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Proteogenomic Elucidation of the Initial Steps in the Benzene Degradation Pathway of a Novel Halophile, *Arhodomonas* sp. Strain Rozel, Isolated from a Hypersaline Environment

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Lately, there has been a special interest in understanding the role of halophilic and halotolerant organisms for their ability to degrade hydrocarbons. The focus of this study was to investigate the genes and enzymes involved in the initial steps of the benzene degradation pathway in halophiles. The extremely halophilic bacteria *Arhodomonas* sp. strain Seminole and *Arhodomonas* sp. strain Rozel, which degrade benzene and toluene as the sole carbon source at high salinity (0.5 to 4 M NaCl), were isolated from enrichments developed from contaminated hypersaline environments. To obtain insights into the physiology of this novel group of organisms, a draft genome sequence of the Seminole strain was obtained. A cluster of 13 genes predicted to be functional in the hydrocarbon degradation pathway was identified from the sequence. Two-dimensional (2D) gel electrophoresis and liquid chromatography-mass spectrometry were used to corroborate the role of the predicted open reading frames (ORFs). ORFs 1080 and 1082 were identified as components of a multicomponent phenol hydroxylase complex, and ORF 1086 was identified as catechol 2,3-dioxygenase (2,3-CAT). Based on this analysis, it was hypothesized that benzene is converted to phenol and then to catechol by phenol hydroxylase components. The resulting catechol undergoes ring cleavage via the meta pathway by 2,3-CAT to form 2-hydroxymuconic semialdehyde, which enters the tricarboxylic acid cycle. To substantiate these findings, the Rozel strain was grown on deuterated benzene, and gas chromatography-mass spectrometry detected deuterated phenol as the initial intermediate of benzene degradation. These studies establish the initial steps of the benzene degradation pathway in halophiles.

Many hypersaline environments, such as natural saline lakes, salt flats, solar salterns, saline industrial effluents, oil fields, and salt marshes, have been shown to be contaminated with high levels of petroleum hydrocarbons. Among these, oil fields pose a special problem due to their sheer numbers worldwide and due to their high salinity caused by produced water (salty brackish water) generated during oil and natural gas extraction. Produced water is highly saline and contains a complex mixture of hydrocarbons, including alkanes (linear or branched), cycloalkanes, mono- and polyaromatics, asphaltenes, heavy metals, and resins.

The ability of microorganisms to degrade aromatic hydrocarbons in terrestrial and marine environments has been studied extensively under oxic conditions (28, 43, 44, 50, 56). On the other hand, little is known about hydrocarbon degradation in hypersaline environments. Bioremediation of polluted hypersaline wastewaters and other environments with nonhalophilic microorganisms is difficult because salt inhibits their growth and the degradation of hydrocarbons (41, 57). Therefore, the cleanup of such environments can only be accomplished by stimulating the growth of indigenous microorganisms capable of degrading petroleum hydrocarbons or through the bioaugmentation of halophilic or halotolerant organisms that degrade hydrocarbons.

There is growing evidence suggesting that microorganisms play a significant role in the fate of hydrocarbons in high-salinity environments. Microcosms established with contaminated soil and sediment samples, enrichment cultures, and pure cultures have convincingly shown the ability of halophiles and halotolerants to degrade a variety of hydrocarbons, including crude oil, aliphatics, and mono- and polyaromatic compounds, as well as phenolics and benzoates, at salinities as high as ≥30% (26, 32, 52). Among bacteria, members of the genera *Halomonas* (15, 16), *Marinobacter* (6, 17), *Bacillus* (33, 46, 49), *Rhodococcus* (46), *Pseudomonas* (46), *Alcaligenes* (5), *Chromohalobacter* (23), *Planococcus* (29), *Streptomyces* (25), *Arthrobacter* (46), and *Actinomyces* (3) have been shown to degrade hydrocarbons at high salinity. Also, examples of pure cultures of archaea, such as *Haloferax* (2, 10, 54), *Haloarcula* (11, 54), *Halococcus*, and many species of *Halobacterium* (2, 24, 32) that have been characterized only phenotypically, were shown to degrade hydrocarbons at high salinity. In addition, a few fungi also were shown to possess the ability to degrade hydrocarbons under hypersaline conditions (39). These reports clearly suggest the potential role of halophilic and halotolerant organisms in the bioremediation of petroleum-impacted saline environments.

Although recent reports dealing with the degradation of hydrocarbons in high-salinity conditions are numerous, little information exists on the genes, pathways, and mechanisms of their degradation. Also, it is not known whether halophilic and halotolerant bacteria degrade hydrocarbons using novel genes and pathways compared to those used by nonhalophiles. Undoubtedly, the discovery of novel genes and pathways is significant, as it could lead to the development of alternative and cost-effective remediation strategies. A few recent studies indicated that the degradation

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of hydrocarbons at high salinity occurs using enzymes described for many nonhalophiles. For example, Hinteregger and Streichsbier (19) have reported catechol 1,2-dioxygenase (1,2-CAT) activity in the phenol-degrading *Halomonas* sp. Garcia et al. (16) have documented the presence of genes encoding 1,2-CAT, catechol 2,3-dioxygenase (2,3-CAT), and protocatechuate 3,4-dioxygenase (3,4-PCA) enzymes in several strains of phenol- and benzoate-degrading *Halomonas* spp. isolated from saline habitats in southern Spain. *Halomonas campisalis* grown on benzoate or salicylate was shown to possess 1,2-CAT (40). Kim et al. (23) showed the presence of 1,2-CAT and 3,4-PCA in a benzoate- and *p*-hydroxybenzoate-metabolizing halophile, *Chromohalobacter* sp. strain HS-2. Moreno et al. (34) have characterized the genes involved in the metabolism of phenol and benzoate in *Halomonas organivorans*. These researchers found that the genes *catA*, *catB*, *catC*, and *catR* encode 1,2-CAT, *cis*-,*cis*-muconate cycloisomerase, muconolactone delta-isomerase, and a transcriptional regulator, respectively. These genes were flanked downstream by the benzoate catabolic genes *benA* and *benB*, which code for the large and small subunits of benzoate 1,2-dioxygenase, respectively. Recently, Dastgheib et al. (9) obtained a phenanthrene-degrading mixed culture (Qphe-SubIV) consisting of two organisms, *Halomonas* sp. and *Marinobacter* sp. Metabolite analysis using high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) showed that 2-hydroxy-1-naphthoic acid and 2-naphthol were among the major metabolites accumulated in the culture media, indicating that the initial dioxygenation might have occurred by a novel mechanism at the C1 and C2 positions (9).

Only two reports exist in the literature on the degradation mechanism of hydrocarbons by archaea in the presence of high salt. For example, an extremely halophilic archaeon, *Haloferax* sp. strain D1227, which degrades benzoate, cinnamate, and phenylpropanoate, was shown to possess gentisate 1,2-dioxygenase. This enzyme was cloned and expressed in *Haloferax volcanii* and shown to be similar to the homologs in bacteria, including *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*), *Comamonas acidovorans* (formerly *Pseudomonas acidovorans*), and *Moraxella osloensis* (10, 14). Fairley et al. (11, 12) also found a closely related gene (*gdoA*) encoding gentisate 1,2-dioxygenase in 4-hydroxybenzoate-degrading *Haloarcula* sp. strain D1. Overall, these studies show that microorganisms in high-salinity environments degrade hydrocarbons using enzymes similar to those from nonhalophiles. However, in-depth studies are needed to obtain greater insights into the molecular mechanisms, intermediates, and pathways of hydrocarbon degradation in hypersaline environments.

In this study, we used phylogenetically closely related novel halophiles: *Arhodomonas* sp. strain Seminole and *Arhodomonas* sp. strain Rozel isolated from enrichments developed using crude-oil-impacted soil and sediment samples from high-salinity environments. A high-quality draft genome sequence of the strain Seminole was used as a guide for elucidating the initial steps of the benzene degradation pathway in these novel halophilic strains. Such information is important for understanding the rate-limiting initial steps that have to be overcome for the efficient removal of toxic compounds.

MATERIALS AND METHODS

Chemicals. Benzene, toluene, benzene-D_6, and phenol-D_6 were purchased from Sigma-Aldrich Co. All the chemicals were of analytical grade and were used without further purification.

Bacterial strains. The *Arhodomonas* sp. strain Seminole (GenBank accession no. JX099567) was isolated from an enrichment developed from an oil-brine soil obtained from an oil production facility in Seminole County, Oklahoma. 16S rRNA gene sequence analysis shows 96% sequence identity with *Arhodomonas aquaeolei* (GenBank accession no. NR_044676). The strain Seminole is able to degrade benzene in the presence of 0.5 to 3 M NaCl, and no degradation occurred in the absence of salt, suggesting that the isolate is a strict halophile. The *Arhodomonas* sp. strain Rozel (GenBank accession no. JX128266) was isolated from an enrichment developed from sediment obtained from Great Salt Lake near Rozel Point, Utah. 16S rRNA gene sequence analysis shows 99% sequence identity with *Arhodomonas aquaeolei* (GenBank accession no. NR_044676). The strain Rozel is able to degrade benzene over a wide range of salinity (0.5 to 4 M NaCl) with optimal degradation at 3 M NaCl (data not shown). 16S rRNA gene sequence alignment of the two isolates shows 98% sequence similarity with a 100% query coverage and an E value of 0, suggesting that these isolates are closely related to each other. The strain Seminole requires >2 weeks to completely degrade 17 to 24 μmol of benzene, while the strain Rozel is able to completely degrade 20 to 25 μmol of benzene in <7 days (these strains will be available from the authors upon request). Both strains were maintained in 1-liter bottles with 500 ml of mineral salts medium (MSM) (37) supplemented with 2.5 M NaCl and 20 to 25 μmol of neat benzene as the sole source of carbon. These bottles served as the mother cultures for all the experiments performed in this study.

To understand the hydrocarbon degradation capacity of the *Arhodomonas* isolates, a draft genome sequence (7× coverage) of the *Arhodomonas* sp. strain Seminole was obtained by pyrosequencing. However, the strain Rozel was selected for proteomic and other studies reported in this article because of its higher benzene degradation rate and broader range of salinity tolerance than those of strain Seminole. To generate sufficient biomass required for proteomic studies, strain Rozel was grown in two sets of 500-ml bottles containing 300 ml MSM supplemented with 2.5 M NaCl and lactate as the sole source of carbon. Bottles were fed 5 mM lactate 2 times consecutively each at the end of log-phase growth. Growth was monitored by measuring both the optical density at 600 nm (OD_{600}) and the total protein by using the Lowry method (30). Once sufficient biomass (114 μg/ml) was generated, approximately 20 to 25 μmol/bottle benzene was added twice to one set of 3 bottles to induce benzene-degrading enzymes. No benzene was added to the other set of 3 bottles as a control. The benzene-amended bottles were closed with rubber septa and aluminum crimps, and headspace samples were withdrawn periodically and monitored for the consumption of added benzene using GC as described previously (37). Cells from both sets of bottles were harvested by centrifugation for 15 min at 10,000 × *g* at 4°C. The cell pellets were immediately frozen and stored at −80°C until further use.

Identification of genes. To identify the putative genes and proteins of interest, we locally installed and used a stand-alone BLAST software package (version 2.2.6) obtained from the NCBI (4). The selected protein sequences were searched for in BLAST against all predicted peptides in the genome of the *Arhodomonas* sp. strain Seminole using a cutoff E value of 1e−45. The relative position within the contig and the putative transcription direction of the predicted genes were determined by using the GeneMark.hmm for Prokaryotes software (31).

Phylogenetic analysis. The predicted amino acid sequences of open reading frames (ORFs) 1080, 1082, and 1084 from the *Arhodomonas* sp. strain Seminole genome were aligned using the ClustalW option in MEGA 5 (53) with closely related and well-characterized phenol hydroxylase, toluene monooxygenase, and benzene monooxygenase components from different aromatic hydrocarbon-degrading nonhalophiles. The sequences for alignment were obtained from the GenBank and UniProtKB database (see Fig. 2). Phylogenetic analysis was performed by using the neighbor-joining algorithm and the Poisson correction method in MEGA 5 (48). Bootstrap values were calculated as a percentage of 1,000 replicates (13, 53).

Preparation of cell extracts. Cell extracts were prepared essentially as described previously (42) with some modifications. Briefly, cell pellets were washed once with 0.14 M NaCl, washed once with Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and then stored as aliquots at −80°C. The pelleted cells were suspended in TE buffer with complete Mini protease inhibitor cocktail (Roche) (1:100 [vol/vol]) and disrupted by using a FastPrep Bio 101 Thermo Savant bead beater (5 cycles of 15 s each). The protein concentration was determined using the 2D Quant kit (GE Healthcare).

Proteomic analysis. The cytosolic proteomes were resolved using 2D gel electrophoresis as described previously (42) with minor modifications. Briefly, aliquots of cell extracts containing 70 μg protein were separated in the first dimension by isoelectric focusing (IEF) in the Ettan IPGphor3 system (GE Healthcare) for a total of 73 kVh at 20°C using 24-cm nonlinear immobilized pH gradients with a pH gradient of 3 to 7. The rehydration solution contained 9.47 M urea, 2.63 M thiourea, 33.4 mM dithiothreitol, 2.4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, and 2% Pharmalyte (broad range, pH 3 to 10). Proteins were separated in the second dimension by using 12% SDS-PAGE and the Ettan DALTsix System (GE Healthcare). Gels were stained using Sypro Ruby and digitally imaged using a Typhoon 9400 (GE Healthcare). Spot detection, matching, abundance quantification, and normalization were performed using Progenesis Workstation software (Nonlinear Dynamics, Durham, NC). The protein patterns of each growth condition were based on gels from three independent cultures. The protein spots of interest were excised and denatured in urea, alkylated, and digested with trypsin, and their trypsinolytic peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Proteins were identified by using Mascot (v2.2.2 from Matrix Science, Boston, MA) and a database generated by *in silico* digestion of the strain Seminole proteome predicted from the genome. Search results were validated by using Scaffold 03 (Proteome Software Inc., Portland, OR), the Peptide Prophet algorithm (22), and Protein Prophet (36). The criteria for accepting each identification will conform to the "Paris" guidelines for proteomics results (http://www.mcponline.org/misc/ParisReport_Final.dtl). A set of stringent criteria for protein identification was used; only protein probability thresholds greater than 99% were accepted, and at least three peptides needed to be identified, each with 95% certainty. Protein candidates containing similar peptides were grouped to satisfy the principles of parsimony. The search results were assessed for false-discovery rates using randomized sequence databases.

Identification of intermediates. The strain Rozel was grown in 160-ml serum bottles filled with 48 ml of MSM supplemented with 2.5 M NaCl and inoculated with 2 ml of actively growing culture from the mother bottle. All bottles were amended with 2 μl of deuterated benzene (benzene-D_6) and 2 μl of unlabeled benzene to achieve an approximate starting concentration of 46 μmol/bottle. These bottles were closed with rubber septa and aluminum crimps and incubated at 30°C for 10 days in an inverted position. Autoclaved control bottles were set up similarly with the labeled and unlabeled benzene. The benzene concentrations were monitored daily by GC. Triplicate active bottles were sacrificed each day (at the end of 24 h), and triplicate control bottles were sacrificed at the end of day 0 and day 10. Bacterial activity was stopped by acidifying the content with 5 N HCl (pH < 2). The entire content of the bottles was extracted with ethyl acetate (10% [vol/vol]; 4 times). The extracts were dried over anhydrous Na_2SO_4, concentrated by rotary evaporation, and reduced further under a stream of N_2 to a volume of 50 μl. The extracts and the phenol-D_6 standard were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, IL) prior to the analyses of the resulting compounds using an Agilent 6890 model GC coupled with an Agilent model 5973 mass spectrometer (MS) as previously described (1). All the identifications were made by comparison to the GC retention times and the mass spectral fragmentation profiles of commercial standards (Sigma-Aldrich, St. Louis, MO) that were similarly analyzed or by comparison with the National Institute of Standards and Technology Mass Spectral Library, version 2.0a.

Nucleotide sequence accession numbers. The nucleotide sequences of the six ORFs (1079 to 1084) corresponding to components of phenol hydroxylase were deposited in the GenBank database under accession numbers JX311705 to JX311710. The nucleotide sequences of ORFs 1078, 1085, 1086, 1087, 1088, 1089, and 1090 were deposited in the GenBank database under accession numbers JX311711 to JX311717.

RESULTS

Genomic analysis. *In silico* analysis of the genome of strain Seminole revealed a number of ORFs predicted to encode enzymes for aromatic hydrocarbon degradation. The genes were clustered on a 32-kb contig. To infer possible catabolic functions of these putative ORFs, BLASTp analyses were performed (E value < 1e−45) against the UniProtKB database (55). We have predicted 13 putative genes that encode enzymes for aromatic compound degradation. These proteins share 44 to 77% sequence identity with proteins previously described in nonhalophilic organisms (Table 1). The products of selected ORFs likely to be involved in both the upper and lower benzene degradation pathways are shown in Fig. 1. For example, we found that six ORFs (1079 to 1084) share a significant identity with components of phenol hydroxylase found in many hydrocarbon-degrading nonhalophiles, including the P0 to P5 components that catalyze phenol-to-catechol conversion in the *Pseudomonas* sp. strain CF600 (38). The deduced amino acid sequence of ORF 1080 is 51% identical to the P1 component in *Pseudomonas* sp. strain CF600, and ORF 1082 is 77% identical to the *phtD*-encoded phenol hydroxylase component in *Wautersia numazuensis* (20), which has a binuclear iron center. ORF 1084 is similar to the P5 component of phenol hydroxylase, which acts as an NADH-ferredoxin oxidoreductase in *Pseudomonas* sp. strain CF600 and other hydrocarbon-degrading organisms (27, 45, 47). Phylogenetic analyses (Fig. 2) also confirmed that ORFs 1080, 1082, and 1084 are closely related to the P1, P3, and P5 components of phenol hydroxylase in *Pseudomonas* sp. strain CF600. The remaining ORFs, 1086 and 1090, code for ring-cleaving 2,3-CAT and 2-hydroxymuconic semialdehyde dehydrogenase, respectively.

Proteomic analyses. To identify the enzymes involved in the initial steps of benzene degradation, a proteomic approach was employed. Two-dimensional gel electrophoresis resolved approximately 1,100 protein spots in the cytosolic proteomes of the strain Rozel cells grown on benzene or lactate as the growth substrate. Quantitative comparison of the resolved proteomes using the Progenesis algorithm revealed significant differences in the protein profiles of cells grown on benzene compared to cells grown on lactate. At least 15 additional proteins were present in benzene-grown cells than in lactate-grown cells. Considering the isoelectric point (pI) and the molecular weight (MW) of the predicted enzymes involved in aromatic catabolism, we targeted 7 of these proteins for identification, 3 of which are shown in Fig. 3. These protein spots were excised and analyzed by using LTQ Orbitrap LC-MS/MS, and the generated peptide mass fingerprints were used for protein identification by using MASCOT and the translated protein database of the strain Seminole genome. Spot 5 was identified as the product of ORF 1086, while spots 6 and 7 matched the products of ORFs 1080 and 1082, respectively, in the strain Seminole genome. ORF 1086 was predicted to encode 2,3-CAT, and ORFs 1080 and 1082 encode phenol hydroxylase compo-

TABLE 1 *In silico* identification of putative ORFs in the benzene degradation pathway in *Arhodomonas* sp. strain Seminole[a]

ORF	Query length[b] (aa)	Putative function[c]	GenBank accession no.	Domain	Organism	% identity[d]	E-value	UniProtKB accession no.
1078	567	Activator of phenol-degradative genes	JX311711	Transcriptional regulator containing PAS, AAA-type ATPase, DNA-binding domains	*Cupriavidus necator* N-1	56	e−174	F8GQJ3
1079	76	Phenol hydroxylase assembly protein (P0)	JX311705	Phenol hydroxylase subunit	*Acinetobacter* sp. RUH2624	46	4e−10	D0C567
1080	**334**	**Phenol hydroxylase P1 protein**	**JX311706**	**Aromatic and alkene monooxygenase hydroxylase, subunit B, ferritin-like diiron-binding domain**	***Pseudomonas* sp. CF600**	**51**	**1e−115**	**P19730**
1081	91	Phenol hydroxylase component (*phyB*)	JX311707	MmoB/DmpM family	*Ralstonia* sp. KN1	66	2e−25	Q9RAF7
1082	**513**	**Phenol hydroxylase component (*phtD*)**	**JX311708**	**Aromatic and alkene monooxygenase hydroxylase, subunit A, ferritin-like diiron-binding domain**	***Wautersia numazuensis***	**77**	**0**	**Q5KT19**
1083	56	Phenol hydroxylase component (*poxE*)	JX311709	Phenol hydroxylase conserved region	*Ralstonia* sp. E2	56	4e−35	O84962
1084	353	Phenol hydroxylase P5 protein	JX311710	Oxygenase reductase FAD/NADH binding domain	*Pseudomonas* sp. CF600	64	3e−161	P19734
1085	115	Plant type ferredoxin-like protein	JX311712	2Fe-2S iron-sulfur cluster binding domain	*Azoarcus* sp. strain BH72	44	3e−15	A1K6K5
1086	**317**	**Catechol 2,3-dioxygenase**	**JX311713**	**Catechol 2,3-dioxygenase**	***Ralstonia metallidurans***	**69**	**e−129**	**Q1LNR9**
1087	194	Uncharacterized protein	JX311714	No putative domains detected	*Magnetospirillum magneticum*	50	8e−139	Q2W7L9
1088	229	Transcriptional regulator	JX311715	Transcriptional regulators	*Azoarcus* sp. strain BH72	48	4e−49	A1K899
1089	141	Putative uncharacterized protein	JX311716	Domain of unknown function	*Pseudomonas putida*	59	2e−27	Q49KG4
1090	486	2-Hydroxymuconic semialdehyde dehydrogenase	JX311717	Aldehyde dehydrogenase family	*Marinobacter* sp. MnI7-9	70	0	G6YS35

[a] Shown are ORFs putatively involved in both upper and lower benzene degradation pathways identified by genomic analysis of the draft genome of *Arhodomonas* sp. strain Seminole.
[b] Amino acid length.
[c] The putative functions of ORFs were predicted using BLASTp with the UniProtKB database. The identification of the proteins in boldface type was verified using proteomic analyses.
[d] Percentage identity was based on BLASTp hits against the UniProtKB database.

nents (Table 2). All three identified proteins were among the most abundant proteins in the cytosolic proteome of the benzene-grown cells. The products of the other predicted ORFs listed in Table 1 were either not resolved well or not translated in appreciable quantities under the conditions used for growing the strain Rozel.

Metabolite detection using GC-MS. During benzene degradation by the strain Rozel, culture fluids collected periodically were extracted, derivatized, and analyzed by GC-MS for the detection of intermediates. Metabolic intermediates were identified by comparing their molecular mass ions (*m*/*z*) and retention times to those of authentic standards. We hypothesized the formation of phenol as a metabolic intermediate by the phenol hydroxylase-like proteins identified in our proteogenomic studies. To confirm that phenol is indeed produced as an intermediate, we grew the strain Rozel in the presence of deuterated benzene (benzene-D_6) and monitored it for the formation of deuterated phenol (phenol-D_6) using GC-MS. A mass spectral profile of a BSTFA-derivatized phenol-D_6 was detected in the inoculated bottles (Fig. 4), and no such peak was detected in the control bottles (data not shown). We monitored the formation of phenol-D_6 on a daily basis for 10 days and were able to detect the GC-MS response for phenol-D_6 in our culture fluids. However, we did not detect catechol in our analyses. The formation of phenol-D_6 was confirmed by injecting the BSTFA-derivatized authentic phenol-D_6 (Fig. 4) and by comparing its mass spectrum and retention time. The mass ions occurring at *m*/*z* 171 and 156 correspond to trimethylsilyl- and dimethylsilyl (formed by the loss of one methyl group from the derivatizing group)-derivatized authentic phenols, respectively. Thus, our finding conclusively shows phenol to be an initial intermediate of aerobic benzene degradation by *Arhodomonas* sp. strain Rozel under hypersaline conditions.

DISCUSSION

Genomic studies provide valuable information regarding degradation pathways as well as the general physiological potential of microorganisms. This study clearly demonstrates how genomic information from one organism can be a useful tool for studying physiology in phylogenetically related organisms. We used a draft

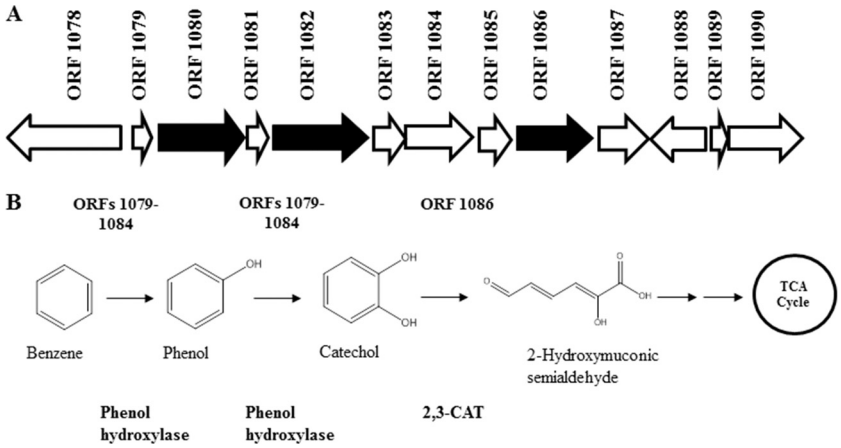

FIG 1 (A) Schematic diagram showing the genetic organization of benzene-degrading ORFs predicted on contig 494 of the *Arhodomonas* sp. strain Seminole genome. These candidate ORFs are involved in the initial steps of the benzene degradation pathway. The putative functions of the candidate ORFs are listed in Table 1. The ORFs with dark arrows were identified by proteomic analysis. The arrowheads indicate the directions of transcription, and the gene sizes are not proportional to the sizes of the arrows. (B) Proposed benzene degradation pathway by a multicomponent phenol hydroxylase-like enzyme in *Arhodomonas* sp. strain Seminole. ORFs and the corresponding putative enzymes in bold were identified by genomic and proteomic analyses. Phenol was confirmed as the initial intermediate of benzene degradation by GC-MS. TCA, tricarboxylic acid.

genome sequence of the strain Seminole as a basis for predicting the early steps of the benzene degradation pathway in a closely related halophile, the strain Rozel. *In silico* analysis of the genome revealed a cluster of 13 genes that encode upper and lower pathway enzymes for aromatic compound degradation located on a 32-kb DNA fragment (Fig. 1A; Table 1). These enzymes share high (44 to 70%) amino acid sequence identity with the enzymes from nonhalophilic microorganisms, thus suggesting that these halophiles use enzymes similar to those found in nonhalophiles.

In this study we used genome-based predictions to target the experimental proteomic analyses and identify the enzymes involved in the initial step of the benzene degradation pathway. Proteomic analyses showed significant differences in the protein profiles of benzene-grown and lactate-grown cells and identified several proteins that were highly abundant and present only in benzene-grown cells. These included two putative phenol hydroxylase components with ferritin-like di-iron-binding domains and a 2,3-CAT. These results indicate that benzene is converted to phenol and then to catechol in two steps by monooxygenase-like enzymes closely related to phenol hydroxylases. Thus, formed catechol is most likely further degraded to 2-hydroxymuconic semi-

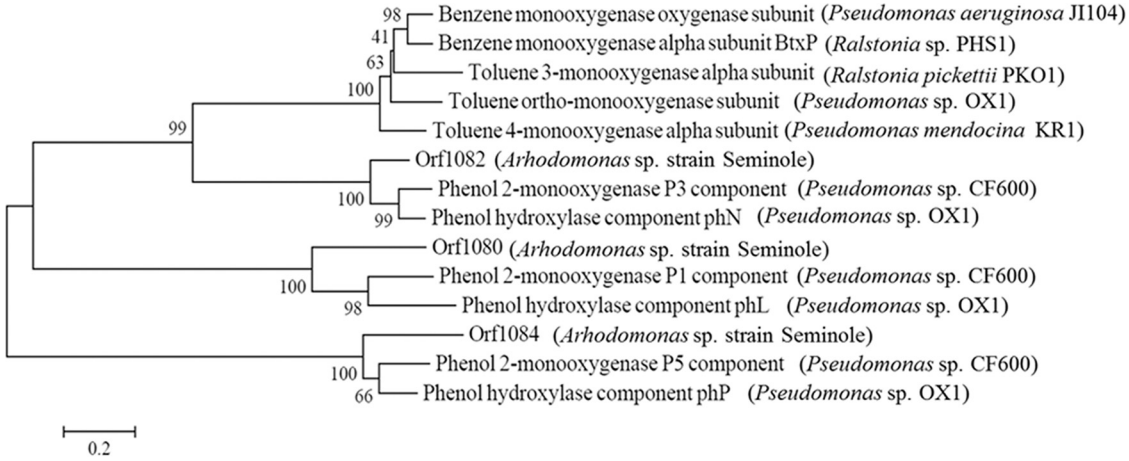

FIG 2 Phylogenetic analysis chart showing the relationships among various monooxygenase components in *Arhodomonas* sp. strain Seminole and in other aromatic hydrocarbon-degrading nonhalophiles. The unrooted neighbor-joining tree was constructed in MEGA 5 by using predicted amino acid sequences of ORF 1080, ORF 1082, and ORF 1084 from the strain Seminole genome and closely related phenol hydroxylase, toluene monooxygenase, and benzene monooxygenase subunits from nonhalophiles. Sequences for the analysis were obtained from the GenBank and UniProtKB database. Bootstrap values were calculated as a percentage of 1,000 replicates and are shown next to the branches. The enzyme components (and the corresponding GenBank accession numbers) are benzene monooxygenase oxygenase subunit (BAA11761), benzene monooxygenase alpha subunit BtxP (ABG82181), toluene 3-monooxygenase alpha subunit (AAB09618), toluene *ortho*-monooxygenase subunit (CAA06654), toluene 4-monooxygenase alpha subunit (AAS66660), phenol hydroxylase phL component (AAO47356), phenol hydroxylase phN component (AAO47358), and phenol hydroxylase phP component (AAO47360). The enzyme components (and the UniProtKB accession numbers) are phenol 2-monooxygenase P1 component (P19730), phenol 2-monooxygenase P3 component (P19732), and phenol 2-monooxygenase P5 component (P19734).

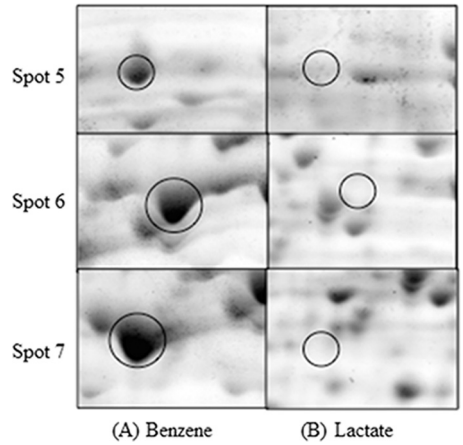

FIG 3 A 2D gel image showing candidate protein spots. Progenesis Workstation software was used for protein spot detection, matching, and abundance quantification. (A and B) Sections of the 2D gels with protein spots of the cytosolic proteome of cells grown on benzene (A) and lactate (B). The protein spots detected only in benzene-degrading cells are circled (lane A). The spots were in-gel digested and analyzed by LTQ Orbitrap LC-MS/MS to create peptide mass fingerprints (PMFs). The PMFs were identified by using MASCOT and the translated protein database of the *Arhodomonas* sp. strain Seminole genome.

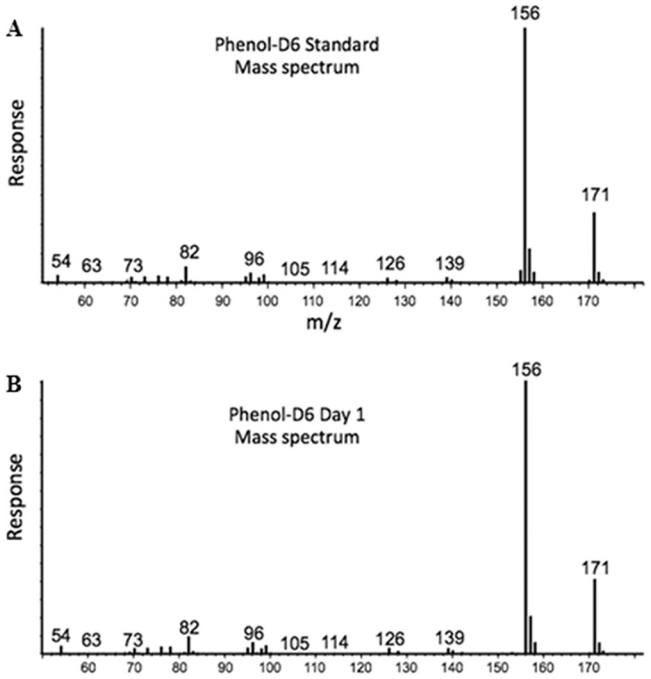

FIG 4 (A) Mass spectrum of a BSTFA-derivatized phenol-D_6 standard. (B) A similar mass spectrum of a BSFTA-derivatized metabolite was found in a deuterated benzene (benzene-D_6)-fed strain Rozel culture on day 1, confirming the metabolite as phenol.

aldehyde via meta-cleavage by ORF 1086, identified as 2,3-CAT (Fig. 1B).

Phenol hydroxylases are multicomponent soluble di-iron-containing monooxygenases that hydroxylate a variety of aromatic hydrocarbons (27). These enzymes are similar to toluene-monooxygenases and have been shown to catalyze the insertion of one oxygen atom not only in phenols but also in a number of hydrocarbons, including benzene, toluene, naphthalene, and trichloroethylene. Our genomic analyses predicted multicomponent phenol hydroxylase-like proteins, including ORFs 1079 to 1084, that are closely related to the *dmp* phenol hydroxylase components P0 to P5, respectively, from the *Pseudomonas* sp. strain CF600 (38, 47, 51). Also, a similar arrangement has been observed in plasmid- or chromosomally encoded phenol hydroxylase complexes, such as the *phy* operon from *Ralstonia* sp. KN1 (35), the *pox* operon from *Ralstonia eutropha* strain E2 (18), the *pht* operon from *Wautersia numazuensis* (20), the *ph* operon from *Pseudomonas stutzeri* OX1 (8), and the gene identifier MpeA2280-85 in *Methylibium petroleiphilum* (21). In addition to those in multicomponent phenol hydroxylases, ORFs 1079 to 1084 also showed significant sequence identity with the polypeptides from toluene/*o*-xylene monooxygenase in *Pseudomonas stutzeri* OX1 (7). Phylogenetic analysis (Fig. 2) of the three major proteins, including ORFs 1080, 1082, and 1084, also showed these ORFs to be closely related (51 to 77% sequence identity) to the major components (P1, P3, and P5) of phenol hydroxylases in *Pseudomonas* sp. strain CF600 and other organisms in comparison to benzene monooxygenases (28% sequence identity). All together, these analyses sug-

TABLE 2 Identification of proteins induced in the proteome of strain Rozel grown on benzene using LC-MS/MS

Protein spot[a]	ORF	pI/MW		Protein	Organism	No. of unique spectra	Sequence coverage (%)	Average signal intensity[c] (SD)	
		Experimental	Theoretical[b]					Lactate	Benzene
5	1086	5.2/35	4.9/35	Catechol 2,3-dioxygenase	*Ralstonia metallidurans*	12	19	ND[d]	2.339 (0.198)
6	1080	5.5/38	5.02/38	Phenol hydroxylase P1 protein	*Pseudomonas* sp. CF600	27	25	ND	1.224 (0.049)
7	1082	6.0/63	5.34/61	Phenol hydroxylase component, phtD	*Wautersia numazuensis*	40	32	ND	0.181 (0.058)

[a] Spot numbers correspond to those in Fig. 3. The protein spots 5, 6, and 7 on the 2D gel were identified using LC-MS/MS, MASCOT, and the translated genome of *Arhodomonas* sp. strain Seminole.
[b] Theoretical isoelectric points and molecular weights of the proteins of interest were calculated using the Compute pI/MW tool from EXPASY. These values were consistent with the experimentally determined pI values and molecular weights.
[c] Spot signal intensities were normalized and averaged over three replica gels (each from independent experiment). These values and SD were calculated using Progenesis algorithms.
[d] Not detected.

gest that ORFs 1079 to 1084 encode functional components of phenol hydroxylase-like proteins in the *Arhodomonas* strains and that these enzyme complexes are composed of hydroxylase, reductase [iron-sulfur flavoprotein that transfers electrons from NAD(P)H to the oxygenase], and a regulator protein that is required for catalysis (27, 45).

The proteogenomic data were further supported by the GC-MS detection of phenol as an intermediate during benzene degradation by the strain Rozel. The analysis showed the formation of phenol within 24 h of incubation, suggesting that benzene is quickly converted to phenol. Thus, these results indicate that the formation of phenol is a result of enzymatic hydroxylation of the benzene ring by phenol hydroxylase-like enzymes. Our GC-MS analyses did not detect the formation of catechol. This could be due to many reasons, including the rapid turnover rate and/or the low concentration of catechol. However, the proteogenomic data clearly suggest catechol as the intermediate. These observations suggest that monooxygenases are versatile catalysts that are not restricted to nonhalophiles but also are employed by halophiles for similar functions, underscoring their diversity, versatility, and ecological spread in microorganisms. In this study we report the biochemical mechanism underlying the initial steps of benzene degradation in the novel Seminole and Rozel halophilic strains.

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