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In silico analysis of a *Rhizobium* sp. RC1 putative haloalkanoic permease sequence motif and classification

Muhammed Adamu Musa and Fahrul Huyop*

Department of Biotechnology and Medical Engineering, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 Johor, Malaysia.
Email: fahrul@utm.my

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ABSTRACT

Aims: The transport of haloalkanoic acids (haloacids) is important in the metabolism of haloacid pollutants by bacteria. In this study, a computational analysis of *Rhizobium* sp. RC1 haloacid permease (DehrP) amino acid sequence was conducted to identify its subfamily, sequence motifs and evolutionary position among closely related transporters.
Methodology and results: Blast search in the Pfam and Transmembrane Classification Databases was used to establish the classification and the subfamily of DehrP. Clustal omega sequence alignment approach and MEME Suite motif-based analysis tools were used to locate the transporter motifs of DehrP. Dotplots of DehrP sequence was computed using the EMBOSS Dotmatcher. MEGA7 software was used to analyze the phylogenetic position of DehrP among closely related symporters in the Transmembrane Classification Database. Comparative analysis by Pfam shows that DehrP is a member of the Major Facilitator Superfamily (#2.A.1). PSI-Blast against the Transmembrane Classification Database shows that DehrP is significantly aligned with a subfamily of transporters called the Metabolite: H^+ Symporters (#2.A.1.6). DehrP has six similar sequence motifs with the Metabolite: H^+ Symporter proteins including the functional motif of GXXXDRXGRR. DehrP is evolutionarily related to *Burkholderia caribensis* MBA4 Haloacid: H^+ Symporters (Dehp2 and Deh4p).
Conclusion, significance and impact study: Based on sequence similarity, DehrP is a Major Facilitator Superfamily protein that belongs to the Metabolite: H^+ Symporter protein subfamily which might coordinate the transport of a haloacid coupled with a proton (H^+). Mutagenesis of DehrP sequence motifs might be useful in the engineering of *Rhizobium* sp. RC1 for efficient uptake and degradation of haloacids.

Keywords: Haloacids, haloalkanoic permease, *Rhizobium* sp. RC1, sequence motif, symporter

INTRODUCTION

A relatively safe and economical method of haloalkanoic acid (haloacid) pollutants removal from the environment is by microbial degradation (Su *et al.*, 2013). However, some haloacids are difficult to degrade (Effendi *et al.*, 2000; Berthiaume *et al.*, 2014), partly due to the slow growth of cells (van der Ploeg and Janssen, 1995), haloacid selectivity (Berry *et al.*, 1979; Janssen *et al.*, 1985; Chaudhry and Chapalamadugu, 1991), and toxicity (Strotmann *et al.*, 1990; Plewa *et al.*, 2010). One of the important steps in the breakdown of haloacids by microorganisms is their ability to transport the pollutants into the cytoplasm (Su *et al.*, 2013). Membrane transport can influence the Monod saturation constant (*Ks*) of microbial growth (van den Wijngaard *et al.*, 1993) and the haloacid dehalogenation by cells (Nijenhuis *et al.*, 2005). The idea of haloacid transporters have been suggested in bacteria such as *Pseudomonas putida* PP3 (Slater *et al.*, 1985), *Xanthobacter autotrophicus* GJ10 (van der Ploeg *et al.*, 1995), *Agrobacterium* sp. NHG3 (Higgins *et al.*, 2005), and *Burkholderia caribensis* MBA4 (Yu *et al.*, 2007). Two distinct haloacid transporters (Deh4p and Dehp2) from *B. caribensis* MBA4 have been demonstrated to have different preferences for monochloroacetate and chloropropionate (Su and Tsang, 2013). However, most of the haloacid transporters are un-characterized and therefore very little is known about the function of the haloacid transport proteins.

Rhizobium sp. RC1, a Gram-negative bacterium, isolated from soil using 2, 2-dichloropropionic acid as a carbon and energy source, have shown its ability to degrade different types of haloacid such as $_{D,\ L}$-2-chloropropionic acid ($_{D,\ L}$-2-CPA), monochloroacetic acid (MCAA) and monobromoacetic acid (MBAA) (Berry *et al.*, 1979; Cairns, 1994; Stringfellow *et al.*, 1997). *Rhizobium* sp. RC1 was reported to be the only organism that synthesized three different dehalogenases ($_D$-2-haloacid

*Corresponding author

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dehalogenase; DehD, L-2-haloacid dehalogenase; DehL and dual isomeric haloacid dehalogenase; DehE) to degrade different haloacid stereoisomers (Leigh *et al.*, 1986; Leigh *et al.*, 1988; Huyop, 2003). *Rhizobium* sp. RC1 haloacid permease (DehrP) has been proposed to be involved in the chloropropionic acid uptake (Jing *et al.*, 2010). The *Rhizobium* sp. RC1 haloacid permease gene *dehrP*, with a 1239 kb open reading frame, is located 511 bases upstream of the D-2-haloacid dehalogenase gene *dehD*, and encodes a 412 amino acids protein (DehrP) that has the Major Facilitator Superfamily (MFS) protein conserved domain (Jing *et al.*, 2010).

In this study, computational analysis of *Rhizobium* sp. RC1 haloacid permease (DehrP) sequence was carried out, with the believe that DehrP is a specialized chloropropionate transporting protein. Comparative analysis of DehrP primary sequence in the Pfam Database and the Transmembrane Classification Database (TCDB) was done to establish its classification and the subfamily of the Major Facilitator Superfamily (MFS) it belongs, based on sequence similarity. Comparative motif analysis of DehrP sequence was done to identify functional sequence motifs using sequence alignment approach and motif-based analysis tools (MEME) (Bailey and Elkan, 1994) and (MAST) (Bailey and Gribskov, 1998). A phylogenetic tree of DehrP and Metabolite: H^+ Symporter family of transporters in the TCDB was constructed to reveal DehrP evolutionary position.

MATERIALS AND METHODS

Sequence retrieval and primary analysis

Rhizobium sp. RC1 haloacid permease (DehrP) (Jing *et al.*, 2010) amino acid sequence was downloaded from the UniProtKB Database (Consortium, 2015) under the accession number Q1M2W6. NCBI-Blastp (Altschup *et al.*, 1990), PSI-Blast (Schaffer *et al.*, 2001) and UniProt-Blastp (Consortium, 2015) were used to analyze the amino acid sequence of DehrP. Pfam v.31.0 (Finn *et al.*, 2016) was used to analyze the transporter classification of DehrP. Major Facilitator Superfamily (MFS) protein sequences for comparative analysis were downloaded from UniProtKB (Consortium, 2015) and the Transmembrane Classification Database (TCDB) (Saier *et al.*, 2016).

Motif analysis

The *Rhizobium sp.* RC1 haloacid permease (DehrP) conserved Major Facilitator Superfamily (MFS) motif was identified by aligning with eleven MFS transporters from *E. coli* (GARP_ECOLI, TCR2_ECOLX, YJHB_ECOLI, YAAU_ECOLI, RAFB_ECOLX, LACY_ECOLI), *C. freundii* (LACY_CITFR), *K. pneumoniae* (LACY_KLEPN), *Synechocystis sp*. (GLCP_SYNY3), *B. subtilis* (ARAE_BACSU, and TCRB_BACSU) and *S. cerevisiae* (HXT2_YEAST) along the region connecting transmembrane helices 2 and 3 as described by Hirai *et al.* (2003) and visualized using WebLogo v.3.5 (Crooks *et al.*, 2004). A comparative analysis of the family-specific motifs of DehrP and the twelve (12) Metabolite: H^+ Symporters (MHS, class: #2.A.1.6) family of proteins in the Transmembrane Classification Database (TCBD IDs: Dehp2; F8SVK1, Deh4p; Q7X4L6, PcaT; Q52000, KgtP; P0AEX3, ShiA; P76350, ProP; P0C0L7, YhjE; P37643, ThiU; P44699, CitH; P16482, TcuC; P0A2G3, MopB; Q45082, and YdfJ; P77228) was carried out using the motif-based analysis tools MEME (Bailey and Elkan, 1994) and MAST (Bailey and Gribskov, 1998) in MEME Suite v.4.11.4.

Sequence alignment

Threshold Dot plots of the full length of DehrP sequence with six Metabolite: H^+ Symporters (MHS) protein sequences in the Transmembrane Classification Database (TCDB IDs: Dehp2; F8SVK1, Deh4p; Q7X4L6, ShiA; P76350, ProP; P0C0L7, YhjE; P37643, and KgtP; P0AEX3) was done using Dotmatcher of the EMBOSS (Rice *et al.*, 2000) server at a window size of 10, threshold of 23 and a default matrix. DehrP sequence was aligned with closely related haloacid transporters from *Agrobacterium* sp. NHG3 (DehP; Uniprot accession number: Q8KLT0) (Higgins *et al.*, 2005) and *B. caribensis* MBA4 (Deh4p; Uniprot accession number: A0A0N7JV68 and Dehp2; Uniprot accession number: A0A0P0R5D5) (Yu *et al.*, 2007; Su and Tsang, 2013) using Clustal omega (Sievers *et al.*, 2011) and visualized using the ESPript v.3.0 (Robert and Gouet, 2014) server.

Phylogenetic tree construction

The 12 MHS sequence from the TCDB (Saier *et al.*, 2016) was used to construct a phylogenetic tree using MEGA7 (Kumar *et al.*, 2016) software with neighbor-joining method (Saitou and Nei, 1987). The MHS amino acid sequences were aligned by ClustalW (Larkin *et al.*, 2007). The bootstrap values were calculated with 500 replicates (Felsenstein, 1985) and those above 50 are shown at the nodes.

RESULTS AND DISCUSSION

Primary analysis of *Rhizobium* sp. Rc1 haloacid permease

Membrane transporters are classified based on their structure, function, mechanisms of transport, and evolutionary position in the transporter classification databases (Finn *et al.*, 2016; Saier *et al.*, 2016). Comparative analysis of *Rhizobium* sp. RC1 haloacid permease (DehrP) primary sequence with transport proteins in the Pfam database (Finn *et al.*, 2016) shows that DehrP is a Major Facilitator Superfamily (MFS_TC2.A.1) protein. MFS is a large group of transporters that are involved in the movement of compounds from the environmental into the cells (Mitchell, 1967; Marger and Saier, 1993; Pao *et al.*, 1998; Reddy *et al.*, 2012). PSI-Blast (Schaffer *et al.*, 2001) of DehrP

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amino acid sequence against the Transporter Classification Database (TCDB) (Saier *et al.*, 2016) turned out twelve (12) Metabolites: H^+ Symporters (MHS) family (#2.A.1.6), a subfamily of the MFS transporters, that had significant sequence alignments with DehrP sequence. The 12 MHS sequences (*B. caribensis* MBA4 haloacid permeases; Dehp2 (class #2.A.1.6.11) (Su and Tsang, 2013) and Deh4p (class #2.A.1.6.8) (Yu *et al.*, 2007), *Escherichia coli* Shikimate transporter; ShiA (class #2.A.1.6.6) (Whipp *et al.*, 1998), *E. coli* proline/betaine transporter; ProP (class #2.A.1.6.4) (MacMillan *et al.*, 1999), *E. coli* Osmoprotectants transporter; YhjE (class #2.A.1.6.10) (Ly *et al.*, 2004), *E. coli* α-ketoglutarate permease; KgtP (class #2.A.1.6.2) (Seol and Shatkin, 1992), *Pseudomonas putida* dicarboxylate transporter; PcaT (class #2.A.1.6.3) (Karimian and Ornston, 1981), *Burkholderia cepacia* Aromatic/4-methyphthalate transporter; MopB (class #2.A.1.6.5) (Saint and Romas, 1996), *Haemophilus influenzae* Thiazole transporter; ThiU (class #2.A.1.6.12) (Rodionov *et al.*, 2002), *Salmonella typhimurium* Citrate-proton symporter; TcuC (class #2.A.1.6.7) (Lewis *et al.*, 2004), *E. coli* K^+ channel; YdfJ (class #2.A.1.6.9) (Tang *et al.*, 2011), and *Klebsiella pneumoniae* Citrate-proton symporter; CitH (class #2.A.1.6.1) (Van der Rest *et al.*, 1991). Score value of the PSI-Blast showed that Dehp2 (score: 475) and Deh4p (score: 473) were more aligned with *Rhizobium* sp. RC1 haloaocid permease; DehrP. Since Dehp2, Deh4p and DehrP can transport chloropropionate, therefore, DehrP might be a MHS class of #2.A.1.6.

The MFS Motifs of *Rhizobium* sp. RC1 haloacid permease

Protein sequences have short motifs, mostly of a specific length, and patterns which may function as binding domains (Liò and Bishop, 2008). Comparative analyses of existing Major Facilitator Superfamily (MFS) proteins have identified specific sequence motif ([RK]XGR [RK]) for the MFS transmembrane proteins (Henderson and Maiden, 1990). The MFS motif was later extended to ten residues (GXXXDRXGRR) by Hirai *et al.* (2003) for the region connecting transmembrane helices (TMs) 2 and 3. Alignment of eleven MFS sequences along the region connecting TMs 2 and 3 with DehrP sequence shows the presence of such a motif (Figure 1) at residues 83 to 92. Replacement of the MFS motif residues in *E. coli* tetracycline resistance efflux transporter (TetA, chain B) (Tamura *et al.*, 2001) and lactose permease (LacY) (Frillingos *et al.*, 1998) was reported to abolish transport activity.

The first-position glycine (G), fifth-position aspartic acid (D), and eighth-position glycine (G) are very important for LacY transport activity in *E. coli* (Jessen-Marshall *et al.*, 1995). Further, comparative motif analysis of DehrP with the 12 MHS members using a motif-based sequence analysis programs MEME (Bailey and Elkan, 1994) and MAST (Bailey and Gribskov, 1998) shows that DehrP has common sequence motifs with the MHS proteins. Both transporters have at least six conserved motifs that are arranged in a similar fashion in all the members (Figure 2). Figure 2B reveals that motifs 2 (residues 10-19) and 6 (residues 9-13) are similar to the MFS signature that is found respectively, between TMs 2 and 3 (GXXXDRXGRR) (Hirai *et al.*, 2003), and TMS 8 and 9 ([RK]XGR [RK]) (Henderson and Maiden, 1990) of the MFS transport proteins. This demonstrates that DehrP has both the MFS and MHS family-specific motifs and therefore is a member of the two groups. Similar motifs have been reported for *Burkholderia caribensis* MBA4 haloacid transporters (Deh4p) by Tse *et al.* (2009).

DehrP full sequence alignment

Comparative analysis of protein sequences by sequence alignment provides useful information during sequence functional analysis by revealing sequence-function relationships (Liò and Bishop, 2008; Shenoy and Jayaram, 2010). Graphical analysis of DehrP primary sequence with six Metabolite: H^+ Symporter (MHS) family protein sequences from *Burkholderia caribensis* MBA4 haloacid permeases (Dehp2 and Deh4p (Yu *et al.*, 2007; Su and Tsang, 2013), *E. coli* Shikimate transporter (ShiA) (Whipp *et al.*, 1998), *E. coli* proline/betaine transporter; ProP (MacMillan *et al.*, 1999), *E. coli* Osmoprotectants transporter (YhjE) (Ly *et al.*, 2004), *E. coli* α-ketoglutarate permease (KgtP) (Seol and Shatkin, 1992), indicates that DehrP sequence is aligned across the full length of each of the transporters (Figure 3). The remaining six MHS also have similar alignment with DehrP (Musa, 2017). Multiple sequence alignment of DehrP with *Agrobacterium* sp. NHG3 haloacid transporter (DehP (98% sequence identity) (Higgins *et al.*, 2005) and *B. caribensis* MBA4 haloacid transporters (Deh4p; 67% identity and Dehp2; 60% identity) (Su and Tsang, 2013; Yu *et al.*, 2007) shows that haloacid transporter sequences are highly conserved (Figure 4) and all have the MFS motifs.

Evolutionary position of DehrP among the MHS transport proteins

The phylogenetic tree (Figure 5) shows the evolutionary position of *Rhizobium* sp. RC1 haloacid permease (DehrP) among the Metabolite: H^+ Symporters (MHS) family of transporters in the Transmembrane Classification Database (TCDB) (Saier *et al.*, 2016), including *Burkholderia caribensis* MBA4 haloacid transporters (Deh4p and Dehp2) (Su and Tsang, 2013; Yu *et al.*, 2007). The *E. coli* α-ketoglutarate permease (KgtP) (Seol and Shatkin, 1992) and putative dicarboxylate transporter (PcaT) (Karimian and Ornston, 1981) from *Pseudomonas putida* are closely related, while the citrate-proton symporters (TcuC and CitH) from *Salmonella typhimurium*(Lewis *et al.*, 2004) and *Klebsiella pneumoniae* (Van der Rest *et al.*, 1991) are closely related since they both transport a similar substrate. The three haloacid transporters (DehrP, Deh4p and Dehp2)

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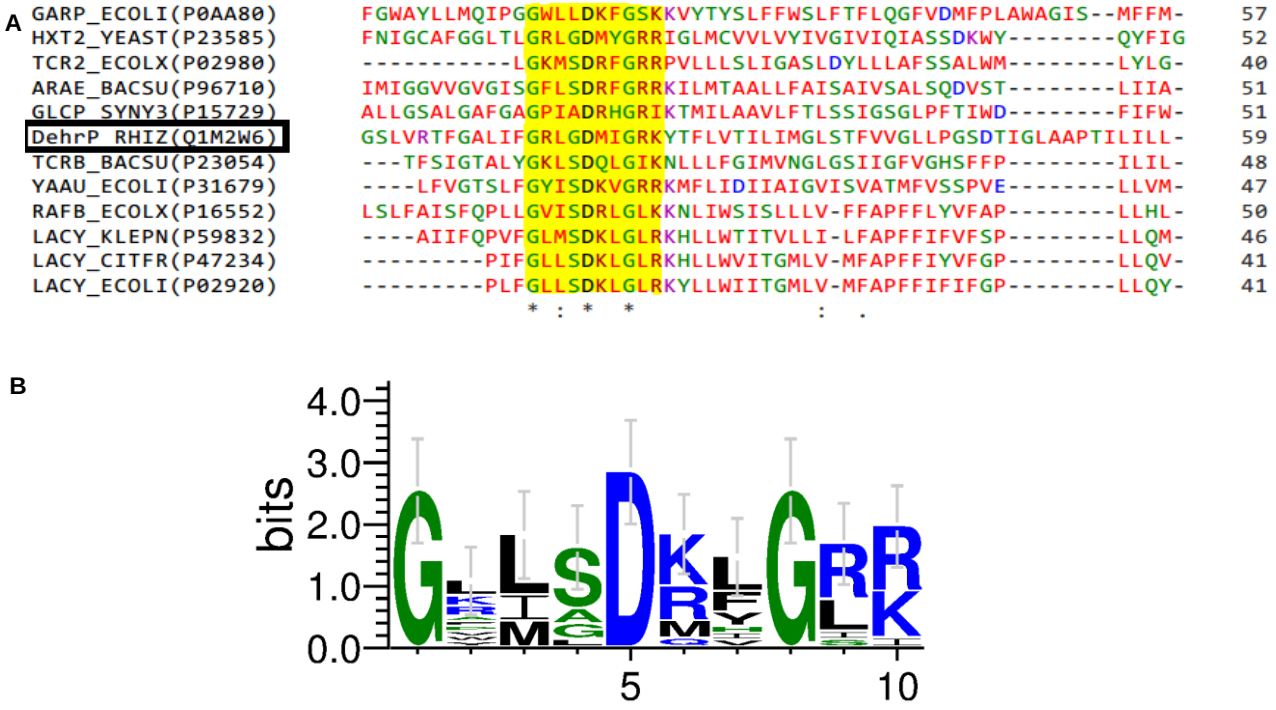

Figure 1: Alignment of *Rhizobium sp.* RC1 haloacid permease (DehrP) Major Facilitator Superfamily (MFS) motif (GXXXDRXGRR) with other MFS transporters in region connecting transmembrane helices 2 and 3. (A) The conserved MFS signature in DehrP and MFS proteins from *E. coli* (GARP_ECOLI, TCR2__ECOLX, YJHB_ECOLI, YAAU_ECOLI, RAFB_ECOLX, LACY_ECOLI), *C. freundii* (LACY_CITFR), *K. pneumoniae* (LACY_KLEPN), *Synechocystis sp*. (GLCP_SYNY3), *B. subtilis* (ARAE_BACSU, and TCRB_BACSU) and *S. cerevisiae* (HXT2_YEAST) is highlighted in yellow and the asterisks (*) indicates conserved residues. The Uniprot accession number of each transporter is shown in bracket. The alignment was done using Clustal omega (Sievers *et al.*, 2011). (B) Logo representation reveals the consensus of the MFS motif among all the MFS proteins as generated by the WebLogo v.3.5 (Crooks *et al.*, 2004).

from *Rhizobium* sp. RC1 (Jing *et al.*, 2010) and *B. caribensis* MBA4 (Yu *et al.*, 2007; Su and Tsang, 2013) then joined the dicarboxylate and citrate proton-symporters to form a clade. It appears that KgtP, PcaT, TcuC and CitH, that transport tricarboxylic acid cycle (TCA) intermediates, are relatively closely related to the haloacid transporters. The chloropropionate-induced DehrP (Jing *et al.*, 2010) is more closely related to Dehp2 (Su and Tsang, 2013) than it is to Deh4p (Yu *et al.*, 2007). This is consistent with the findings in a kinetics study by Su and Tsang (2013) that Dehp2 prefers chloropropionate to monochloroacetate while Deh4p has higher affinity for monochloroacetate.

Members of the second clade that are relatively close to the first clade include the *E. coli* osmoregulatory proline/glycine betaine transporter ProP (MacMillan *et al.*, 1999) and the transporter of aromatic compounds, phthalate and 4-methyphthalate, MopB from *B. cepacian* (Saint and Romas, 1996). These are followed by the *E. coli* transporter aromatic compound shikimate transporter (ShiA) (Whipp et al., 1998), K+ channel (YdfJ) (Tang et al., 2011), putative osmoprotectants transporter (YhjE) (Ly et al., 2004), and the *Haemophilus influenza* putative hydroxyethylthiazole transporter (ThiU) (Rodionov et al., 2002). Members of the second clade appear to transport diverse substrates and have the ability to transport aromatic compounds. The proton(s) symport activity of CitH (Van der Rest et al., 1991), KgtP (Seol and Shatkin, 1992), and ProP (MacMillan et al., 1999) have been established. On the other hand, the pH-dependent activity of Deh4p and Dehp2 and the inhibition of their MCA transport activity by the un-coupler of transmembrane potential such as protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) have been reported (Su and Tsang, 2013).

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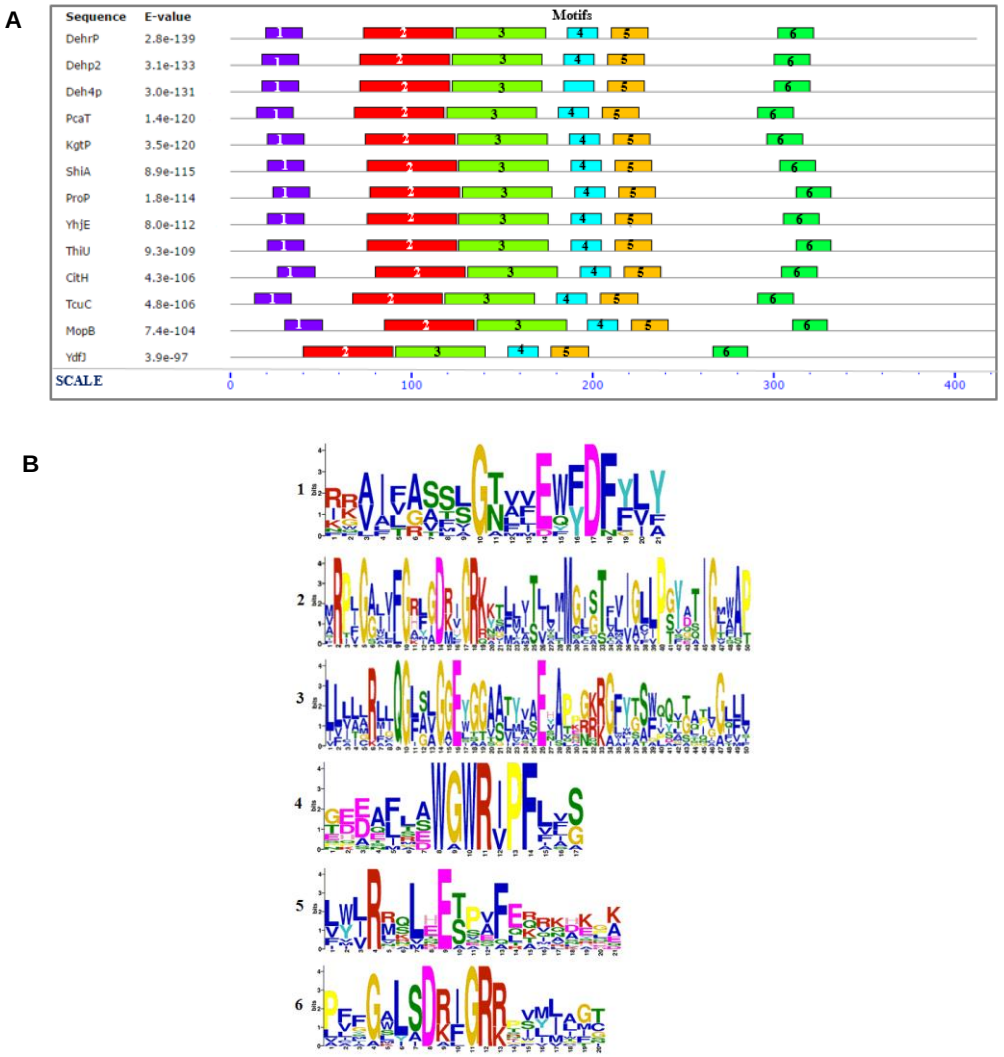

Figure 2: The presence of Metabolite: H^+ Symporter (MHS) family-specific motifs in DehrP. (A) The relative locations of DehrP six conserved MHS motifs compared with twelve (12) MHS family (#2.A.1.6) proteins in the Transmembrane Classification Database (TCDB). (B) The signature sequences of the six motifs in logo format. The protein sequences of DehrP (Uniprot ID: Q1M2W6) and the 12 MHS members (TCBD IDs: Dehp2; F8SVK1, Deh4p; Q7X4L6, PcaT; Q52000, KgtP; P0AEX3, ShiA; P76350, ProP; P0C0L7, YhjE; P37643, ThiU; P44699, CitH; P16482, TcuC; P0A2G3, MopB; Q45082, and YdfJ; P77228) in the TCDB were analyzed with the motif-based analysis tools MEME (Bailey and Elkan, 1994) and MAST (Bailey and Gribskov, 1998) in the MEME Suite v.4.11.4.

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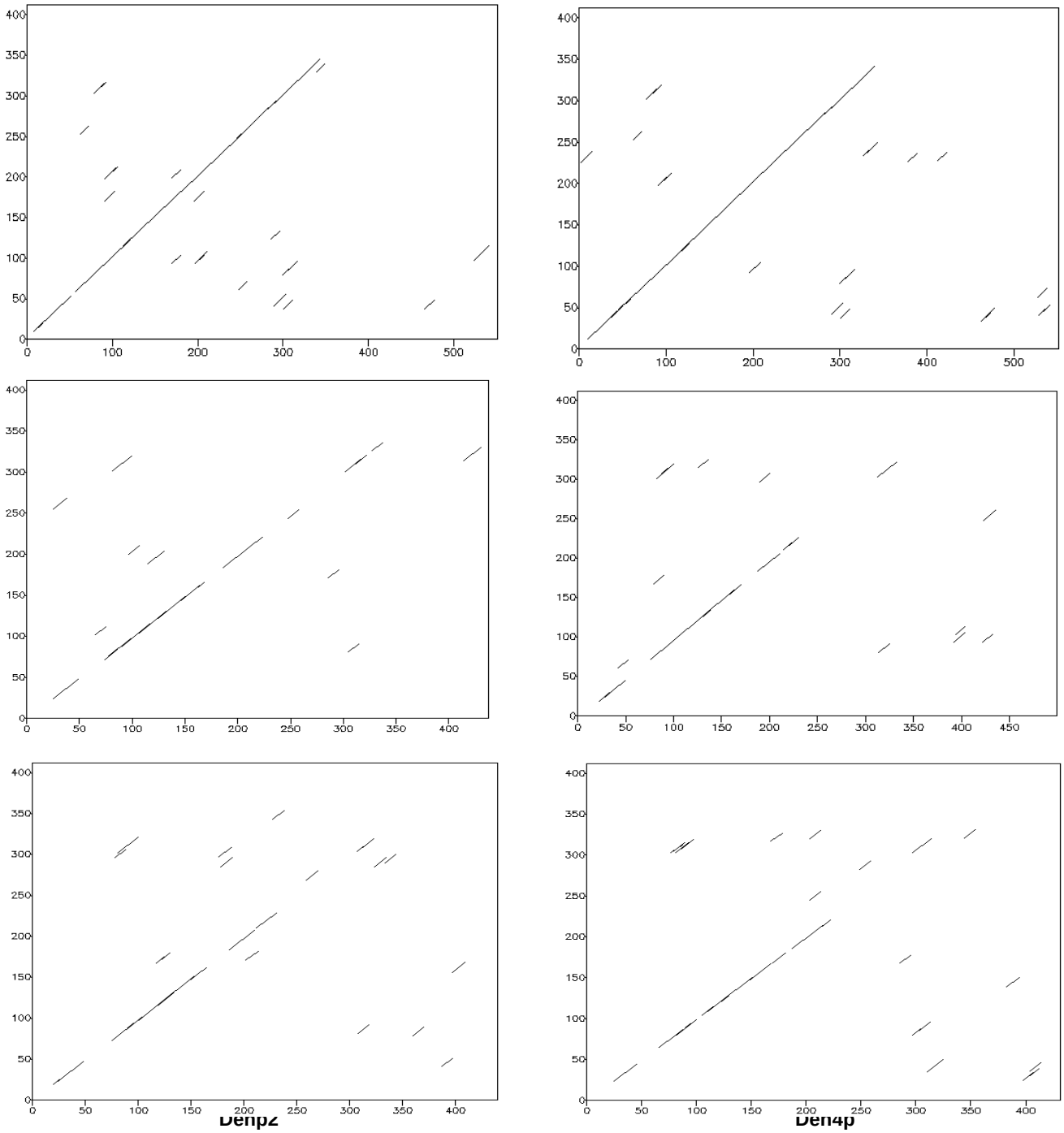

Figure 3: Comparison of DehrP 412 amino acid sequence with the Metabolite: H^+ Symporter (MHS) family transport protein sequences. DehrP sequence (Uniprot ID: Q1M2W6, x-axis) was compared with six MHS proteins (y-axis) in the Transmembrane Classification Database (TCDB) with the TCDB IDs: Dehp2; F8SVK1, Deh4p; Q7X4L6, ShiA; P76350, ProP; P0C0L7, YhjE; P37643, and KgtP; P0AEX3). The dot plots were generated using Dotmatcher of the EMBOSS (Rice *et al.*, 2000) server with a window size of 10, a threshold of 23 and a default matrix was used.

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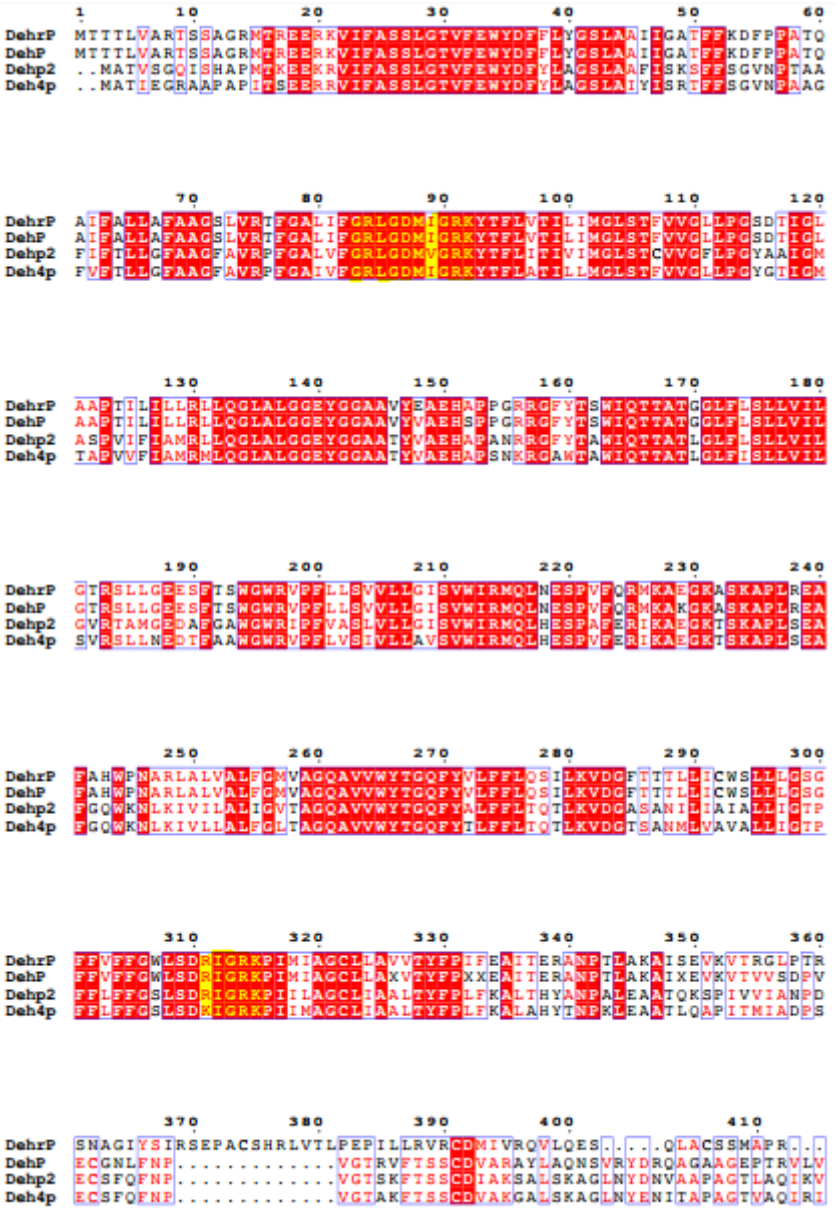

Figure 4: The conserved regions of DehrP and closely related haloacid transporters. DehrP (Uniprot accession number; Q1M2W6 (Jing *et al.*, 2010)) share conserved regions with *Agrobacterium* sp. NHG3 haloacid transporter (DehP, Uniprot accession number; Q8KLT0 (Higgins *et al.*, 2005) and *B. caribensis* MBA4 haloacid transporters (Deh4p, Uniprot accession number; A0A0N7JV68 (Yu *et al.*, 2007) and Dehp2, Uniprot accession number; A0A0P0R5D (Su and Tsang, 2013). The highly-conserved regions (red) contains the conserved MFS signature motifs of GXXXDRXGRR and [RK]XGR [RK] shown in yellow highlights. The alignment was done using Clustal Omega (Sievers *et al.*, 2011) and visualized by ESPript v.3.0 (Robert and Gouet, 2014).

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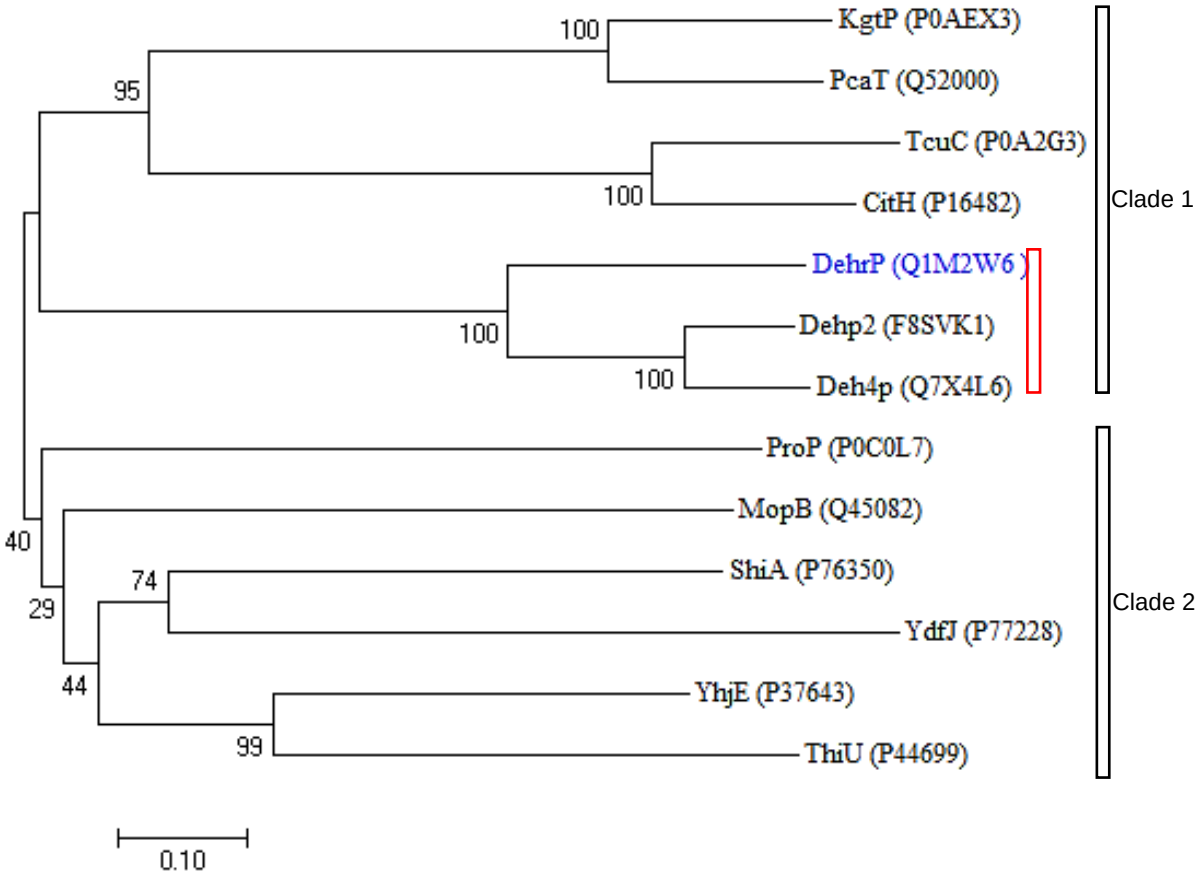

Figure 5: Phylogenetic relationship of DehrP and other Metabolite: H^+ Symporters (MHS) family members. Haloacid transporters from *Rhizobium* sp. RC1 (DehrP in blue font) and *B. caribensis* MBA4 (Dehp2 and Deh4p) are indicated by a red bar. The 12 amino acid sequences were obtained from the transmembrane classification database (TCDB) with the accession numbers shown in brackets. The MHS amino acid sequences were aligned by ClustalW (Larkin *et al.*, 2007), and the tree was constructed by MEGA7 (Kumar *et al.*, 2016) with a neighbor-joining method (Saitou and Nei, 1987). The bootstrap values were calculated with 500 replicates and those above 50 are shown at the nodes. The scale bar represents substitutions per site.

CONCLUSION

The use of comparative sequence analysis to provide information about sequence-function relationship has become an essential part of bioinformatics. Based on sequence similarity, *Rhizobium* sp. RC1 haloacid permease (DehrP) is a major facilitator superfamily protein that belongs to the Metabolite: H^+ Symporter (#2.A.1.6) subfamily and is closely related to *Agrobacterium* sp. NHG3 and *B. caribensis* MBA4 haloacid transporters. DehrP has transporter motifs that matches the major facilitator superfamily transport protein motifs. These motifs might function in haloacid transport in *Rhizobium* sp. RC1. Analysis of the effects of transmembrane electrochemical gradient and pH on haloacid transport can be used to confirm the symporter activity of DehrP. Threading the sequence of DehrP unto the MFS transport proteins of known crystal structure might provide insight as to whether these motifs are part of the binding site(s) of *Rhizobium* sp. RC1 haloacid permease.

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