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Chai, Benli, Tsoi, Tamara, Sallach, J. Brett orcid.org/0000-0003-4588-3364 et al. (10 more authors) (2019) Bioavailability of clay-adsorbed dioxin to *Sphingomonas wittichii* RW1 and its associated genome-wide shifts in gene expression. *Science of the Total Environment*. 135525. ISSN 0048-9697

<https://doi.org/10.1016/j.scitotenv.2019.135525>

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1 **Bioavailability of clay-adsorbed dioxin to *Sphingomonas wittichii***
2 **RW1 and its associated genome-wide shifts in gene expression**

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1 **Abstract**

2 Polychlorinated dibenzo-*p*-dioxins and dibenzofurans are a group of chemically-related
3 pollutants categorically known as dioxins. Some of their chlorinated congeners are among the
4 most hazardous pollutants that persist in the environment. This persistence is due in part to the
5 limited number of bacteria capable of metabolizing these compounds, but also to their limited
6 bioavailability in soil. We used *Sphingomonas wittichii* strain RW1 (RW1), one of the few
7 strains able to grow on dioxin, to characterize its ability to respond to and degrade clay-bound
8 dioxin. We found that RW1 grew on and completely degraded dibenzo-*p*-dioxin (DD)
9 intercalated into the smectite clay saponite (SAP). To characterize the effects of DD sorption
10 on RW1 gene expression, we compared transcriptomes of RW1 grown with either free
11 crystalline DD or DD intercalated clay, i.e. sandwiched between the clay interlayers (DDSAP).
12 Free crystalline DD appeared to cause greater expression of toxicity and stress related
13 functions. Genes coding for heat shock proteins, chaperones, as well as genes involved in DNA
14 repair, and efflux were up-regulated during growth on crystalline dioxin compared to growth
15 on intercalated dioxin. In contrast, growth on intercalated dioxin up-regulated genes that might
16 be important in recognition and uptake mechanisms, as well as surface
17 interaction/attachment/biofilm formation such as extracellular solute-binding protein and
18 LuxR. These differences in gene expression may reflect the underlying adaptive mechanisms
19 by which RW1 cells sense and deploy pathways to access dioxin intercalated into clay. These
20 data show that intercalated DD remains bioavailable to the degrading bacterium with
21 implications for bioremediation alternatives.

22 **Key words:** dioxin, bioavailability, clay-adsorbed, *Sphingomonas wittichii*, toxicity,
23 transcriptome

1 **Introduction**

2 The ubiquitous occurrence of polychlorinated dibenzo-*p*-dioxins and dibenzofurans
3 (PCDD/Fs) is a result of their widespread formation and distribution as an unintentional
4 chemical byproduct of industrial and incineration processes as well as through natural
5 formation during forest fires and volcanic activity (US EPA, 2006). While the contribution of
6 anthropogenic PCDD/Fs sources has decreased markedly since the 1980's, they remain a
7 significant contaminant of concern based on their extreme environmental persistence and
8 toxicity at low exposure dosage (Alcock and Jones, 1996; Van den Berg et al., 1998). Efforts to
9 remediate highly contaminated sites, including many Superfund sites in the United States,
10 typically involve remediation technologies characterized by high cost and high environmental
11 impact. The remedies most commonly employed include excavation and dredging followed by
12 landfilling of contaminated soils and sediments (Kulkarni et al., 2008; Bridges et al., 2010).
13 For this reason, researchers have been motivated to develop new remediation technologies for
14 the cleanup of these sites that are less costly and minimize habitat destruction. Two of the most
15 promising include the application of sorbent amendments to sequester PCDD/Fs in forms that
16 reduce or eliminate their bioavailability, as well as microbiologically mediated biodegradation
17 of PCDD/Fs (Kulkarni et al., 2008; Ghosh et al., 2011; Chai et al., 2016).

18 A significant challenge in microbiologically mediated biodegradation has been the
19 isolation of bacteria and/or bacterial communities that can detoxify PCDD/Fs in the
20 environment (Moreno-Forero et al., 2015). While a number of isolated bacterial strains have
21 shown the ability to grow on dibenzofuran (DF) or co-metabolize specific PCDD/F congeners,
22 few isolates have been discovered with the ability to utilize dibenzo-*p*-dioxin (DD) as a sole
23 carbon source (Field and Sierra-Alvarez, 2008). *Sphingomonas wittichii* strain RW1 (RW1),

1 isolated from the Elbe River in Germany, is one of the few bacterial strains with the ability to
2 grow on PCDD/Fs, specifically DD (Wittich et al., 1992), DF, and 4-chloro-dibenzofuran
3 (Field and Sierra-Alvarez, 2008; Change, 2008; Wilkes et al., 1996). It is also able to co-
4 metabolize PCDD/F congeners with up to six Cl substituents (Wilkes et al., 1996).
5 Furthermore, RW1 is to our knowledge the only such strain with a sequenced genome.

6 Previous studies have investigated the specific metabolic pathways used by RW1 in the
7 oxidation of DD and DF including the common upper pathway responsible for transforming
8 DF into salicylate and DD into catechol followed by further catabolism into aliphatic
9 compounds prior to entering the TCA cycle for complete oxidation (Chai et al., 2016). The
10 RW1 genome consists of one chromosome and two mega plasmids (Wittich et al., 1992).
11 Recent gene-knockout studies show that degradation of DD, but not DF, by RW1 requires at
12 least one chromosomally-encoded upper pathway gene in addition to the plasmid-encoded
13 upper pathway genes (Thamer and Zylstra, 2016). This indicates that conjugal plasmid transfer
14 alone would be insufficient to confer DD degradation ability and leads to the question of
15 whether RW1 contains other chromosomally-encoded features optimized for DD degradation.

16 Our previous work used transcriptomic analysis to outline the differences in RW1's
17 transcriptional responses to DD and DF (Chai et al., 2016). The stress response was stronger
18 with DD, suggesting higher toxicity compared to DF. Furthermore, it was found that either
19 DD or clay resulted in the category-wide down-regulation of genes associated with cell
20 motility and chemotaxis.

21 The effect of clay exposure on RW1 represents an environmentally significant finding
22 for a number of reasons. First, clays are a major class of geosorbents in soils and as such are a
23 major component of soil microbes' microenvironment. Smectite clays are also important

1 components of clay landfill liners and slurry walls. Smectite clays possess high sorptive
2 affinity for dioxins, and have been suggested as potential sorbent amendments aimed at
3 reducing contaminant bioavailability (Liu et al., 2009; US EPA, 2013). While the recently
4 demonstrated bioavailability of clay-intercalated PCDD/Fs to a mammalian (mouse) model has
5 raised concerns regarding its utility in remediation efforts (Boyd et al., 2011), the
6 bioavailability of clay-sorbed dioxin to bacteria remains unknown. Therefore, our objectives
7 were first to determine the bioavailability of intercalated DD to RW1, and after finding
8 intercalated DD bioavailable to RW1, to examine differences in RW1 gene expression during
9 growth on DD as sole carbon source with DD either in crystalline form or intercalated into the
10 smectite clay mineral saponite (SAP).

11 **Materials and Methods**

12 RW1 strain

13 *Sphingomonas wittichii* strain RW1 was kindly provided by Dr. R. Halden of Arizona
14 State University. Sequences of RW1 chromosome (NC_00911) and plasmids (NC_00907 and
15 NC_00908), and corresponding annotations were downloaded from Joint Genome Institute
16 (JGI)¹ where the genome was sequenced and assembled. Pathway information for RW1 was
17 obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG)².

18 Analytical materials

19 DD with 97% purity was purchased from Wako Pure Chemical Industries Ltd (Osaka,
20 Japan). Cesium chloride and other inorganic salts with 99% purity were obtained from Fisher

¹ <http://genome.jgi-psf.org/sphwi/sphwi.home.html>

² <http://www.kegg.jp/kegg/>

1 Scientific (Pittsburgh, Pa). All chemicals were used as received. SapCa-2, a saponite clay
2 mineral from Ballarat, CA was acquired from the Source Clays Repository of the Clay
3 Minerals Society (Purdue University, West Lafayette, IN). The physical/chemical properties
4 for the SapCa-2 are given in Table 1.

5 Preparation of the Cs saturated SapCa-2

6 Preconditioning of the SAP mineral was necessary to ensure intercalation of DD, i.e.
7 the positioning of dioxin molecules between the planar 2:1 (silicon tetrahedral: aluminum
8 octahedral: silicon tetrahedral) clay layers; dioxin was oriented parallel to the planar clay
9 layers. Cs⁺ was selected for use as the exchangeable cation as it is less hydrated than other
10 monovalent cations (Na, K) and far less hydrated than divalent cations. The reduced hydration
11 is important because it allows the negatively charged oxygen in DD to interact with the
12 exchangeable cation Cs⁺, and creates larger absorption domains between exchangeable cations
13 that are unobscured by water. Furthermore, the lower hydration resulting from the use of Cs⁺
14 results in limited water molecules in the clay interlayers creating a subaqueous environment
15 that is energetically favorable for the hydrophobic DD molecules. Previous studies have shown
16 the efficacy of DD intercalation by Cs-saponite (Liu et al., 2009; Rana et al., 2009).

17 The preparation of homoionic Cs⁺-SapCa-2 followed the method of Arroyo et al.
18 (2004). Briefly, the clay suspension was first titrated with 0.5 M sodium acetate buffer (pH 5)
19 until a stable pH at 6.8 was reached to remove carbonate impurities. Clay-sized particles (<2
20 μm) were obtained by low-speed centrifugation and then re-suspended in 0.1 M CsCl solution
21 four times to ensure complete Cs⁺ saturation. The resultant Cs- SapCa-2 was washed using
22 Milli-Q water until free of chloride as indicated by a negative test with AgNO₃, then quick
23 frozen, freeze-dried and stored.

1 Loading of dibenzo-*p*-dioxin on Cs- SapCa-2

2 Sorption of dibenzo-*p*-dioxin to Cs-SapCa-2 was performed using a method similar to
3 Liu et al. (2009) and Rana et al. (2009). Briefly, for each reaction a 28 ml aliquot of 1000 ppm
4 stock DD solution in methanol was added to 35 L of Milli-Q water containing 0.1 M cesium
5 for a DD concentration of 0.8 mg/L. Then Cs-saturated SAP clay (600 mg) was then added to
6 the DD solution, agitated to equilibrate for 24 h, after which the clay particles were allowed to
7 settle by gravity for 24-48 h. The supernatant was removed and the remaining clay suspension
8 was collected, concentrated by centrifugation and the pellets were quick-frozen, dried and
9 stored prior to use.

10 Confirmation of DD loading using TOC analysis

11 To measure the initial concentration of DD sorbed to the Cs-SAP, total organic carbon
12 (TOC) analysis was performed using a Shimadzu SSM-5000A analyzer following the
13 EPA/600/8-87/020 method. Briefly, total carbon content (TC) was measured by catalytically
14 aided combustion oxidation at 900°C and inorganic content was measured by pre-acidification
15 at 250°C. TOC was then calculated as the difference between TC and inorganic carbon (IC).
16 Analysis was performed on 1 g samples both pre and post loading of DD and the
17 concentrations of DD were determined by the increase in TOC between the two samples. Prior
18 to loading with DD, IC content and TOC content of the purified Cs-SAP clay, were determined
19 to be below their respective detection limits. After loading, the dioxin concentration on Cs-
20 SAP was determined by the increase in TOC to a value of $0.53 \pm 0.01\%$ (w/w). DD consists of
21 78.26 % carbon, thus the percentage of DD in SAP is 0.67 % (6700 mg/Kg). This is in
22 agreement with values reported by Liu et al. (Liu et al., 2009). Furthermore, the intercalation of
23 DD by SAP from aqueous solution was supported by three lines of evidence (Liu et al., 2009;

1 Rana et al., 2009): First, the interlayer basal spacing between clay layers increased consistently
2 as DD sorption increased (Table 1). Second, Fourier-transform infrared (FTIR) spectroscopy of
3 rinsed and air-dried clay films showed a similar steady increase in FTIR intensities of DD
4 vibrational bands. Third, the vast majority of Cs⁺ cations are in the interlayer, and several
5 cation-dependent FTIR vibrational shifts provided strong evidence for direct DD-Cs⁺
6 coordination complexes. The dioxin-loaded clay was added to bacterial cultures to final
7 concentration of 1%, with a corresponding dioxin concentration of 365 μM (67.2 ppm).

8 Extraction and quantification of DD from bacteria plus clay culture

9 The amount of DD in the bacteria clay culture was measured by High Performance
10 Liquid Chromatography (HPLC). DD was extracted from cultures containing 1 ml of medium
11 and 10 mg of Cs-SAP by dual extractions using 0.5 ml dimethylsulfoxide (DMSO) in 25 ml
12 corex glass centrifuge tubes. Samples were mixed with DMSO for 30 min prior to
13 centrifugation at 3300g. Combined supernatants were subject to HPLC analysis using a Perkin-
14 Elmer series 200 (Norwalk, CT) with a UV detector set at a wavelength of 223 nm and a C18
15 HPLC column (Supelcosil Discovery, 15cm×4.6mm, 5μm) with a mobile phase mixture
16 consisting of 80% methanol and 20% water at a flow rate of 1.0 ml/min. Matrix matched
17 calibrations were prepared at 5, 10, 20, 40, and 60 ppm and a coefficient of correlation of
18 0.9994 was achieved. The percent recovery of the extraction and analytical method was 92.4%
19 determined by repeat measurements of DD from 10 mg fortified Cs-SAP with the known
20 concentration of DD in the SAP having been confirmed through TOC analysis described
21 above.

22 Culture conditions

1 RW1 was grown at 30°C using defined mineral DSMZ medium 457 (Brunner medium)
2 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH³). Carbon substrates were
3 added as follows: succinate (SUC) was added to a final 20 mM concentration, dibenzo-*p*-
4 dioxin (DD) (365 μM) was added to sterile flasks from acetone stock and the flasks were left
5 open in a sterile hood for 5 h to allow acetone to completely evaporate. Brunner medium was
6 then added and flasks were sonicated for 10 sec to dislodge and break up substrate crystals.
7 Clay-sorbed-dioxin (DDSAP) was added to 1% (w/v) final concentration (1% DDSAP
8 corresponded to 365 μM DD) in Brunner medium and sonicated to homogenize clay particles.
9 The DD concentration in both DD and DDSAP cultures were equivalent at 365 μM (67.2
10 ppm). Scanning electron microscopy was used to visualize RW1 growth under the two
11 different culture conditions (Figure 1). Cell density of cultures (CFU, colony forming units)
12 was determined by plating serial dilutions on nutrient agar for succinate-only or Brunner agar
13 with DF crystals (added onto Petri dish lid) for dioxin and DDSAP cultures.

14 RNA isolation for transcriptomes

15 Total RNA was isolated from early-to-mid log phase cultures (CFU of 1.0x10⁹ to
16 1.5x10⁹ cells/mL) using PureLink Mini Kit (Life Technologies, Carlsbad, CA) with lysis step
17 modified as per the RiboPure Kit instructions (Life Technologies, Carlsbad, CA). To ensure
18 complete removal of DNA, RNA samples underwent a two-step DNase I treatment, first with
19 DNase I (amplification grade, Life Technologies) followed by TURBO DNase I (Life
20 Technologies). RNA concentration was measured using Qubit (Life Technologies) and its
21 quality was validated using BioAnalyzer (Agilent Technologies, Santa Clara, CA).

³ <http://www.dsmz.de/>

1 Transcriptomes were sampled in triplicate from cultures grown under three different
2 conditions: succinate (SUC), dibenzo-*p*-dioxin (DD), and clay-sorbed dibenzo-*p*-dioxin
3 (DDSAP).

4 Transcriptome sequencing and data processing

5 Enrichment of mRNA was performed by removing ribosomal RNA at the Michigan
6 State University Research Technology Support Facility (RTSF) using Duplex Specific
7 Normalization (DSN), in which cDNA libraries were treated with duplex-specific nuclease to
8 deplete ribosomal RNAs and increase the abundance of mRNA-derived cDNAs. Library
9 preparations and Illumina sequencing (GA II) were performed at RTSF. Sequence reads that
10 passed the purity filter (Illumina chastity filter) were retained, processed and mapped to the
11 RW1 genome as in our previous transcriptome study (Chai et al., 2016). Differentially
12 expressed genes were called at a False Discovery Rate (FDR) of 5% using DESeq2 (Love et
13 al., 2014) with R (R Core Team, 2014).

14 Gene set enrichment analysis

15 Enrichment analysis aims to identify differentially expressed groups of functionally
16 related genes, such as genes whose products are involved in the same metabolic pathway. This
17 was achieved by determining whether a functional gene set is statistically over-represented, i.e.
18 enriched among all gene sets. Enrichment tests were performed using a Python script
19 (“functional_enrichment.py”) written by Yulia Mostovoy⁴ and Biocyc⁵.

⁴ <https://cgrlucb.wikispaces.com/Functional+Enrichment+Analysis> (this web site terminated its service in September, 2018 due to the funding problem.)

⁵ <https://biocyc.org/>

1 Genomic comparisons

2 To find other organisms sharing genes in the Swit_0683 to Swit_0703 genomic region
3 regardless of synteny, we conducted tBLASTn searches on GenBank's nt database at cutoffs of
4 60% sequence identity and 60% of gene length aligned. *Sphingomonas sp.* strain DC-6 shared
5 all 21 genes with RW1, while the next closest genome (*Sphingobium yanoikuyae* strain S72)
6 shared only seven genes. The RW1 genome was compared to DC-6 genome using JGI's
7 neighborhood ortholog tool⁶ while average amino acid identity (AAI) and average nucleotide
8 identity (ANI), along with percent shared genome and percent shared ORFs were calculated
9 using MIGA⁷ (Rodriguez-R et al., 2018).

10 Results

11 We found that RW1 was able to grow in DDSAP cultures with intercalated dioxin as
12 the sole carbon source (Table 1), at the same growth rate as for the cultures with free
13 (crystalline) dioxin (Fig.2). HPLC analysis of the culture extract showed that the reduction in
14 DD concentration corresponded with the growth of RW1 (Fig. 2).

15 We performed pairwise differential expression tests between transcriptomes from the
16 three culture conditions, i.e. dioxin-alone (DD), dioxin intercalated in clay (DDSAP), and
17 succinate control (SUC) and identified 1151 genes including 248 hypothetical protein-coding
18 genes (1000 for DDSAP vs SUC, 695 for DD vs SUC and 86 for DDSAP vs DD) as
19 differentially expressed between at least two different culture conditions (Table S1, S2),
20 representing 20 COG categories. Expression profiles of these genes are presented with log₂ of
21 scaled normalized abundance values (Fig. 3). Based these three comparisons, we found:

⁶ <https://img.jgi.doe.gov/>

⁷ <http://microbial-genomes.org/>

1 (1). RW1's response to dioxin compared to SUC reconfirmed what we found in our
2 previous study, including up-regulation of catabolic pathways for dioxin, its intermediate
3 metabolite catechol, and the more general aromatic compounds (Table 2, Table S2) as well as
4 down-regulation of flagellar assembly pathway and genes for chemotaxis (Table 3). With
5 many more mapped reads in this study than our previous study, we were able to detect a higher
6 number of differentially expressed genes (695; 462 up-regulated and 233 down-regulated vs
7 183 up-regulated and 137 down-regulated). Although 496 of the 695 genes found in this study
8 did not reach our statistical criteria for differential expression in our previous study, the
9 majority of these (410 out of 496) were positively correlated in expression pattern between the
10 two studies. Only three genes (Swit_3144, Swit_3190, Swit_2651) showed statistically
11 significant opposite expression patterns between these two studies, well within the 5% false
12 discovery rate used in both studies. Gene set enrichment analysis indicated up-regulation of
13 degradation pathways of aromatic compounds including dioxin and down-regulation of
14 flagellar assembly and chemotaxis (Table 2, 3).

15 (2). Differences in expression between DDSAP and DD were found for a relatively
16 small number (86) of differentially expressed genes (Table S1). The majority (19/21) of the
17 genes up-regulated in DDSAP vs DD were also up-regulated in DD vs SUC, including 14
18 genes from the Swit_0683-Swit_0694 and Swit_0702-Swit_0703 gene clusters, which span six
19 transcription units (according to our RNA-Seq assemblies, data not shown). Three TonB-
20 dependent receptor genes were up-regulated including one (Swit_0687) in the gene clusters.
21 One LysR transcriptional regulator gene (Swit_4320) was up-regulated but did not respond to
22 DD in this and our previous study.

1 Sixty-five genes were up-regulated in DD compared to DDSAP. They include several
2 gene clusters, e.g. Swit_1152-Swit_1154, Swit_1712-Swit_1714, Swit_4786-Swit_4792, and
3 Swit_5364-Swit-5371.

4 **Discussion**

5 Our study demonstrated that dioxin intercalated into clay is bioavailable and sufficient
6 as the sole carbon source for RW1 growth. Further, the similar growth rates between the
7 sorbed and free dioxin suggests that bioavailability was not rate limiting. This is congruent
8 with studies involving biphenyl which showed that rates of mineralization could not be
9 explained by desorption into the liquid phase even if instantaneous desorption was assumed
10 (Feng et al. 2000). Desorption of DD into bulk culture solution is expected *a priori* to be
11 insignificant. The low hydration of Cs⁺ provides subaqueous conditions in the Cs⁺ saturated
12 clay interlayers, and FTIR data from a similarly prepared DD intercalated Sap (Liu et al, 2009;
13 Rana et al., 2009) indicates the formation of Cs⁺-DD complexes. Both factors provide
14 favorable energetics for DD sorption, especially in the clay interlayers. The relative
15 concentrations of DD in the aqueous phase vs. clay-sorbed DD is defined by the sorption
16 isotherm (Liu et al., 2009), and by far most of the DD mass is in the clay-sorbed state. Also,
17 desorption hysteresis is common over the time scale of the experiments, which would further
18 suppress any dissolved DD.

19 The access to intercalated dioxin may be facilitated by hydration of the exchangeable
20 cation, which controls the spacing between clay interlayers. If the Cs⁺ ion is replaced by Na⁺
21 for instance, the layers expand substantially and this would make it easier to access dioxin.
22 However, Cs⁺ sorption to smectite clays is highly selective (Maes et al., 1985) and desorption
23 has been shown to be very low even under aggressive acid dissolution treatments (Zachara et

1 al., 2002). The exact mechanisms microbes use to access intercalated substrates remains
2 unclear. In clays, the only known abiotic degradation processes involving dioxin required the
3 presence of a transition metal as an exchangeable cation and occurs only in the absence of
4 water and therefore not possible under our experimental conditions (Boyd, S.A. and M. M.
5 Mortland. 1985).

6 We detected 10 genes known to be directly involved in stress/toxicity response up-
7 regulated in DD vs DDSAP, most of which were also up-regulated in DD compared to SUC,
8 consistent with our previous study (Chai et al., 2016). These included high temperature stress
9 genes, e.g. heat shock sigma factors σ^{32} RpoH gene (Swit_0060) and two heat shock protein
10 genes: chaperon protein DnaK (Hsp70) (Swit_1250) and Hsp20 (Swit_0619), RND efflux
11 pump genes (Swi_1152, Swit_1153, Swit_1154) belonging to an outer-membrane protein
12 family in Gram-negative bacteria known for removing antimicrobials and toxins (Venter et al.,
13 2015), a gene (Swit_3457) from the Glutathione S-transferase superfamily best known for
14 detoxification of xenobiotics (Allocati et al., 2008), a gene (Swit_3139) from the a
15 Bacterioferritin family, which was reported to provide bacteria cells protection against acid,
16 oxidative and other environmental stresses (Choi et al., 2000; Calhoun and Kwon, 2011), a
17 Rhodanese family genes (Swit_3732) encoding a cyanide detoxification enzyme (Chaudhary
18 and Gupta, 2012) and a gene from the TetR transcriptional regulator family (Swit_4362),
19 known to interact with a wide diversity of other small molecule inducing ligands, including
20 toxic chemicals, and to regulate genes nearby or at different genome locations (Cuthbertson
21 and Nodwell, 2013). Three other stress response genes, i.e. phage shock proteins, PspA and
22 PspC (Swit_2937, Swit_2939), known to be induced by various membrane stresses in bacteria

1 (Kleerebezem et al., 1996), and a glutathione S-transferase family gene (Swit_0145) also
2 showed higher expression levels in DD than DDSAP, although short of statistical significance.

3 Another interesting finding is the strong up-regulation of some bacterial conjugative
4 type IV secretion system genes (Swit_5364-Swit_5369, Swit_5371 on plasmid pSWIT01) in
5 DD vs DDSAP. Their expression levels follow DD > DDSAP > SUC, sharing the same trend
6 with stress/toxicity genes. The type IV secretion system is generally associated with
7 pathogenicity of Gram-negative bacteria (Wallden et al., 2010), however it is possible that the
8 up-regulation of these genes is also linked to a stress response.

9 Regarding effects from clay, we showed in our previous study that RW1's response to
10 clay alone (SAP) was limited to down-regulation of genes in cell motility pathways and for
11 these genes there were no detectable differences between DDSAP and DD. This suggests that
12 it was the interaction of dioxin with clay in the intercalated state that mainly contributes to the
13 responses unique to DDSAP, while the simple additive effect from clay alone was negligible.
14 Since expression profiles of DDSAP and DD were in general very similar (Table 2, Fig. 3), the
15 effect of dioxin intercalation, and not the presence of clay, was likely the driving factor for the
16 observed expression differences.

17 We hypothesized that dioxin intercalated into clay creates a unique challenge for RW1
18 in accessing the carbon source, compared to the freely available dioxin in DD, and therefore
19 may require a different set of genes to be up-regulated. Hence, we sought to determine what
20 genes and pathways are important in enabling RW1 to utilize dioxin in this deemed less
21 accessible, clay-sorbed state. Genes up-regulated in DDSAP compared to DD are the logical
22 candidates. Of these 21 genes, the majority were those that responded to both DDSAP and DD
23 but significantly stronger to DDSAP than DD (Fig. 4). This created an apparent differential

1 gradient of DDSAP > DD > SUC and most belong to the gene cluster Swit_0683-Swit_0703.
2 Interestingly, nine genes from this cluster (Swit_0684, Swit_0687, Swit_0688, Swit_0689,
3 Swit_0690, Swit_0692, Swit_0693, Swit_0702, Swit_0703) also showed very uniform
4 expression patterns in our previous study, i.e. they were strongly up-regulated in DF vs DD
5 (and DF vs SUC). This suggests that while these genes were induced by dioxin, the lower
6 toxicity of DF and intercalated DD in DDSAP may be responsible for their stronger
7 expression, and further suggests that the elevated expression of these genes may be required
8 for unimpeded growth with dioxin (DD or DF). The fact that Swit_0683-Swit_0703 spans
9 several confirmed transcription units (RNA-Seq assembly data not shown) suggests their up-
10 regulation in DDSAP and, to a lesser level DD, is a response regulated at the transcriptional
11 level. Considering that most of these genes are known for encoding membrane-associated
12 proteins such as porin, TonB-dependent receptors, LuxR, amino acid permease, and
13 extracellular solute-binding protein, it is possible that the form and intensity of cell surface
14 level interactions, e.g. the possible increased access to dioxin through sensing (LuxR quorum
15 sensor, Swit_0694), channeling (porin, Swit_0689), and binding (extracellular solute-binding
16 protein, Swit_0692) between dioxin and RW1 constitutes what differentiates the RW1
17 response to dioxin in DDSAP compared to other states. Apart from this gene cluster, another
18 LuxR gene (Swit_5012) was also more strongly up-regulated in DDSAP than in DD.

19 In a search for genomes that share a similar Swit_0683 to Swit_0703 genomic region
20 with JGI's neighborhood ortholog tool, we found *Sphingomonas sp.* DC-6 (GenBank
21 accession: CP021181.1) (DC-6) to be the only genome that shares with RW1 the complete
22 gene synteny in this region, while the next closest organism (*Sphingobium yanoikuyae* strain
23 S72) only shared seven genes with RW1. This synteny comprises 35 genes, well beyond

1 Swit_0679 and Swit_0713, in both directions (Fig. 5). In fact DC-6 is also the closest relative
2 to RW1 at the whole genome level. Using MiGA (Rodriguez-R et al., 2018) we calculated
3 97.08% Average Amino Acid Identity (AAI) and 98.87% Average Nucleotide Identity (ANI)
4 between their chromosomal genomes. These two genomes share 82.7% of open reading frames
5 and 75.3% of the total genome. The next closest relative to RW1 was *S. sanxanigenens* with a
6 considerably lower AAI of only 60.3% and sharing only 16 of the genes in the cluster. DC-6
7 shared with RW1 most lower pathway genes (16 of 24) for dioxin degradation. However, it
8 lacks at least two key genes (3-oxoadipate CoA-transferase) in the catechol pathway and is
9 missing the entire gene set for the upper pathway. *Sphingomonas sp.* DC-6 was isolated for its
10 ability to degrade chloroacetanilide herbicide acetochlor (Chen et al., 2013) and, as far as we
11 are aware, has not been tested for ability to metabolize DD or DF.

12 In related work, Thamer and Zylstra (2016) found from gene knockout-
13 complementation experiments that in addition to plasmid-borne genes several chromosomal
14 genes are required for dioxin upper pathways. Our transcriptomic data indicated that some of
15 those genes (Swit_0910, Swit_3046, and Swit_3055) were expressed in all three growth
16 conditions. Among these, Swit_0910, Swit_3055 were up-regulated by dioxin (DDSAP and
17 DD compared to SUC). Additional analysis of RNA-seq reads mapped to Swit_0910 and its
18 nearly identical homolog Swit_0886 confirmed that only Swit_0910 was highly expressed and
19 up-regulated by dioxin while Swit_0886 was hardly active, consistent with the observation that
20 Swit_0886 in its native form did not enable dioxin degradation unless fused to a strong
21 promoter. These up-regulated genes are candidates for future knockout experiments.

22 In summary, RW1 was able to access and utilize dioxin intercalated between clay
23 layers. While the exact mechanism used by RW1 to access intercalated DD is not known, the

1 up-regulation of genes associated with recognition, uptake, and surface
2 interactions/attachment/biofilm formation suggest these processes facilitate the accessibility of
3 RW1 to dioxin in the intercalated state. Further, the intercalation of DD into clay reduced
4 toxicity and stress to the cells as compared with exposure to fully available crystalline DD.
5 These results suggest that sequestration via intercalation by clay minerals do not eliminate the
6 bioavailability of DD to the degrader RW1 and may support the efficacy of remedial efforts
7 combining geosorbent amendments with microbial bioremediation. Our studies were with
8 freshly prepared clay-DD but historically DD contaminated sites could have aged clay-DD and
9 hence its biodegradability would need to be evaluated.

10 **Data Availability**

11 The data discussed in this publication have been deposited in NCBI's Gene Expression
12 Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number
13 GSE115658 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115658>). (access
14 token for editors and reviewers: mvepiagubhktjx)

15 **Author Contributions**

16 TT, BT, SB, and JT designed the study. TT, CL, MB and JL carried out the experiment
17 work. BC, BS, TT, HL, CJ, JC, and GZ carried out the data analysis and discussions. BC, BS,
18 TT and JC, SB, and JT wrote the manuscript, with contributions from all co-authors.

19 **Funding**

20 Research reported in this publication was supported by the National Institute of
21 Environmental Health Sciences of the National Institutes of Health under Award Number

1 P42ES004911. The content is solely the responsibility of the authors and does not necessarily
2 represent the official views of the National Institutes of Health.

3 **Conflicts of Interest Statement**

4 The authors declare that the research was conducted in the absence of any commercial
5 or financial relationships that could be construed as a potential conflict of interest.

6 **Acknowledgements**

7 The authors thank Michigan State University Research Technology Support Facility
8 (RTSF) for sequencing the transcriptomes.

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Supplementary Material: Table S2. Differentially expressed genes in DDSAP vs SUC (1000) and DD vs SUC (659).

Figure 1. Scanning Electron Microscope images of *Sphingomonas wittichii* Strain RW1 cultures growing on 365 μ M dibenzo-*p*-dioxin introduced as crystals (A) or intercalated in saponite clay interlayers with bacteria cells identified (B & C).

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Figure 4. Genes up-regulated in DDSAP compared to DD and SUC conditions

Figure 5. The gene synteny in Swit_0683-Swit_0703 genome neighborhood shared between RW1 and *Sphingomonas sp.* DC-6