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1 **Proteomic analysis of *Marinobacter hydrocarbonoclasticus* SP17 biofilm formation at the**
2 **alkane-water interface reveals novel proteins and cellular processes involved in**
3 **hexadecane assimilation.**

4
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27

28 **Abstract**

29 Many hydrocarbon degrading bacteria form biofilms at the hydrocarbon-water interface to
30 overcome the low accessibility of these poorly water-soluble substrates. In order to gain
31 insight into the cellular functions involved, we undertook a proteomic analysis of
32 *Marinobacter hydrocarbonoclasticus* SP17 biofilm developing at the hexadecane-water
33 interface. Biofilm formation on hexadecane led to a global change of the cell physiology
34 involving modulation of the expression of 573 out of 1144 detected proteins when compared
35 with planktonic cells growing on acetate. Biofilm cells overproduced a protein, encoded by
36 MARHY0478 that contains a conserved domain belonging to the family of the outer
37 membrane transporters of hydrophobic compounds. Homologs of MARHY0478 were
38 exclusively found in marine bacteria degrading alkanes or possessing alkane degradation
39 genes and hence presumably constitute a family of alkane transporter specific to marine
40 bacteria. Interestingly, we also found that sessile cells growing on hexadecane overexpressed
41 type VI secretion system components. This secretion system has been identified as a key
42 factor in virulence and in symbiotic interaction with host organisms. This observation is the
43 first experimental evidence of the contribution of a type VI secretion system to environmental
44 adaptation and raises the intriguing question about the role of this secretion machine in alkane
45 assimilation.

46

47 *Keywords:* *Marinobacter hydrocarbonoclasticus* SP17; biofilm; alkane degradation;
48 proteomic; type VI secretion system

49

50 **1. Introduction**

51

52 Biofilm formation at the hydrocarbon-water interface has been observed with various
53 alkane degrading strains, e.g. *Rhodococcus* sp. Q15 [34], *Acinetobacter venetianus* RAG-1 [2]
54 and *Oleiphilus messinensis* [9] as well as polycyclic aromatic hydrocarbons (PAHs)
55 degrading strains, e.g. *Pseudomonas* sp. strain 8909N [19], *Sphingomonas* sp. CHY-1 [35] and
56 *Mycobacterium frederiksbergense* LB501T [3].

57 The ecological significance of these biofilms has been demonstrated by a study devoted to the
58 diversity of biofilm communities developing on PAHs [27]. It has been shown that biofilm
59 communities contained a greater diversity of active species and of PAH degradation genes
60 than the planktonic communities enrichment. Furthermore the diversity of active species
61 found in the biofilm closely matched that of the PAH-contaminated soil used as inoculum.
62 These findings suggest that biofilm formation on hydrocarbons is a probable lifestyle in
63 natural ecosystems.

64 Biofilms developing on hydrocarbons present two properties distinguishing them from
65 other biofilms which are their substrate-substratum specificity and their capacity to overcome
66 the low accessibility of hydrophobic substrates. It was observed in the case of PAH-degrading
67 strains that the less soluble the hydrocarbon was, the more cells grew at the PAH-water
68 interface [12,23]. Biofilm formation has been shown to promote growth on hydrocarbons by
69 facilitating interfacial access. Kinetic studies demonstrated that growth at the interface occurs
70 faster than mass transfer rate of hydrocarbons in the absence of bacteria would suggest
71 [4,5,10,13]. Thus, biofilm formation constitutes an efficient adaptive strategy to assimilate
72 hydrocarbon. However, the genetic determinants and the molecular mechanisms underlying
73 the functioning of these biofilms remain poorly understood. In order to identify the proteins

74 and cellular functions involved, we undertook a proteomic analysis of the biofilm of
75 *Marinobacter hydrocarbonoclasticus* SP17 growing at the hexadecane-water interface. This
76 marine alkane degrading bacterium readily forms biofilms on metabolizable hydrophobic
77 organic compounds including *n*-alkanes from 8 to 28 carbon atoms, whereas in the same
78 condition this strain does not develop biofilms on an inert substratum such as plastic or glass
79 [15]. The doubling time of biofilm cells growing on hexadecane (between 3 and 5 hours
80 depending on the development stage of the biofilm) is similar to that observed on water-
81 soluble substrates such as acetate, pyruvate and lactate (P-J Vaysse, unpublished results).
82 Furthermore, the rate of hexadecane degradation dramatically decreases when the biofilm is
83 disorganized by strong shaking, indicating a strong link between biofilm formation and the
84 utilization of alkanes [15]. These observations indicate that the biofilm lifestyle must provide
85 *M. hydrocarbonoclasticus* SP17 with efficient mechanisms to access the hexadecane. In the
86 current study, we undertook a proteomic analysis of a biofilm growing at hexadecane-water
87 interface. The results obtained indicate that adaptation to alkane utilization as carbon and
88 energy source involves a global change in cell physiology. Novel proteins and cellular
89 processes involved in alkane assimilation were revealed.

90 2. Material and methods

91

92 2.1 Growth conditions and preparation of protein extracts

93

94 The bacterial strain used in this study was *Marinobacter hydrocarbonoclasticus* SP17
95 (ATCC 49840). Cultures were carried out in synthetic sea water (SSW) [8].

96 All chemicals used were from Sigma-Aldrich unless otherwise specified. Biofilm cultures
97 were inoculated with cells growing exponentially on acetate (20 mM) washed twice with one
98 volume of SSW and resuspended to a final optical density at 600nm of 0.1 in 300 ml SSW
99 medium supplemented with 0.2% (v/v) hexadecane in 1 l Erlenmeyer flasks. Biofilms were
100 grown at the hexadecane-SSW interface at 30 °C, under slow shaking (50 rpm). After 35
101 hours incubation, the medium was carefully discarded, the biofilms resuspended in 30 ml of
102 SSW and centrifuged for 20 min at 20,000 g at room temperature. Biofilms were harvested
103 above the supernatant while residual planktonic cells were pelleted. Cells grown on acetate
104 (100 ml) were harvested during exponential growth phase (OD_{600nm}= 0.3) by centrifuging
105 20 min at 20,000 g at room temperature.

106 For protein extraction, cell pellets or biofilms were washed twice with 5 ml of acetone,
107 resuspended in 5 ml of water containing a protease inhibitor cocktail and sonicated on ice for
108 1 min with 500 ms/s pulses at 35 W. Sonicated cell suspensions were then incubated with 40
109 µg of DNase I, 10 µg of RNase A plus 0.01% (v/v) Triton X100 for 30 min at room
110 temperature. Proteins were precipitated for 30 min at 4 °C by adding 500 µl of 100%
111 trichloroacetic acid and then centrifuged 10 min at 20,000g at 4 °C. Protein extracts were
112 washed twice with 1 ml of trichloroacetic acid 10% (v/v) and twice with 1 ml of acetone.
113 Proteins were air dried and dissolved in IEF buffer (urea 8 M, 3-[(3-
114 Cholamidopropyl)dimethylammonio]-1-propanesulfonate 4% (w/v), dithiothreitol 60 mM,

115 Pharmalyte 3-10 2% (v/v) (Amersham Biosciences) and bromophenol blue 0.0002% (w/v)).
116 Protein concentration was estimated using Biorad Protein assay and finally adjusted to 1 µg/µl
117 with IEF buffer.

118

119 *2.2 2D electrophoresis and gel analysis*

120

121 Two hundred and thirty micrograms of protein were applied to a 24 cm Immobiline
122 Dry strip with a 3-7 non linear pH gradient (GE Healthcare). Isoelectric focusing and SDS-
123 PAGE (12.5%) were carried out using the Multiphor II and Ettan DALTsix systems,
124 respectively (GE Healthcare) according to the manufacturer's instructions. Gels were stained
125 with Deep Purple Total Protein Stain (GE Healthcare) and then scanned using a Typhoon
126 9200 fluorescent scanner (GE Healthcare). Image analysis, spot detection and matching were
127 carried out using Image Master Platinum software (GE Healthcare) and checked manually.

128 Three replicate gels from three independent experiments were run for each growth condition.
129 The normalized protein amount for each protein spot was defined as the fraction of that spot
130 volume to the total spot's volume of the gel. Student t-test ($P < 0.05$) and a threshold of 2-fold
131 change were used to determine the proteins significantly differentially expressed between the
132 two conditions.

133

134 *2.3 Protein identification by nanoLC-MS/MS*

135

136 Protein spots were excised from gels, destained in ammonium bicarbonate 50 mM
137 50% acetonitrile (ACN), rinsed twice in ultrapure water and dehydrated in 100% ACN. After
138 ACN removal by evaporation, gel pieces were dried, rehydrated with a trypsin solution (10
139 ng/ml in 50 mM ammonium bicarbonate) at 4 °C for 10 min and finally incubated overnight

140 at 37 °C. The supernatant was collected by two successive extractions with
141 H₂O/ACN/HCOOH (47.5/47.5/5), pooled, and concentrated in a vacuum centrifuge to a final
142 volume of 25 µl. The peptide mixture was analyzed by on-line capillary HPLC (LC Packings,
143 Amsterdam, The Netherlands) coupled to a nanospray LCQ Deca XP Ion Trap mass
144 spectrometer (Thermo-Finnigan, San Jose, CA). MS/MS data were acquired using a 2 *m/z*
145 units ion isolation window and a 35% relative collision energy. Data were searched using
146 SEQUEST through Bioworks 3.3.1 interface (ThermoFinnigan) against the *M.*
147 *hydrocarbonoclasticus* SP17 whole genome sequence. Gene numbers, gene function, and
148 functional category are presented according to the unpublished annotated genome.

149

150 *2.4 Database searching and sequence analysis*

151

152 Sequences similarities were searched against the translation of the non-redundant
153 GenBank CDS database, using the BLAST program [1]. Homologs were defined as proteins
154 giving an alignment with a bits score above 100 (scoring matrix BLOSUM62) and an
155 expected value (E-value) below 1.10^{-25} . Protein domain searches and multiple sequence
156 alignment were carried out using the NCBI-Conserved Domain Database search program with
157 CDD v2.16-27036 PSSMs database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>)
158 [18]. Synteny similarities were searched using the MAGE microbial genome annotation
159 system from the Genoscope Evry France
160 (<https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/MageHome>).

161

162 **3. Results and discussion**

163

164 *3.1. Comparison of protein patterns of biofilm cells growing on hexadecane with planktonic*
165 *cells growing on acetate*

166

167 In order to characterize the molecular mechanisms involved in the development of
168 biofilm on alkanes, we compared the proteomes of biofilm growing on hexadecane and
169 planktonic cells growing on acetate, hereafter referred to as BH and PA respectively (Fig. 1).
170 A total of 1144 spots appearing on all three replicates of at least one growth condition were
171 detected. 576 spots (50%) were found to change significantly between PA and BH. Among
172 these proteins, 245 were overexpressed in BH and 331 in PA. 81% of the differentially
173 expressed protein had an induction ratio above 10. This high proportion of differentially
174 expressed proteins is unusual when compared to other similar proteomic analyses. For
175 instance, a study of *Alcanivorax burkomensis* SK2 comparing growth on hexadecane with
176 growth on pyruvate revealed 97 proteins differentially expressed [24]. However, *A.*
177 *burkomensis* SK2 protein extracts were prepared from whole cultures consisting of a mixture
178 of biofilm cells and detached planktonic cells. Therefore, the expression protein profiles
179 might reflect the average protein expression levels from different cellular states, thus
180 lessening the actual expression fold-change of one specific condition. Furthermore, in our
181 study, proteins were revealed using a fluorescent dye with a higher sensitivity and larger
182 dynamic range than Coomassie blue.

183 The high number of differentially expressed proteins between the biofilm cells
184 growing on hexadecane and those growing on acetate signify that growth on hexadecane
185 involve a global change in the cell physiology, requiring numerous cellular functions. This

186 could be explained by the change of two major factors between the compared conditions: the
187 lifestyle (biofilm or planktonic) and the carbon source (hexadecane or acetate). In many
188 species, biofilm formation has been shown to require a large number of protein functions. The
189 biofilm lifestyle induces changes in the environmental conditions encountered by cells, such
190 as the formation of nutrient microgradients. The adaptation to these new conditions requires
191 most likely a great number of proteins [28]. On the other hand, the response to alkanes
192 involves the modulation of the expression of numerous proteins required for the transport, the
193 metabolism or to avoid toxic effects of these compounds [31]. A total of 58 spots, that could
194 unambiguously be assigned to a single protein, were kept for further analysis (Table 1). The
195 major type IV pilus subunit, PilA (encoded by MARHY2564) was under-produced in BH.
196 Type IV pili have been shown to be involved in host cell adhesion, biofilm formation, DNA
197 uptake and twitching motility [21]. A DNA microarray analysis showed that pilin genes were
198 repressed in the biofilm of *Pseudomonas aeruginosa* [33]. Furthermore, the type IV pilus of
199 *P. aeruginosa* has been shown to have multiple effects on biofilm formation mainly through
200 twitching motility and adhesion [11,20].

201 Proteins involved in nutrient transport across cellular envelopes constitute a large class
202 of proteins overproduced in BH. This includes components of phosphate (encoded by
203 MARHY3535 *pstS*) and thiosulfate (encoded by MARHY2019 *cysP*) ABC transporters. Iron
204 uptake and transport proteins were represented by FhuE, FbpA and CirA encoded by
205 MARHY1035, MARHY2192 and MARHY3135 respectively. Four other proteins coding for
206 putative ABC transporters and porins (encoded by MARHY0256, MARHY0299,
207 MARHY3277, MARHY34332 and MARHY3166) were also found, although their actual
208 function remain uncertain. Increased capacities in solute transport may reflect an adaptation to
209 the constraints imposed by the biofilm lifestyle. Indeed, the increase in biofilm thickness is
210 thought to hinder nutrient penetration into the deepest layers of the biofilm. Thus, cells would

211 require an increased capacity in nutrient uptake and transport. This is particularly important in
212 seawater where iron and phosphate are in limiting concentrations. Micronutrients such as iron
213 and inorganic phosphate are also known to strongly influence biofilm development by acting
214 as environmental cues regulating biofilm formation [25].

215

216 3.2. Redirection of carbon flux in biofilm cells growing on hexadecane

217 Most of the enzymes corresponding to the CO₂-releasing steps of the tricarboxylic acid
218 cycle (TCA cycle) (encoded by MARHY0078 *idh*, MARHY2120 *sucD*, MARHY2121 *sucC*,
219 MARHY2126 *sdhA*) were found to be down regulated in BH. In addition, the gene encoding
220 malate synthase (MARHY1458 *glcB*), catalyzing the conversion of glyoxylate into malate,
221 was upregulated. This modulation of TCA cycle enzymes suggests a stimulation of the
222 glyoxylate bypass in BH. This anaplerotic pathway allows the replenishment of cells in the
223 metabolic intermediates necessary for the synthesis of their cellular components when acetyl-
224 CoA is the only carbon source available in the cell [16]. The activation of the glyoxylate
225 bypass under a hexadecane diet compared to a diet of pyruvate or glucose has been already
226 observed in proteomic analyses on *A. burkomensis* SK2 and *Geobacillus thermodenitrificans*
227 [6,24]. In our study, stimulation of the glyoxylate bypass was observed under hexadecane
228 conditions compared with acetate. In hexadecane and acetate conditions, the intracellular
229 carbon source is acetyl-CoA hence the glyoxylate pathway is required in both conditions. The
230 explanation of the glyoxylate bypass stimulation could lie in the fact that hexadecane is a
231 more energetic substrate than acetate. In fact the breakdown of hexadecane to acetyl-CoA
232 produces energy whereas the conversion of acetate to acetyl-CoA does not. Augmenting the
233 flux of carbon through the glyoxylate bypass on hexadecane could allow cells to restore the
234 balance between carbon assimilation and energy production. However interpretation of

235 proteomic data with regard to metabolic flux should be taken with caution, since metabolic
236 pathways can be regulated at the level of enzyme activity, in addition to gene expression.

237 Fatty acid biosynthesis genes *fabA*, *fabB* and *fabF* (MARHY3086, MARHY3087 and
238 MARHY1438, respectively) were found to be down regulated in BH. This corroborates the
239 fact that in cells growing on hexadecane the main fatty acids of cellular lipids were derived
240 from the oxidation of alkanes [26].

241

242 3.3. A type VI secretion system is overproduced by biofilm cells growing on hexadecane

243

244 Three proteins sharing similarities with type VI secretion system (T6SS) subunits,
245 encoded by MARHY3623, MARHY3634 and MARHY3635, were overexpressed in BH.
246 MARHY3634 and MARHY3635 were among the most abundant protein detected in BH, with
247 0.2 and 0.7 % of total protein respectively. MARHY3634, MARHY3635 and MARHY3623
248 are localized within a cluster of 16 genes (from MARHY3635 to MARHY3620) that are
249 transcribed in the same direction. All members of the *M. hydrocarbonoclasticus* SP17 cluster
250 share similarity with genes found in T6SS clusters from other bacteria including *Vibrio*
251 *cholerae*, *P. aeruginosa* and *Escherichia coli*. The best synteny conservation with a
252 characterized T6SS was observed with *Vibrio cholerae* N16961 T6SS gene cluster (Fig. 2).

253 Homologs of component of T6SS already characterized, i.e. Dot, IcmF, and ClpV (a
254 subfamily of ClpB ATPase), were also found in *M. hydrocarbonoclasticus* SP17 cluster
255 (MARHY3626, MARHY3621 and MARHY3625). Vgr and Hcp are two proteins secreted
256 through T6SS. One homolog of Vgr is found (MARH3620) within this cluster while other
257 homologs of Vgr and Hcp are found elsewhere on the *M. hydrocarbonoclasticus* SP17
258 chromosome [7] (Fig. 3). Experimental evidence of the functionality of homologs of
259 MARHY3635 and 3634 was provided by studies of the pathogen *Edwardsiella tarda* [22].

260 Deletion mutants of *evpA* and *evpB* (MARHY3635 and MARHY3634, respectively) were
261 impaired in protein secretion and showed reduced virulence in blue gourami fish. Based on
262 the high degree of sequence similarity and synteny conservation between characterized T6SS
263 clusters, we propose that the genes from MARHY3635 to MARHY3620 constitute a T6SS
264 gene cluster. The functionality of this T6SS is supported by the detection of three proteins
265 encoded by this cluster.

266 Although type VI secretion systems have been identified as key factors in virulence of
267 pathogenic bacteria and in symbiotic interaction with host organisms, *in silico* analyses have
268 revealed their presence in many species that are not considered as pathogens or symbionts.
269 This led to the hypothesis that T6SS may also contribute to environmental adaptation [29].
270 Our data provide the first experimental evidence of the production of a T6SS during biofilm
271 development by an environmental bacterium. The role of T6SS in biofilm formation during
272 growth on hexadecane by *M. hydrocarbonoclasticus* SP17 remains puzzling. T6SS may be
273 required to secrete proteins needed for biofilm formation or for efficient assimilation of
274 carbon source difficult to access.

275

276 3.4. Evidence for an alkane transporter family specific to marine bacteria

277

278 Two proteins produced in large quantities in BH, encoded by MARHY0478 and
279 MARHY0477, drew our attention because their sequences were found to be conserved
280 exclusively in marine species capable to use alkanes as a carbon source or possessing at least
281 one copy of an alkane hydroxylase gene. The phylogenetic distribution of MARHY0478 and
282 MARHY0477 homologs is rather restricted since they are found only in two orders of the
283 Gammaproteobacteria: the *Oceanospirillales* and the *Alteromonadales*. The degree of peptide
284 sequence identity varies between 29% and 98%. Interestingly, these proteins are found in

285 *Alcanivorax borkumensis* SK2, a key alkane degrader in polluted seawater. Several strains
286 possess multiple homologs of MARHY0478 and MARHY0477 with a maximum of six in
287 *Alcanivorax* sp DG881 (Table 2).

288 In *M. hydrocarbonoclasticus* SP17, MARHY0478 and MARHY0477 are separated by only
289 11 base pairs and are transcribed in the same direction. This spatial organization is indicative
290 of a putative operon. Such an operon would suggest that the products of these genes are
291 involved in the same function. An operon structure is likely to be conserved in *M.*
292 *hydrocarbonoclasticus* VT8 and *A. burkumensis* SK2 since these strains contain homologs of
293 MARHY0477 and MARHY0478 that are adjacent and transcribed in the same direction.

294 The strong expression of MARHY0478 and MARHY0477 in biofilm on hexadecane indicates
295 that they might play an important role in alkane assimilation. In order to gain more insight
296 into their function, conserved domains in the two proteins were searched against the NCBI
297 Conserved Domain Database using RPS-BLAST [18]. Only the protein encoded by
298 MARHY0478 gave a positive hit (E-value = 10^{-13}) with the pfam03349 domain family. This
299 conserved domain is found in outer membrane proteins transporting hydrophobic compounds
300 out of Gram negative bacteria. This family includes the monoaromatic hydrocarbon transport
301 proteins TodX from *Pseudomonas putida* F1 and TbuX from *Ralstonia pickettii* PKO1, and
302 the long chain fatty acid transporter FadL from *Escherichia coli* [14,30,32]. Fig. 3 shows the
303 multiple peptide sequence alignment for the conserved domain including the amino acid
304 sequence deduced from MARHY0478, TodX, TbuX and FadL. This alignment is based upon
305 the frequency of each amino acid substitution in the set of protein used to create the conserved
306 domain model [17]. Peptide encoded by MARHY0478 exhibited a clear sequence similarity
307 with the four hydrophobic compounds transporters. In view of this significant domain
308 conservation, it is very likely that MARHY0478 encodes for a hydrophobic compound
309 transporter. Given that MARHY0478 was induced during growth on hexadecane, and that

310 homologs are only found in marine alkane degrading strains or putative alkane degrading
311 strains, we hypothesized that MARHY0478 and its homologs would constitute a family of
312 alkane transporter specific to marine bacteria. The production in large quantity of an alkane
313 transporter in biofilm cells would contribute to the high growth rate observed on alkane
314 despite its low solubility.

315 Polypeptides encoded by MARHY0477 and their homologs define a protein family
316 whose function is likely to be related to MARHY0478, as members of the two families co-
317 occur in the same strains and occasionally form a putative operon. The ecological significance
318 of the MARHY0478 and MARHY0477 families certainly deserve closer inspection as they
319 are present in genera known to be the main actors in hydrocarbon degradation in marine
320 environments.

321

322

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430

431 **Legends to figures**

432

433 Fig. 1. Two-dimensional gel electrophoresis of protein extracts of *M. hydrocarbonoclasticus*
434 SP17. Planktonic cells grown on acetate (top image) and biofilm cells grown on hexadecane
435 (bottom image). Spots of identified proteins are numbered. Molecular weights (in kDa) are
436 indicated on the left side.

437

438 Fig. 2. Organization of the genes encoding for type VI secretion system of *M.*
439 *hydrocarbonoclasticus* SP17 (top) and *Vibrio cholerae* N16961 (bottom). The values between
440 homologous genes refer to the percent peptide sequence identity of their products. Accession
441 number : MARHY3635 to MARHY3620 (FP475883 to FP47598), MARHY0176
442 (FP475953), MARHY1076 (FP475938) and MARHY2494 (FP475918)

443

444 Fig. 3. Multiple sequence alignment of FadL, TbuX, TodX and translated MARHY0478.
445 Sequence alignment was performed using the web-based tool CD search using the PSSM
446 112176 scoring matrix. Conserved residues are shown in grey boxes and identical residues in
447 black boxes. Secondary structures elements of FadL were retrieved from the PDB database
448 (PDB ID:1t16) and are shown above the sequences.

449

Tables

Table 1

Identification of proteins differentially expressed in biofilm on hexadecane compared to planktonic cells on acetate.

Spot NO.	CDS	Gene	Product	Differential abundance ^a	Amount in planktonic/acetate condition ^b	Amount in biofilm/hexadecane condition ^b	Gene accession number ^c
Transport and binding protein							
1	MARHY1035	<i>fhuE</i>	Outer membrane receptor for ferric iron uptake	+5.01	0.0102	0.0513	FP475940
2	MARHY2192	<i>fbpA</i>	Iron(III) ABC transporter, periplasmic iron(III)-binding protein	+3.89	0.1233	0.4793	FP475923
3	MARHY3135	<i>cirA</i>	Ferric iron-catecholate outer membrane transporter	BH	ND	0.079	FP475909
4	MARHY2019	<i>cysP</i>	Thiosulfate transporter subunit	+3.01	0.1170	0.3525	FP475928
5	MARHY3535	<i>pstS</i>	ABC phosphate transporter, periplasmic component	+10.74	0.1018	1.0934	FP475901
6	MARHY3277	–	ABC-type metal ion transporter, periplasmic component	BH	ND	0.0831	FP475905
7	MARHY1478	–	ABC-type branched-chain amino acid transporter, periplasmic component	-4.32	0.0882	0.0204	FP475934
Cellular processes							
8	MARHY2564	<i>pilA</i>	Fimbrial protein precursor	-2.44	0.6983	0.2861	FP475917
9	MARHY2994	<i>tpm</i>	Thiopurine methyltransferase	PA	0.0551	ND	FP475913
Information transfer							
10	MARHY3200	<i>greA</i>	Transcription elongation factor	BH	ND	0.0843	FP475907
Protein fate							
11	MARHY0922	–	FKBP-type peptidyl-prolyl cis-trans isomerase	PA	0.0418	ND	FP475944
12	MARHY0942	<i>dsbC</i>	Protein disulfide isomerase II	+4.44	0.0363	0.1611	FP475943
13	MARHY0958	<i>mucD</i>	Serine protease	+4.05	0.0517	0.2091	FP475942
14	MARHY1798	<i>slyD</i>	FKBP-type peptidyl-prolyl cis-trans isomerase	PA	0.0584	ND	FP475931

Spot NO.	CDS	Gene	Product	Differential abundance ^a	Amount in planktonic/acetate condition ^b	Amount in biofilm/hexadecane condition ^b	Gene accession number ^c
Stress response							
15	MARHY3764	<i>ahpF</i>	Alkyl hydroperoxide reductase f-subunit	BH	ND	0.0369	FP475881
Cofactor biosynthesis							
16	MARHY2568	<i>coaE</i>	Dephospho-CoA kinase	PA	0.0533	ND	FP475916
Aminoacid biosynthesis							
17	MARHY1723	<i>asd</i>	Aspartate-semialdehyde dehydrogenase	PA	0.0655	ND	FP475932
18	MARHY3112	<i>hisH</i>	Glutamine amidotransferase	BH	ND	0.1733	FP475910
19	MARHY0406	<i>lysA</i>	Diaminopimelate decarboxylase	PA	0.0901	ND	FP475949
20	MARHY2259	<i>serC</i>	3-phosphoserine/phosphohydroxythreonine aminotransferase	BH	ND	0.1327	FP475920
Nucleotide biosynthesis							
21	MARHY2255	<i>cmk</i>	Cytidylate kinase	BH	ND	0.0691	FP475921
22	MARHY2158	<i>ndk</i>	Multifunctional nucleoside diphosphate kinase and apyrimidinic endonuclease and 3'-phosphodiesterase	BH	ND	0.0836	FP475924
23	MARHY3664	<i>pyrC</i>	Aspartate carbamoyltransferase, non-catalytic chain	BH	ND	0.0533	FP475882
24	MARHY2249	<i>pyrF</i>	Orotidine-5'-phosphate decarboxylase	+3.35	0.0582	0.1948	FP475922
Central intermediary metabolism							
25	MARHY3262	<i>exaC</i>	NAD ⁺ dependent acetaldehyde dehydrogenase	-3.44	0.0968	0.0281	FP475906
Energy metabolism							
26	MARHY1444	<i>acnB</i>	Bifunctional aconitate hydratase 2 and 2-methylisocitrate dehydratase	-2.89	0.9104	0.3147	FP475936
27	MARHY0078	<i>idh</i>	Isocitrate dehydrogenase	-3.13	0.2014	0.0643	FP475954
28	MARHY2120	<i>sucD</i>	Succinyl-CoA synthetase, NAD(P)-binding, alpha-subunit	-3.2	0.8657	0.2708	FP475927
29	MARHY2121	<i>sucC</i>	Succinyl-CoA synthetase, beta-subunit	-6.97	0.1894	0.0272	FP475926
30	MARHY2126	<i>sdhA</i>	Succinate dehydrogenase, flavoprotein subunit	-4.35	0.3639	0.0837	FP475925
31	MARHY0774	<i>petA</i>	Ubiquinol-cytochrome c reductase iron-sulfur subunit	+6	0.0449	0.2696	FP475945
32	MARHY1458	<i>glcB</i>	Malate synthase G	BH	ND	0.0203	FP475935
33	MARHY1802	<i>etfA</i>	Electron transfer flavoprotein subunit, FAD-binding	+3.13	0.1707	0.5347	FP475930
34	MARHY1815	<i>paaG</i>	Enoyl-CoA hydratase	PA	0.073	ND	FP475929

Spot NO.	CDS	Gene	Product	Differential abundance ^a	Amount in planktonic/acetate condition ^b	Amount in biofilm/hexadecane condition ^b	Gene accession number
Fatty acid biosynthesis							
35	MARHY1009	<i>acs</i>	Acetyl-CoA synthetase	PA	0.0435	ND	FP475941
36	MARHY3086	<i>fabA</i>	Beta-hydroxydecanoyl thioester dehydrase	PA	0.1247	ND	
37	MARHY3087	<i>fabB</i>	3-oxoacyl-[acyl-carrier-protein] synthase I	-2.54	0.5215	0.2056	FP475911
38	MARHY1438	<i>fabF</i>	3-oxoacyl-[acyl-carrier-protein] synthase II	-4.04	0.1566	0.0388	FP475937
39	MARHY1579	<i>prpE</i>	Propionate-CoA ligase	-5.75	0.1203	0.0209	FP475933
Uncertain or unknown function							
40	MARHY0256	–	Probable ABC transporter, ATPase subunit	PA	0.2227	ND	FP475952
41	MARHY0299	–	Probable TRAP dicarboxylate transporter	BH	ND	0.123	FP475951
42	MARHY3613	–	Probable PspA protein	+3.5	0.1716	0.6	FP475899
43	MARHY3634	–	Probable type VI secretion system subunit	BH	ND	0.2073	FP475884
44	MARHY3635	–	Probable type VI secretion system subunit	+6.68	0.1118	0.7467	FP475883
45	MARHY3432	–	Probable TonB-dependent receptor	+10.99	0.0193	0.2118	FP475902
46	MARHY3289	–	Probable porin	PA	0.4185	ND	FP475904
47	MARHY0478	–	Probable hydrophobic compounds transporter	+11.23	0.0369	0.4144	FP475946
48	MARHY0477	–	Conserved protein	+5.62	0.0369	0.2076	FP475947
49	MARHY0460	–	Conserved protein	BH	ND	0.696	FP475948
50	MARHY1073	–	Conserved protein	PA	0.0385	ND	FP475939
51	MARHY2963	–	Conserved protein	+9.21	0.0326	0.3001	FP475914
52	MARHY3166	–	Conserved protein	-14.81	3.612	0.2445	FP475908
53	MARHY3295	–	Conserved protein	BH	ND	0.0961	FP475903
54	MARHY0333	–	Conserved protein	BH	ND	0.1249	FP475950
55	MARHY3623	–	Probable type VI secretion system subunit	+4.71	0.0142	0.0671	FP475895
56	MARHY3550	–	Conserved protein	PA	0.1351	ND	FP475900
57	MARHY2686	–	Conserved protein	+22.01	0.0924	2.0331	FP475915
58	MARHY2432	–	Conserved protein	PA	0.0642	ND	FP475919

^aPositive values represent overexpression in biofilm/hexadecane condition. Negative values represent underexpression in biofilm/hexadecane condition. BH means that the protein is solely detected in biofilm/hexadecane condition. PA means that the protein is solely detected in planktonic/acetate condition. ^bAverage percent of spot volume relative to total spot volume. ND, not detected.

Table 2

Phylogenetic distribution of MARHY0477 and MARHY0478 homologs.

Strain	Provenance	Alkane degradation ^a	MARHY0477 homologs ^b	MARHY0478 homologs ^b	Alkane degradation genes ^c
<i>Marinobacter hydrocarbonoclasticus</i> SP17	Marine sediment, Mediterranean Sea	yes	-	-	2 alk, 2 P450
<i>Marinobacter hydrocarbonoclasticus</i> VT8	Oil well off the Vietnamese coast	yes	2 (74%, 33%)	3 (98%, 74%, 74%)	3 alk, 1 P450
<i>Marinobacter algicola</i> DG893	Culture of a dinoflagellate	yes	1 (59%)	1 (83%)	2 alk
<i>Alcanivorax borkumensis</i> SK2	Sea water, North Sea	yes	2 (34%, 32%)	1 (40%)	2 alk, 1 P450
<i>Alcanivorax</i> sp. DG881	Culture of marine algae	yes	6 (35%, 32%, 33%, 31%, 31%, 29%)	6 (54%, 54%, 50%, 48%, 46%, 42%)	2 alk, 2 P450
<i>Bermanella marisrubri</i>	Sea water, Red Sea	ND	1 (31%)	1 (45%)	1 alk
<i>Moritella</i> sp. PE36	Deep sea floor, Pacific Ocean	ND	1 (29%)	1 (39%)	1 alk

Sequences similar to translated MARHY0477 and MARHY0478 were searched against the non-redundant GenBank CDS translations database, using the BLAST program. ^aND not determined. ^bNumber of homologous genes and their percent peptide sequence identity in brackets. ^calk and P450 indicate the presence of alkane hydroxylase and cytochrome P450 alkane monooxygenase genes respectively.

Figures

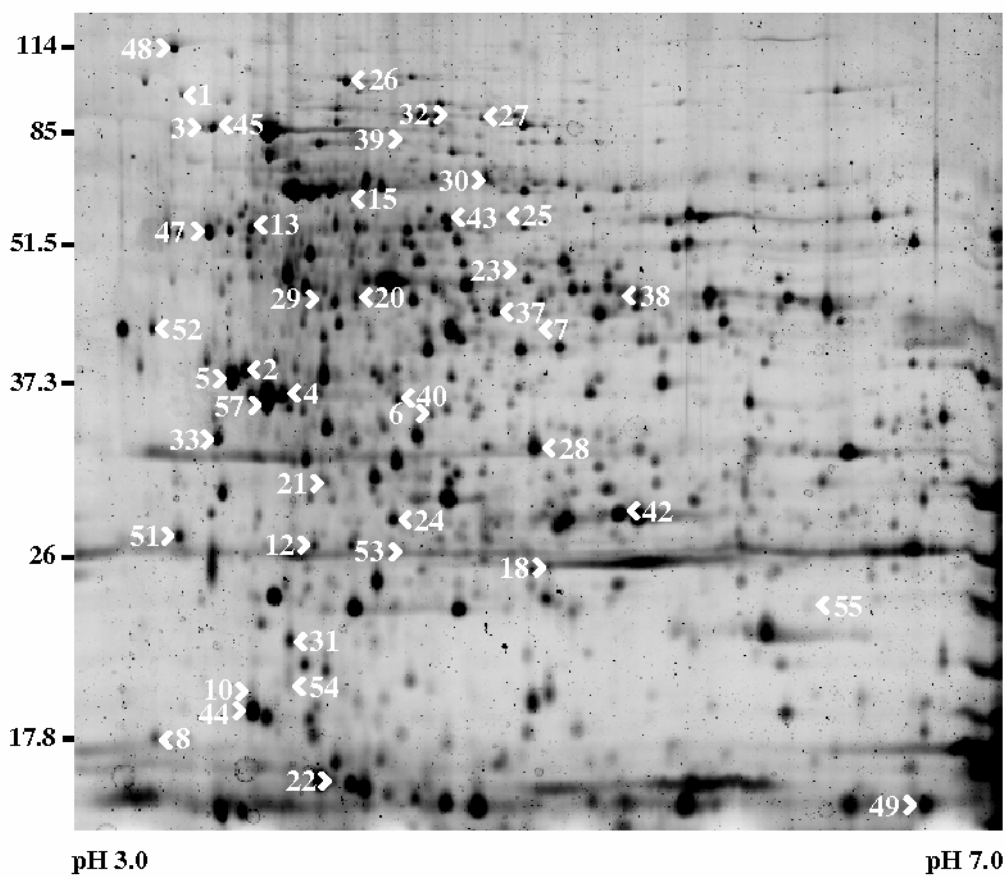
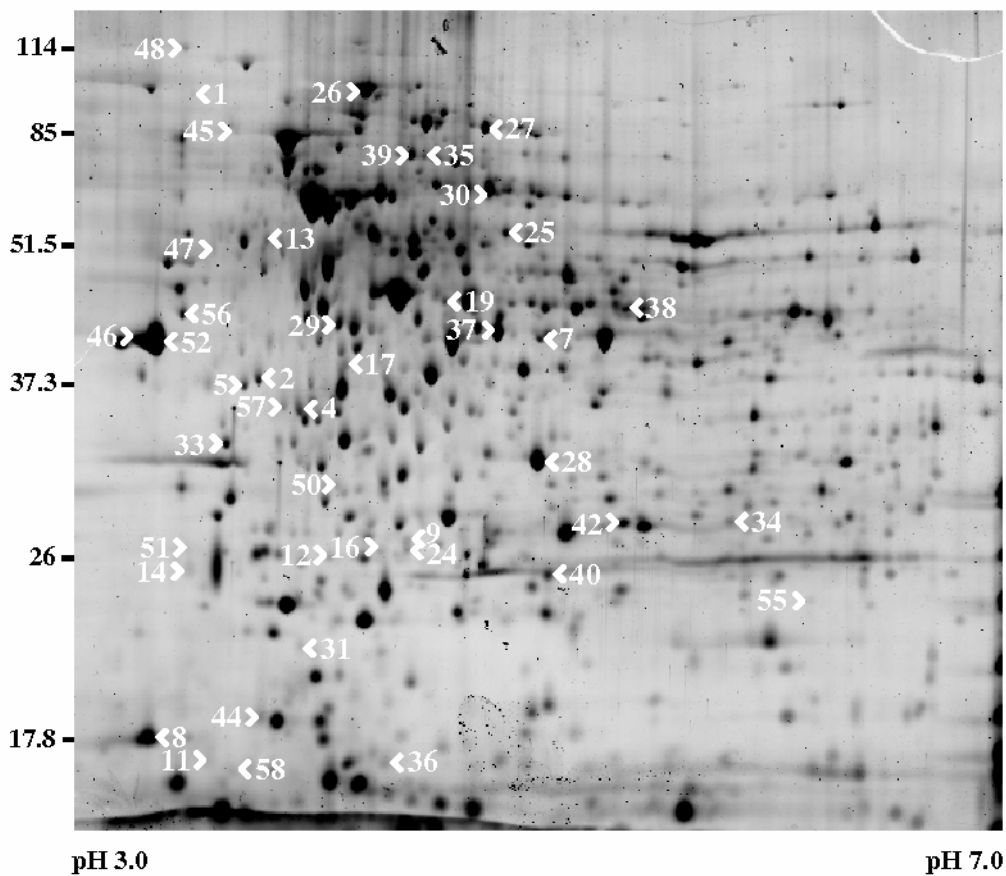


Fig. 1

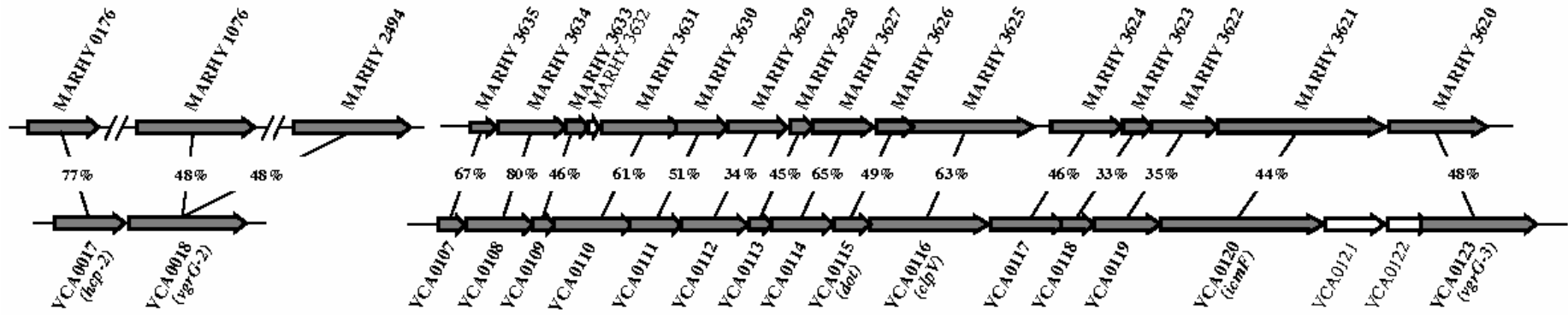


Fig. 2

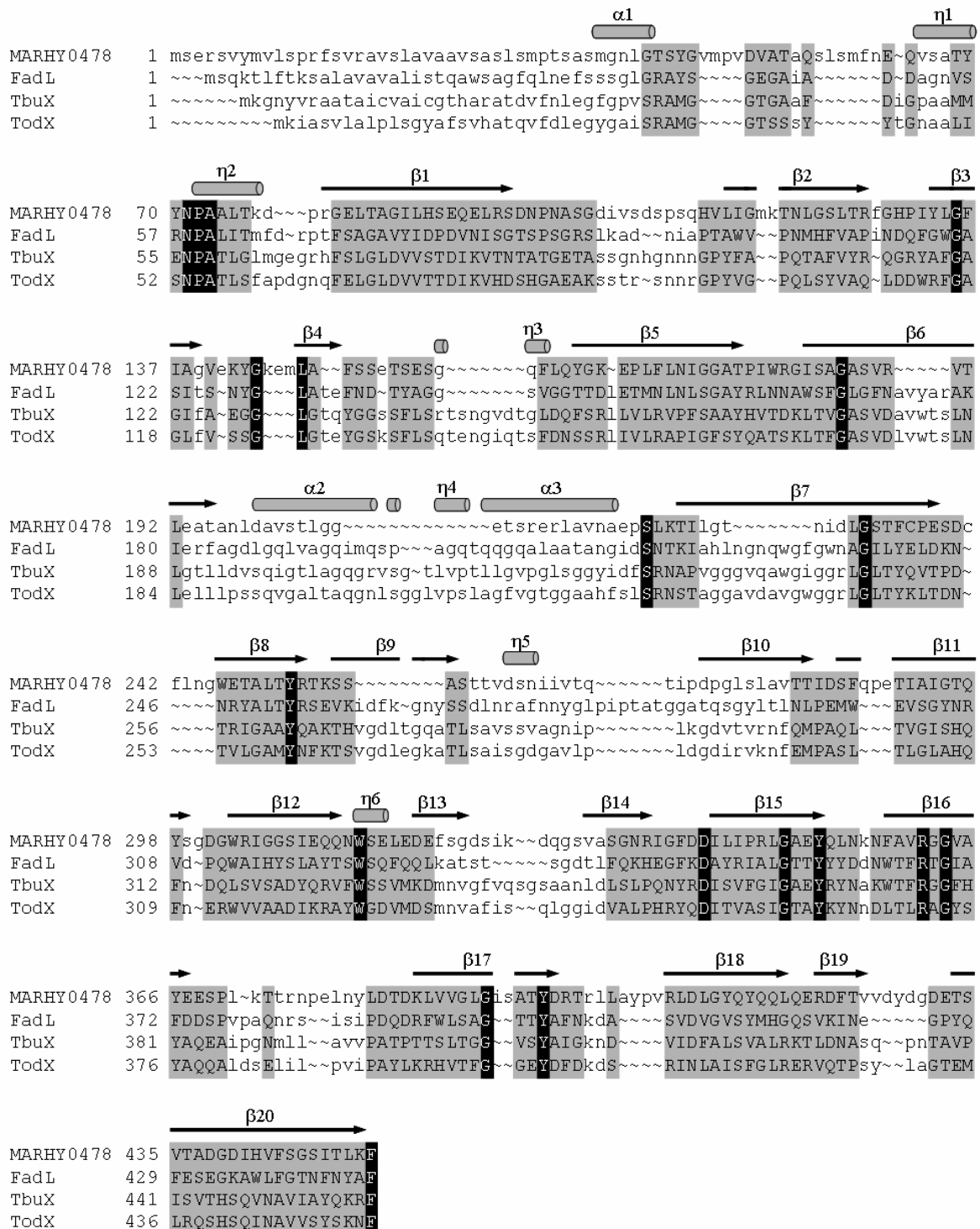


Fig. 3