenAB* involved in the utilization of benzoate and catechol was isolated from

H. organivorans [23]. Furthermore, Kin *et al*. [31], using a combination of molecular and biochemical approaches have elucidated the catabolic pathways for benzoate and 4-HBA in *Chromohalobacter* sp. strain HS-2, however, the complete genetic pathway was not revealed.

In the genomic sequence of *H*. sp. KHS3 genes homologous to CatR, PobR, and PcaR transcriptional factors were identified. These transcriptional factors modulate the β-ketoadipate pathway in several HC-degrading species. Future work has to be done to reveal its role in *H*. sp. KHS3, as possible biotechnological targets to improve the ability of aromatic compounds degradation.

The *ben* and *cat* genes are contiguous and organized in the *catBCAbenABCDE* cluster in the genome of *H*. sp. KHS3. This *cat-ben* gene arrangement is also present in *Acinetobacter* sp. ADP1, *Chromohalobacter* sp. HS-2, *Ralstonia metallidurans* DSM 2839, and *P. fluorescens* [31, 57]. Interestingly, the organization of genes in this *H*. sp. KHS3 cluster is similar to *Chromohalobacter* sp. HS-2 and *H. organivorans*, being all members of *Halomonadaceae* family.

Concerning *pca* genes, in *H*. sp. KHS3 genome, we identified a single *pcaBIJFLHG* cluster, a similar arrangement to that described for *pca* genes in other *Halomadaceae* family members as *H. titanicae* BH1 and *C. salexigens* DSM3043, and to that reported in *Rhodococcus opacus, Acinetobacter* sp. ADP1, *Caulobacter crescentus*, and *Agrobacterium tumefaciens* [57], although with a different gene organization in the cluster. In summary, the *cat-ben* and *pca* gene organization in *H*. sp. KHS3 is similar to that of other HC-degrading bacteria and seems to be similar in members of *Halomonadaceae* family, in contrast to several *Pseudomonas* species where *ben, cat*, and *pca* genes are scattered over the genome [57, 58].

Bioinformatics analysis (ANI values; Genome to genome distances, DSMZ; tetranucleotide signature frequency correlation coefficient) [59] previously obtained by members of our group indicate that *H*. sp. KHS3 is a strain of *H. titanicae* (personal communication). The presence of β-ketoadipate whole pathway in *H*. sp. KHS3 could indicate that this strain is more specialized in aromatic compound degradation by *ortho*-cleaving than *H. titanicae* BH1, where a fewer *ortho*-cleaving genes were identified [60], and the degradation ability has not been characterized.

Typical genes for upper degrading pathways of PAHs have not been identified in the genome of *H*. sp. KHS3 indicating that maybe an alternative upper pathway could be found in this strain. As many of the genes involved in the upper degradation pathways of PAHs are often located on plasmids [61], the absence of these genes in *H*. sp. KHS3 genome could be attributed to a loss of degrading plasmids during bacterial cultivation in a non-selective carbon source for DNA extraction. Bacteria can also degrade PAHs via the cytochrome P450-mediated pathway, with the production of trans-dihydrodiols [62, 63]. Some genes of naphthalene degradation pathway by cytochrome p450 were found in the genome of *H*. sp. KHS3 exploring the KEGG map of metabolism of xenobiotics by cytochrome p450 (10 genes of glutathione s-transferase, EC: 2.5.1.18, and one gene of epoxide hydrolase, EC: 3.3.2.9).

On the other hand, some *Halomonas* strains have been described to use aliphatic hydrocarbons as sole carbon source [21, 64]. *Halomonas* sp. KHS3 did not grow in presence of alkanes, although different chain length alkanes were tested as the sole carbon and energy source. This fact was supported by the absence of genes related to aliphatic hydrocarbon degradation. However, when searching in IMG annotated genome of the described alkane degrading *Halomonas* sp. MCTG39a strain [64], no genes of alkane dioxygenases (a key enzyme in the alkanes degradation pathways) could be found. Further experiments should be done to shed light on the ability of MCTG39a to grow using alkanes and to get information on aliphatic degrading pathways. *Halomonas* sp. C2SS100 has also been isolated as an alkane degrading strain [21], but there is no genomic sequence available.

The knowledge of the ability of microorganisms capable of degrading aromatic compounds in saline and hypersaline environments has been accumulating significantly in the past two decades. In the present work, we increase the knowledge of our recent *H*. sp. KHS3 isolation, demonstrating its ability to produce biosurfactants, degrade, and exhibit chemotactic responses to different aromatic compounds. The strain possess a variety of genes related to aromatic compounds degradation, been β-ketoadipate pathway completely represented. Due to the recognized importance of Mar del Plata harbor (where this microorganism was isolated) and the evidence of aromatic compounds and HC contaminated water in this place [65], decontamination of sea water by biodegradation with indigenous microorganisms represents a great challenge. More exhaustive subsequent studies from the results presented here could be useful for developing a bio-treatment process of this marine water as well as for other saline waters.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interests.

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