ISOLATION AND CHARACTERIZATION OF HALOTOLERANT 2,4-DICHLOROPHENOXYACETIC ACID DEGRADING BACTERIA FROM SULFIDIC, LOW SALINITY SALT SPRINGS

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ABSTRACT

The bacterial communities at two sulfidic, low salinity springs with no history of herbicide contamination were screened for their ability to grow on 2,4-dichlorophenoxyacetic acid (2,4-D). Nineteen isolates, closely matching the genera *Bacillus*, *Halobacillus*, *Halomonas*, *Georgenia* and *Kocuria*, showed diverse growth strategies on NaCl-supplemented and NaCl-free 2,4-D medium. The majority of isolates were halotolerant, growing best on nutrient rich broth with 0% or 5% NaCl; none of the isolates thrived in medium with 20% NaCl. The *tfdA* gene, which codes for an α – ketoglutarate dioxygenase and catalyzes the first step in 2,4-D degradation, was detected in nine of the salt spring isolates. The *tfdAa* gene, which shows ~60% identity to *tfdA*, was present in all nineteen isolates. Many of the bacteria described here were not previously associated with 2,4-D degradation suggesting these salt springs may contain microbial communities of interest for bioremediation.

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KEYWORDS

- 2,4-D
- *tfdA*
- *tfdAa*
- salt springs
- halotolerant bacteria

INTRODUCTION

Bacteria have tremendous potential to degrade organic compounds and study of the metabolic pathways involved is a key component to more efficient environmental remediation. One well-studied organic acid degradation pathway is that of 2,4–dichlorophenoxyacetic acid (2,4-D) (4, 12, 13, 15, 19, 23). The broadleaf herbicide 2,4-D was developed during World War II by British scientists to help boost production of grass crops like corn, wheat, and rice (23). Since the late 1940's it has been one of the most popular herbicides used by industrial farms and homeowners for the control of broadleaf weeds (30).

The 2,4-D degradation pathway of *Cupriavidus necator* JMP134 (B-*Proteobacteria*; formerly *Ralstonia eutropha*) has received considerable attention and serves as a model system for microbial degradation (2, 3, 26). The first gene in the catabolic pathway, *tfdA*, codes for an α – ketoglutarate dioxygenase (6, 7). The remainder of the pathway is comprised of at least five genes, e.g. *tfdBCDEF*, that continue to break down the molecule for use in central metabolism (29). Members of the *a-*, *B-* and γ-*Proteobacteria* are known to degrade 2,4-D and several unique degradation pathways have been identified. In the B- and γ- *Proteobacteria* three classes of *tfdA* genes are found while the *a-Proteobacteria* carry *tfdAa* that has ~60% identity to the *tfdA* gene of JMP134 (9, 30). A novel *cadABC*-based degradation

pathway which codes for 2,4-D oxygenase subunits (15) has also been found in *a-Proteobacteria* (10).

Although there has been extensive research conducted on 2,4-D degraders in the soil (12 ,13 ,15, 23), there is less work on the removal of 2,4-D in aquatic environments of varying salinity. Maltseva *et al.* (18) isolated three 2,4-D degrading bacteria from the family *Halomonadaceae* in the soil of a high salinity, alkaline lake that was heavily polluted with 2,4-D and other organic acids. Although these isolates came from an extreme environment, and were moderately halophilic and alkaliphilic, they still utilized the well-known *tfdA*-based degradation pathway of JMP134 (18).

The objective of this study was to isolate and characterize 2,4-D degrading bacteria from salt springs at Big Bone Lick State Park in Boone County, KY. The park and surrounding area contain numerous natural springs but this area does not have a history of 2,4-D contamination. The isolates obtained, including some genera not previously associated with 2,4-D degradation, were examined for their ability to grow on 2,4-D in the presence and absence of NaCl and screened for commonly occurring 2,4-D degradation genes. Isolation and characterization of 2,4-D degrading bacteria from novel environments, such as the salt springs in Big Bone Lick State Park, not only expands information on the diversity of herbicide degrading bacteria but also provides useful information for bioremediation of contaminated saline environments.

MATERIALS AND METHODS

SITE DESCRIPTION AND SAMPLE COLLECTION

Salt spring samples were collected from Big Bone Lick State Park located in Boone County, KY. The area surrounding the park is a rural semi-agricultural region; there are several salt-springs inside the park's boundary and many more springs throughout the surrounding area. Two springs were the focus of this study because they are easily accessed from the park visitor center. Spring samples representing both spring water and water mixed with spring sediment were collected from the small spring (N 38° 53' 4.582", W 84° 45' 10.648") and large spring (N 38° 53" 5.899", W 48° 45" 9.928") in sterile polypropylene containers, transported to IU Southeast in a cooler (~1.5 h) and used immediately for plating experiments.

PLATING AND ISOLATE PURIFICATION

A dilution series of the spring samples was done using sterile phosphate buffered saline with dilutions spread onto Artificial Sea Water nutrient broth (ASW) (17) agar plates with 5, 10 or 20% NaCl to assess levels of halotolerant heterotrophs and MMO minimal medium plates (13) supplemented with 125 ppm 2,4-D / 5% NaCl to assess levels of 2,4-D degraders. Plates were incubated for two weeks at 28°C. Single colonies were transferred to MMO 125 ppm 2,4-D / 5% NaCl for purification and after several plate transfers they were grown in Difco Marine broth (Beckton Dickinson & Co., Sparks, MD) and stored at -80°C in 15% glycerol.

GROWTH ASSESSMENT ON 2,4-D

Purified isolates were streaked to MMO 2,4-D plates with and without 5% NaCl and incubated at 28°C for 14 days. At regular intervals growth of the isolates was assessed visually by two independent observers and growth was scored as none (-), poor (+), moderate (++) or good (+++).

DNA EXTRACTION, PCR AND 16S rRNA GENE CHARACTERIZATION

Genomic DNA from individual isolates was extracted using the MoBio Microbial DNA Extraction Kit (mobio.com). The 16S rRNA gene was amplified using 27f and 1492r primers (25), and isolates were screened for *tfdA* using the TVU and TVL primers of Hogan *et al.* (8). Screening for *tfdAα* was done using Alpha1 (5'-ACS GAG TTC KSC GAC ATG CG-3') and Alpha2 (5'-GCG GTT GTC CCA CAT CAC-3') primers using the PCR conditions described by Itoh *et al.* (9). Promega GoTaq® Green Master Mix was used for PCR with reaction mixtures prepared according to manufacturer's protocols and thermal cycling was performed using a TECHNE Flexigene thermal cycler. The 16S rRNA gene products were purified using the MoBio Ultraclean™ PCR Cleanup Kit and DNA sequencing using primers 27f, 1492r and 519f (25) was performed at the IUPUI Core Sequencing Facility using Applied Biosystems 3100 Genetic Analyzers and Big Dye Terminator chemistry v3.1 (perkinelmer.com). Sequences were edited using Chromas (http://technelysium.com.au) and compiled in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Full or partial sequences were used to query the Genbank database using the Basic Local Alignment Search Tool (BLAST) (1).

SALT TOLERANCE

Each isolate was transferred to 2ml of ASW nutrient broth (17) containing NaCl ranging from 0, 5, 10, 15 or 20% and incubated at 25°C at 200 rpm for 48 hours. Longer incubations of 72 and 96 hours did not yield increases in optical density. Optical density was measured using a Biotek ELx 800 plate reader at 630nm.

SALT SPRING CHEMISTRY

Spring water temperature, pH, salinity, total dissolved solids and conductivity were measured using a Hach SensION 156 multi-parameter meter on 9 Sept 2006, 8 Mar 2007, 30 Sept 2009, 16 Oct 2011, 5 Feb 2010 and 10 Oct 2010. No pH data was collected from the springs during the 5 Feb 2010 sampling trip as the pH probe malfunctioned on this date.

RESULTS

SALT SPRING CHARACTERISTICS

This study focused on two salt springs located within the borders of Big Bone Lick State Park in Boone County, KY. During six visits to the springs between 2006-2010 spring characteristics and water chemistry such as pH, temperature and salinity were measured (Table 1). The springs are shallow (~13 cm at the deepest point) and located no more than 100 meters from one another. In general, chemistry between the two springs was similar which, along with their proximity, suggests a common groundwater source.

	Small spring	Large spring
Temperature (°C)	16.10 ± 3.28	13.88 ± 2.42
pH	7.34 ± 0.22	7.49 ± 0.35
Salinity (‰)	7.32 ± 0.53	7.42 ± 0.27
TDS (mg/L)	6.91 ± 1.16	7.18 ± 0.23
Conductivity (µs/cm)	10.76 ± 0.86	10.14 ± 0.70

[1]Values are mean ± SEM. All measurements n=6 except pH where n=5.

Table 1. Water characteristics of two Big Bone Lick State Park salt springs.

ASSESSMENT OF CULTURABLE BACTERIAL COMMUNITIES OF THE SPRINGS

Spring samples representing water only and water mixed with sediment were plated onto artificial seawater medium (ASW) supplemented with 5, 10 or 20% NaCl to assess heterotrophic halotolerant bacterial populations (Table 2). CFUs were observed at all three salt concentrations although there was generally a reduction in CFU/mL as the salt concentration increased. Sediment samples from both springs contained larger bacterial populations at each salt concentration compared to water-only spring samples. Samples from both springs yielded CFUs on MMO 2,4-D medium supplemented with 5% NaCl (Table 2). The sediment containing small spring sample had the most 2,4-D degrading bacteria with counts ranging from 1-2 orders of magnitude more than the other three spring samples.

ISOLATION AND IDENTIFICATION OF PUTATIVE 2,4-D DEGRADING ISOLATES

Nineteen isolates transferred from the original 2,4-D plates and which produced small transparent colonies on media with 2,4-D as the sole carbon source at 28°C were chosen for further characterization. Using 16S rRNA gene sequence data the closest match to each isolate was determined from Genbank (Table 3). Eleven of 19 isolates closely matched the genus *Bacillus* with *Halobacillus* as the next most common genus (4 isolates) followed by *Halomonas* (2 isolates). Eleven of the sequences were nearly full length (1389 nts or greater) and all identities to database sequences were 99% with the exception of isolate 19 (*Halomonas ventosae*) which showed 98% identity.

GROWTH ASSESSMENT ON 2,4-D

To assess growth on 2,4-D as a sole carbon

Table 2. CFU/mL estimates of halotolerant bacteria in two Big Bone Lick State Park salt springs.

	Small spring		Large spring	
Growth medium	Water	Water + Sediment	Water	Water + Sediment
	CFU/mL	CFU/mL	CFU/mL	CFU/mL
5% NaCl ASW[1]	570	1.4×10^5	3.1×10^5	4.2×10^5
10% NaCl ASW	100	3.1×10^4	160	1.6×10^4
20% NaCl ASW	330	7.9×10^5	40	170
MMO 2,4-D 5%NaCl	60	8.1×10^5	70	730

[1]Artificial sea water agar-based medium supplemented with NaCl.

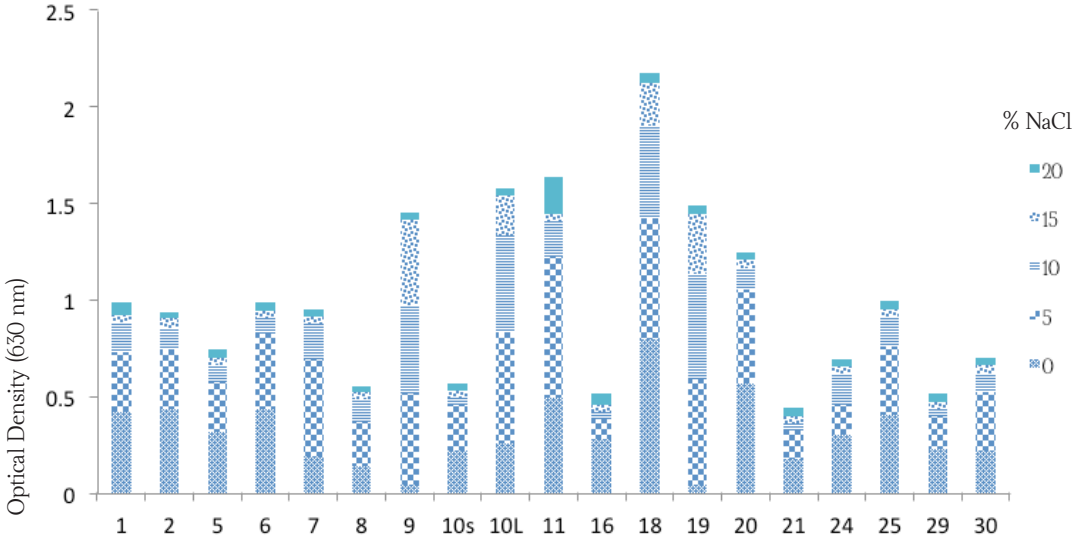

Fig. 1. Salt tolerance estimates of isolates from Big Bone Lick State Park. Each bar shows the optical density at 630nm across five salt concentrations in ASW broth.

Table 3. Identity, *tfdA* gene presence and growth characteristics of 2,4–D degrading halotolerant isolates.

Isolate number	Species/Accession number	Sequence length	% Identity	*tfda*[1]	*tfda–α*[2]	Growth 2,4–D	Growth 2,4–D + 5% NaCl
1	*Bacillus hwajinpoensis* / FR695443	1454	99		*	+++	+++
2	*Georgenia muralis* / AB455495	1406	99	*	*	+++	+++
5	*Bacillus hwajinpoensis* / FR695443	1469	99	*	*	++	++
6	*Bacillus hwajinpoensis* / FR695443	1440	99	*	*	++	++
7	*Bacillus baekryungensis* / AY505507	1452	99	*	*	++	++
8	*Bacillus aquamaris* / KF443807	958	99	*	*	+	++
9	*Halomonas sp.* / GU212640	1450	99	*	*	+	++
10s	*Halobacillus trueperi* / KJ174505	1470	99	*	*	+++	+++
10L	*Halobacillus trueperi* / DQ157162	1461	99	*	*	+	–
11	*Halobacillus alkaliphilus* / JQ068929	1467	99	*	*	+	+
16	*Bacillus sp.* / DQ084469	1453	99		*	+++	++
18	*Kocuria sp.* / HM579811	1389	99		*	–	++
19	*Halomonas ventosae* / GQ903444	795	98		*	–	++
20	*Bacillus baekryungensis* / JN210568	913	99		*	++	++
21	*Halobacillus alkaliphilus* / JQ068929	1465	99		*	+++	+
24	*Bacillus aquimaris* / KF769538	1436	99		*	+++	++
25	*Bacillus baekryungensis* / JN210568	1437	99		*	+	–
29	*Bacillus sp.* / HM587911	629	99		*	+++	+++
30	*Bacillus sp.* / HQ677199	687	99		*	+++	+++

[1]Amplified with primers from Hogan *et al.* (8).[2] Amplified with primers from Itoh *et al.* (9).

source each of the nineteen isolates was streaked onto MMO medium containing 125 ppm 2,4-D with or without 5% NaCl. Over the course of 14 days each isolate was visually examined independently by two investigators and the level of growth was scored as none (–), poor (+), moderate (++) or good (+++). Five isolates (represented by the genera *Bacillus*, *Georgenia*, *Halobacillus*) showed good growth (+++) on MMO 2,4-D plates with or without NaCl and four isolates showed moderate growth (++) on both medium types (Table 3). Interestingly, isolates 18 & 19 (represented by *Kocuria* and *Halomonas*, respectively) showed no growth (–) on MMO 2,4-D medium but good growth (+++) on MMO 2,4-D medium supplemented with NaCl. Numerous attempts to grow the nineteen isolates in MMO 2,4-D broth failed to yield observable growth. Additional attempts to encourage growth with casamino acids and to design liquid medium optimized to spring conditions also failed.

PRESENCE OF *TFDA* AND *TFDA*-LIKE GENES

The nineteen spring isolates were screened by PCR for two types of genes commonly found in 2,4-D degrading bacteria. DNA extracts from nine of the isolates were positive for the presence of *tfdA* and all nineteen isolates were positive for amplification of *tfdAa* (Table 3).

SALT TOLERANCE OF THE ISOLATES

Salt tolerance of each isolate was determined by inoculating ASW medium supplemented with 0, 5, 10, 15 or 20% NaCl. Fig. 1 shows the optical density at 630nm for each isolate across the five salt concentrations. Eighteen of the nineteen isolates showed a preference for media with 0% or 5% NaCl. ASW without added NaCl supported growth of all the isolates above 0.1 OD units with the exception of isolates 9 and 19 (both *Halomonas*). The two *Halomonas* isolates showed a similar growth pattern with good growth at 5, 10 and 15% NaCl. Although isolate 18 (*Kocuria* sp.) grew poorly on MMO 2,4-D medium without salt (Table 3), it grew the best of the nineteen isolates in 0% ASW and also had the highest combined optical density across the salt concentrations of any isolate (Fig. 1).

DISCUSSION

The objective of this study was to undertake a culture-based approach to examine 2,4-D degrading bacteria in salt springs at Big Bone Lick State Park. The springs in the park offer a unique environment for microbial growth. Low salinity, sulfur-enriched groundwater feeds the springs throughout the year and mineral deposits regularly appear in the spring's water and outer edges as white precipitate. This work represents the first attempt to examine 2,4-D degrading microbial communities in these springs. Although the springs sampled are of low salinity previous culture-based studies have identified large populations of moderate halophilic and halotolerant bacteria in spring water and sediment samples. With this in mind, the current study was designed to isolate 2,4-D degrading bacteria that could tolerate at least 5% NaCl.

Although the salt springs sampled in this

study have no known history of 2,4-D contamination, samples from two salt springs at Big Bone Lick State Park showed an abundance of colonies on agar-based plates with 2,4-D as the sole carbon source. Ka *et al.* (12) demonstrated that successive 2,4-D applications in agricultural soil can greatly enrich the soil microbial community for 2,4-D degrading bacteria. Although the Big Bone Lick springs were not obviously treated or heavily contaminated with 2,4-D, low level 2,4-D groundwater contamination from agricultural runoff cannot be ruled out as an enrichment source.

Varied growth levels on 2,4-D medium with and without salt were observed in the nineteen spring isolates chosen for further characterization. While some isolates grew equally well on 2,4-D medium with or without NaCl, others showed a clear preference for salt-free or salt-containing medium. These diverse growth strategies make sense given that the isolates described here were enriched on 2,4-D agar-based medium rather than 2,4-D supplemented broth which may select only the fastest growing isolates. Allowing the initial 2,4-D containing plates to incubate for 14 days may have also provided slow growing isolates time to become established. Novel slow growing 2,4-D degrading bacteria have been found in pristine environments using an enrichment strategy involving organic supplements and low initial levels of 2,4-D (14).

Two of the nineteen isolates described here were identified as closely matching the genus *Halomonas*. Maltseva *et al.* (18) previously isolated three moderately halophilic, alkaliphilic bacteria from alkali lakes heavily contaminated with 2,4-D and numerous other aromatic compounds. These isolates were members of the Halomonadaceae family and contained 2,4-D degradation pathways that matched the *tfdA* pathway from *Cupriavidus necator* JMP134. As *tfdA* was found in only one of the two *Halomonas* isolates described here, more work is clearly needed to determine the nature of degradation in the non-*tfdA* containing *Halomonas* isolate. *Halomonas* is a metabolically diverse genus well known for its halotolerance and biodegradative potential. For example, *Halomonas organivorans*, isolated from the soil of Isla Cristina, Spain grows on phenol and a wide-range of other organic acids (5).

Interestingly, seventeen of the nineteen isolates described here were Gram positive bacteria. *Bacillus* was the most common genus found (eleven isolates) and several of these contained the *tfdA* gene. *Bacillus* sp. have been previously reported to carry *tfdA* (8) but these isolates did not degrade 2,4-D. The *Bacillus* isolates in this study closely match recently described halotolerant species such as such as *Bacillus aquamaris*, *Bacillus hwajinpoensis* and *Bacillus baekryungensis* (27, 28) and thus these isolates should be screened more fully for their degradative potential.

Isolates closely matching *Halobacillus* were also identified as 2,4-D degraders in this study and three out of the four isolates contained the *tfdA* gene. The genus *Halobacillus* was first proposed in 1996 (24) and now contains nearly 20 species with interesting properties such as production of protective carotenoids and novel salt-tolerant enzymes of interest to biotechnology (11). This is the first report identifying 2,4-D degradation genes in this genus.

Two additional Gram positive isolates closely matched *Kocuria* sp. and *Georgenia muralis*. *Kocuria* are commonly found on the skin of humans and some species have recently emerged as pathogens in immunocompromised patients (22). *Georgenia muralis* was first isolated from

a medieval wall painting in the church St. Georgen in Austria in 2002 (16). A novel finding of this study is that *Georgenia muralis* and *Kocuria* sp. have not previously been shown to degrade 2,4-D. The *Georgenia muralis* isolate described here not only grew well on 2,4-D medium with or without added NaCl but also contained the *tfdA* gene, which has not previously been reported.

All nineteen of the isolates from Big Bone Lick State Park were positive for *tfdAa*. *tfdAa* is found in *Proteobacteria* such as *Bradyrhizobium* and *Sphingomonas* sp. (9, 10) and thus it is interesting that the diverse collection of isolates described here, none of which are *a-Proteobacteria*, carry this gene. Additional primer sets designed to detect novel *tfdA*-like genes were recently described (30) and it would be informative to screen the isolates described here with these primers to further examine *tfdA* gene diversity.

Nine of the nineteen isolates in this study contained both *tfdA* and *tfdAa* raising the possibility that multiple degradation pathways exist in these isolates. It has been suggested that the *tfdA* gene products serve other cellular roles besides the degradation of herbicides (8) and it would be worthwhile to screen the Big Bone Lick salt spring isolates for other degradation abilities.

One area to enhance this study lies in designing minimal broth that will support growth of the salt spring isolates. Numerous attempts to grow the isolates in minimal liquid medium with 2,4-D as the sole carbon source failed to stimulate growth. Additionally, liquid medium designed to replicate spring water chemistry also yielded poor growth. Development of minimal broth supporting growth of the isolates described here will allow confirmation of 2,4-D degradation and provide a means for quantitative determination of 2,4-D disappearance (14).

In summary, this study has identified the salt springs at Big Bone Lick State Park as a potentially novel source of 2,4-D degrading bacteria. Since degradation pathways in the salt spring isolates appear to match those associated with *tfdA* and *tfdAa*, it would be informative to sequence these genes to determine their relationship to other known 2,4-D degradation pathways. Horizontal gene transfer of 2,4-D degradation pathways has been proposed based on incongruent phylogenies of 16S rRNA genes compared to *tfdA* (20). More recently, a transposon containing both *cadAB* and tfd associated genes, but not *tfdA* or *tfdAa* was found in a 2-methyl-4-chlorophenoxyacetic acid (MCPA) degrading *Sphingomonas* isolate (21). Understanding the role gene transfer plays in the evolution of moderately halophilic bacteria would be an exciting area of future research.

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