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Proteomic characterisation of polyethylene terephthalate and monomer degradation by *Ideonella sakaiensis*

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ABSTRACT

Synthetic plastics, like polyethylene terephthalate (PET), have become an essential part of modern life. Many of these products are remarkably persistent in the environment, and the accumulation in the environment is recognised as a major threat. Therefore, an increasing interest has been focusing on the screening for organisms able to degrade and assimilate the plastic. *Ideonella sakaiensis* originally isolated from a plastisphere has been reported as a bacterium that was solely thriving on the degradation on PET films. The processes affected by the presence of PET and its monomeric substances terephthalic acid, ethylene glycol, ethyl glycolate, and sodium glyoxylate monohydrate were elucidated by analysis of differential protein expression. The exposure of PET and its monomers induced the MHETase and affect two major pathways: the TCA cycle and the β-oxidation pathway. The increased expression of proteins directly or indirectly involved in these pathways suggests their underlying importance in the degradation of PET by *I. sakaiensis* since these proteins are mechanistically supporting the enzymes involved in the degradation of PET and its monomers.

1. Introduction

Synthetic plastics, such as polyethylene terephthalate (PET), have become an essential part of modern life, including, but not limited to, healthcare, technology, and food and drinking packaging. Many of these products are remarkably persistent in the environment due to the lack or low activity of enzymes that can break down the plastic [1,2].

In the year 2021 80.9 million tons of PET were produced worldwide, and approximately 50% of these were used for food and drinking packaging [3]. Due to the plastics' low cost and disposable nature, large quantities of PET have been introduced into the environment, resulting in accumulation of PET in multiple ecosystems [2]. Despite of PET being 100% recycleable, plastic is the third most collected waste, e.g. in beach clean-ups [4]. The accumulation of these polymers in the environment is recognised as a major threat to both aquatic and terrestrial ecosystems [5–7]. Therefore, increasing interest has been focusing on the screening for organisms able to degrade and assimilate the plastic.

Ideonella sakaiensis was isolated from a plastisphere as a bacterium that was solely thriving on the degradation of PET films. Multiple studies have investigated this organism, demonstrating its ability to degrade and assimilate PET (e.g., [1,8]). This organism has since obtained much attention, and the important mechanisms of enzymatic PET degradation have been associated with its surface hydrophilization and PET hydrolases. *I. sakaiensis* possesses two unique enzymes capable to hydrolyse PET [9]. PET hydrolase (PETase) hydrolyses PET into Mono(2-hydroxyethyl) terephthalate (MHET), and MHET hydrolase (MHETase) hydrolyses MHET into terephthalic acid and ethylene glycol. These enzymes belong to the group of cutinases [10]. However, the exact degradation pathway of PET is still not fully understood, and the vast majority knowledge on the enzymatic degradation of PET originates from bioinformatic studies based on homology.

Ethylene glycol and terephthalic acid are the building blocks of PET, linked by ester bonds [11]. These ester bonds can be hydrolysed by various hydrolytic enzymes. More than five groups of enzymes have been reported to hydrolyse PET, e.g., PETases [1,12], cutinases [13], lipases [14], carboxylesterases [15], and polyester hydrolases [16]. Although a variety of different enzymes has been associated with degradation of PET naturally, the enzymes are frequently limited to initial steps in the pathway.

The aim of this study was to elucidate the expression of proteins when *I. sakaiensis* is exposed to PET and its monomers. Through application of proteomics, the physiological changes will provide insight into the expression of enzymes directly or indirectly involved in the degradation processes. The approach makes it possible to identify the

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processes affected by presence of PET, terephthalic acid, ethylene glycol, ethyl glycolate, and sodium glyoxylate monohydrate. The findings provide novel information on the biodegradation of PET and its monomers, and a better understanding of the physiological responses of *I. saikaiensis* that can provide important tools for designing new strategies and technologies for the bioremediation of this type of plastic.

2. Material and methods

2.1. Polyesters, monomers, organism, and media

Amorphous and cryo-milled PET (<100 μm) was provided by AIMPLAS (2.78% crystallinity, T_g of 77.26 °C, T_c of 153.11 °C, T_m of 244.72 °C, ΔH_c of −38.20 J•g^{-1}, and ΔH_m of 42.09 J•g^{-1}). The four monomers terephthalic acid (TPA), ethylene glycol (EG), ethyl glycolate (EGL), and sodium glyoxylate monohydrate (SGM) used for the incubations were purchased from Merck Life Science.

A cryo-stock of the strain *Ideonella sakaiensis* strain 201-F6 (Bioresource Collection and Research Center) was activated and grown in liquid NBRC 982 media (10 g•L^{-1} peptone, 2 g•L^{-1} yeast extract, 1 g•L^{-1} $MgSO_4$•7H_2O) at 37 °C and agitated at 200 rpm for 2 days. Incubations for analysis of differential protein expression were grown in yeast sulphate and vitamin (YSV) media (containing 0.1 g•L^{-1} yeast extract, 0.2 g•L^{-1}, 1 g•L^{-1} ammonium sulphate, 0.1 g•L^{-1} calcium carbonate, 1% (*v*/v) trace elements (consisting of 0.1% (*w*/*v*) $FeSO_4$•7 H_2O, 0.1% (w/v) $MgSO_4$•7 H_2O, 0.01% (*w*/*v*) $CuSO_4$•5 H_2O, 0.01% (*w*/*v*) $MnSO_4$•7 H_2O, and 0.01% (*w*/*v*) $ZnSO_4$•7 H_2O), and 0.1% (*v*/v) vitamin mixture (consisting of 0.25% (w/v) thiamine-HCl, 0.005% (w/v) biotin, and 0.05% (w/v) vitamin B12) made in 10 mM phosphate buffer (pH 7, 1.5 g•L^{-1} Na_2HPO_4•H_2O and 0.58 g•L^{-1} NaH_2PO_4•2H_2O). This media was chosen because it repressed starvation and stress responses but allowed *I. sakaiensis* to be prone for expression of proteins as a response to the presence of PET/monomers.

Growth monitoring was performed using an automated image acquisition platform, the oCelloScope system (BioSense Solutions ApS). The bacteria were grown in YSV medium, with and without PET, starting with approximately $1{\cdot}10^4$ cells, estimated from the OD_{600} [17]. Both conditions were tested in 8 replicates. The camera focus and illumination level were set to 2790 mm and 300, respectively. Image acquisition was performed in one scan area in the centre of each well, which was scanned every 30 min for 16 h. The growth kinetics were determined by image stack processing using the segmentation extracted surface area (SESA) algorithm, in the UniExplorer software (BioSense Solutions ApS).

2.2. Proteomic analysis of Ideonella sakaiensis exposed to PET and PET monomers

Biological quadruplicates of 25 mL were run in parallel under the same conditions for the incubations with PET, the four monomers, and controls without added plastic or monomers. PET and the monomers were added to a final concentration of 1% (*w*/*v* for TPA, EGL, and SGM or *v*/v for EG). Cellular growth in the presence/absence of PET and its monomers was stopped by snap-freezing all cultures in liquid nitrogen after 8 doubling times after reaching exponential growth, ensuring that the cells were growing in the presence/absence of the plastic/monomers for multiple generations.

2.3. Protein extraction and quantitative spectrometry

The protein extraction was performed as previously described [18]. In brief, the pelleted culture was washed in ddH_2O, pelleted, and resuspended in TEAB and BPER buffer, followed by ultrasonication (four rounds of 75 W, 6 °C and 180 s).

Supernatant from pelleted cultures (control and exposed to PET) were used to extract secreted/extracellular proteins, as previously described method [19]. In brief, the proteins were precipitated by TCA and recovered by centrifugation.

The iST sample preparation kit (PreOmics GmbH, Germany) was used for preparing the extracted proteins, for liquid chromatography-tandem mass spectrometry (LC-MS/MS), following manufacturer's recommendations. Tryptic peptides were analysed by automated LC–electrospray ionization (ESI)–MS/MS, as described elsewhere [18].

2.4. Bioinformatic processing

The raw data were analysed with MaxQuant (v.1.6.10.43) [20], using the search engine Andromeda [21], with carbamidomethylation set as a fixed modification and methionoine oxidation as a variable modification and with a false discovery rate (FDR) of 1%, using label-free quantification (LFQ) as implemented in MaxQuant. Data were cross-referenced with a downloaded proteome of *I. sakaiensis* strain 201-F6 (UP000037660), downloaded from UniProt (accessed 22nd of July 2022).

The output file from MaxQuant was analysed in Perseus (v.1.6.15.0) [22], and the differential protein expression was investigated using student's *t*-test, performed on $\log_2$-transformed LFQ values with a significance level of an FDR value of ≤0.1 and a s0 of ≥0.1. Fold change was expressed as the ratio of averaged LFQ values of a protein across at least 3 out of 4 replications of *I. sakaiensis* exposed to PET or one of its monomers and *I. sakaiensis* grown in YSV media. When >10 proteins were statistically differentially expressed, they were uploaded to DAVID bioinformatics resources [23,24] for functional enrichment analysis, using a customized background, based on the detected proteins, and otherwise standard settings.

3. Results

3.1. Effect of polyethylene terephthalate on Ideonella sakaiensis

The automated image acquisition system, oCelloScope, was used to detect bacterial growth and determine if the PET had any inhibitory or stimulating effect on the growth. After monitoring the growth of *I. sakaiensis* over a period of 16 h it was observed that the presence of PET in the media (n = 8) did not influence the growth significantly compared to a control without PET (n = 8) (Fig. 1).

3.2. Proteomic analysis of exposure to polyethylene terephthalate

Analysis of differential *I. sakaiensis* protein expression upon exposure to PET was conducted in biological quadruplicates in order to identify the physiological response. The analysis of differential protein expressions of *I. sakaiensis* exposed to PET resulted in detection of 1072 different proteins, with a total of 6 differentially expressed proteins (p-value <0.1; $\log_2 < -0.5$ or $\log_2 > 0.5$). Of the differentially expressed proteins 4 were found to have an increased abundance and 2 with a decreased abundance relative to the control. The proteins differentially expressed due to the presence of amorphous and pulverized PET were identified and classified to several biochemical pathways, including the β-oxidation of fatty acid metabolism, the tricarboxylic acid cycle (TCA cycle), and proton transportation.

The protein with the highest differential increase in expression upon exposure to PET was ATP synthase subunit c (A0A0K8P094) (p-value = 0.0013 and $\log_2$ = 7.2), while the protein with the second highest increase in expression when exposed to PET was enoyl-CoA hydratase (A0A0K8P1Z2) (p-value = 0.036 and $\log_2$ = 2.5) (Table 1).

The two proteins with decreased abundance upon exposure to PET were fumarate hydratase class II (A0A0K8NZK0) (p-value = 0.038 and $\log_2 = -2.3$) and propionyl-CoA carboxylase (A0A0K8P2T8) (p-value = 0.012 and $\log_2 = -1.9$), connected to the TCA cycle and involved in the conversion of propionyl-CoA into succinyl-CoA, respectively (Table 1).

Incubations for analysing the secreted proteins upon exposure to PET were also performed in biological quadruplicates. A total of 1019 secrete

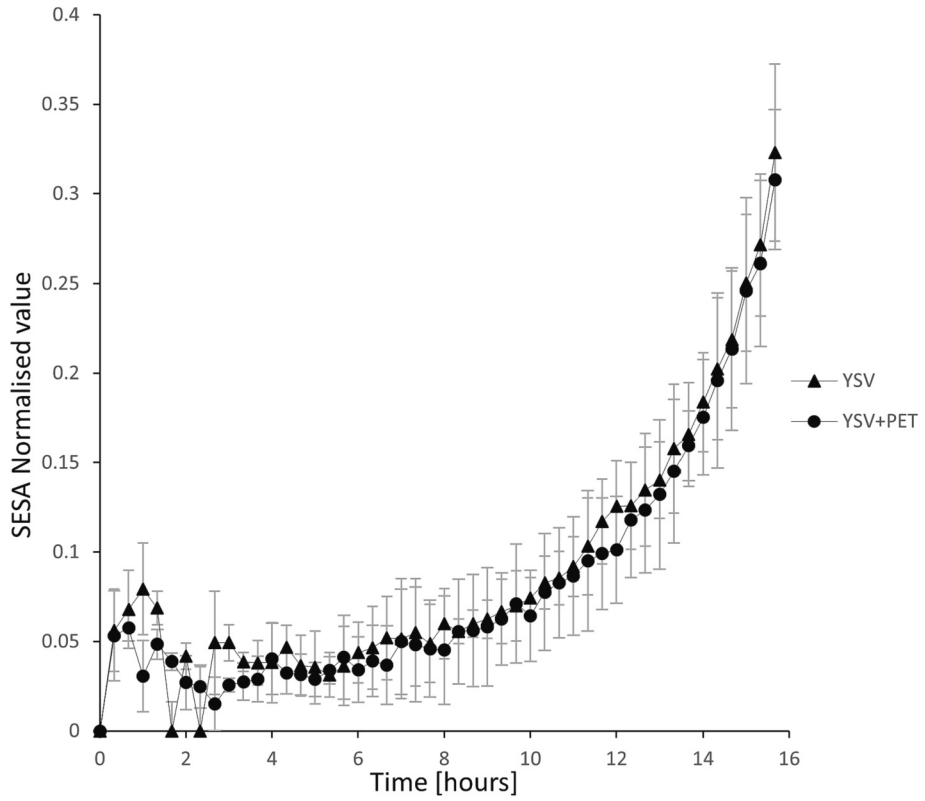

Fig. 1. Bacterial density (SESA: Segmentation extracted surface area) was measured using real time microscopy (oCelloScope) for growth of *I. sakaiensis* in YSV media with and without PET (n = 8).

proteins were detected, and hereof 6 proteins were differentially expressed (Table 2). Of the differentially expressed proteins 4 were found to have an increased abundance and 2 with a decreased abundance. The two proteins with differential decreased expression were both membrane proteins (Putative membrane protein (A0A0K8P727) and Capsular polysaccharide export system inner membrane protein KpsE (A0A0K8NXQ1), *p*-value = 0.00054 and $\log_2 \leq$ -0.91).

Looking into the secreted proteins with increased abundance upon exposure to PET, revealed that the protein with the highest increase was the Mono(2-hydroxyethyl) terephthalate hydrolase (MHETase) (A0A0K8P8E7) (p-value = 0.00015 and $\log_2 = 8.6$). The other 3 secreted proteins showing a differential increase in expression were 4-hydroxythreonine-4-phosphate dehydrogenase (A0A0K8P8F3), Putative exported protein (A0A0K8P8D2), and Putative transmembrane protein (A0A0K8NZY0) (Table 2).

The results from *I. sakaiensis* exposed to PET provided an understanding of the physiological state of *I. sakaiensis* when exposed to PET. However, to obtain deeper insight into the potential pathways involved in the degradation of the depolymerized PET, further exposures to individual monomers of PET were also tested.

The expressions of both PETases and MHETases were also investigated for secreted and intracellular proteins when exposed to PET and were identified in all samples. PETases were not found to be differentially expressed in any of the samples, but MHETases were found to be differentially expressed when comparing the secreted proteins.

3.3. Proteomic analysis of exposure to monomers of polyethylene terephthalate

Proteomic analysis yielded a total of 1226 proteins across the four treatments, which could be quantified in at least 3 of 4 biological replicates from *I. sakaiensis* exposed to monomers derived from PET (EG (*n* = 4), TPA (n = 4), SGM (n = 4), and EGL (n = 4)) and control without the monomers (n = 4). Differential protein analysis showed that exposure to EG resulted in 24 differentially expressed proteins, of which 17 showed increased abundance and 7 with decreased abundance (Table 1, for full list of differentially expressed proteins, see Table S1). In *I. sakaiensis,* cells exposed to TPA were found to significantly alter the expression of 85 proteins, 5 of which showed increased expression, and 80 with decreased expression, while 147 proteins were found differentially expressed when exposed to SGM, with 31 of them having increased expression and 116 with decreased expression (Table 1, for full list of differentially expressed proteins, see Table S1). For the last tested monomer, only one protein was seen to have a significantly increased expression and 72 with decreased expression (Table 1, for full list of differentially expressed proteins, see Table S1).

For pathways that were found to have an increased expression upon exposure to EG, more than one enzyme in the pathway were seen to have a significant increase in expression. Some of the most significantly increased proteins in incubations with EG exposure were related to the TCA cycle (Succinate–CoA ligase [ADP-forming] subunit beta (A0A0K8P1C5) and phosphoenolpyruvate carboxykinase [GTP] (A0A0K8P2T9), *p*-value $\leq$ 0.04 and $\log_2 \geq$ 0.99), respiratory metabolism (Putative iron-sulfur cluster insertion protein ErpA (A0A0K8NVX2) and ferredoxin–NADP(+) reductase (A0A0K8NY35), p-value $\leq$ 0.04 and $\log_2 \geq$ 0.94), and energy (Fructose-1,6-bisphosphate aldolase (A0A0K8P546), and Electron transfer flavoprotein, beta subunit (A0A0K8P8V4), *p*-value $\leq$ 0.008, and $\log_2 \geq$ 0.58) (Table 1, for full list of differentially expressed proteins, see Table S1).

The incubations with TPA exposure resulted in an increased expression of proteins mainly related to the protein biosynthesis (Peptide chain release factor RF-3 (A0A0K8NXZ6), Lysine–tRNA ligase (A0A0K8NTY4), and Tyrosine–tRNA ligase (A0A0K8NVW4), *p*-value $\leq$ 0.05 and $\log_2 \geq$ 1.6) (Table 1, for full list of differentially expressed proteins, see Table S1). Proteins with decreased abundance upon exposure to TPA belonged to the categories of protein folding (Co-

Table 1
Top 5 proteins which were found to be differentially expressed in *I. sakaiensis* strain 201-F6 upon exposure to polyethylene terephthalate (PET) terephthalic acid (TPA), ethylene glycol (EG), ethyl glycolate (EGL), and sodium glyoxylate monohydrate (SGM). The roman number in superscript refers to Fig. 2.

Carbon source	Increased abundance				Decreased abundance			
	p-value	Difference	Protein ID	Protein name or description	p-value	Difference	Protein ID	Protein name or description
PET	1.3E-03	7.2	A0A0K8P094	ATP synthase subunit c[I]	0.038	−2.3	A0A0K8NZK0	Fumarate hydratase class II[III]
	0.036	2.5	A0A0K8P1Z2	Enoyl-CoA hydratase[II]				
	0.018	2.4	A0A0K8P073	ATP synthase subunit b[I]				
	0.037	2.3	A0A0K8NUL6	RND efflux system, outer membrane lipoprotein CmeC	0.012	−1.9	A0A0K8P2T8	Propionyl-CoA carboxylase[III]
	0.045	2.9	A0A0K8NVX2	Putative iron-sulfur cluster insertion protein ErpA	0.018	−1.4	A0A0K8P4T9	TerB domain-containing protein
	0.022	2.1	A0A0K8NYZ2	Cold shock protein CspA	9.8E-04	−1.2	A0A0K8P8R8	Hemolysin-type calcium-binding region
EG	0.045	2.0	A0A0K8P1C5	Succinate–CoA ligase [ADP-forming] subunit beta[V]	0.015	−0.82	A0A0K8NTX5	Uncharacterized protein
	7.3E-04	1.5	A0A0K8NZW8	Cold shock protein CspA	2.2E-04	−0.78	A0A0K8NTU7	TonB-dependent receptor
	9.5E-03	1.1	A0A0K8P7H3	2-isopropylmalate synthase	3.3E-03	−0.75	A0A0K8NTC6	Outer membrane protein A
	0.019	2.5	A0A0K8NXZ6	Peptide chain release factor RF-3[IV]	2.1E-05	−7.8	A0A0K8NWU1	Chaperonin GroEL
	0.010	2.2	A0A0K8NVV1	RND efflux system, membrane fusion protein CmeA	7.5E-04	−6.0	A0A0K8P1H2	TonB-dependent receptor
TPA	0.055	1.9	A0A0K8P359	50S ribosomal subunit assembly factor BipA	5.7E-04	−5.7	A0A0K8NX31	Co-chaperonin GroES
	0.053	1.8	A0A0K8NTY4	Lysine–tRNA ligase[IV]	2.6E-03	−5.4	A0A0K8P4K4	Adenosylhomocysteinase
	0.024	1.6	A0A0K8NVW4	Tyrosine–tRNA ligase[IV]	2.6E-03	−5.3	A0A0K8P6K4	50S ribosomal protein L7/L12
	6.9E-06	5.5	A0A0K8P7X7	Uncharacterized protein	2.2E-05	−5.4	A0A0K8NYT9	Chaperone protein ClpB
	1.1E-04	5.3	A0A0K8NXT4	Ferrichrome-iron receptor	1.6E-05	−4.9	A0A0K8P1D7	2,4-dienoyl-CoA reductase [NADPH]
SGM	4.4E-04	4.7	A0A0K8P207	Peptidoglycan-associated protein	2.5E-05	−4.2	A0A0K8NZ70	Granule-associated protein
	9.6E-04	4.6	A0A0K8P7A3	Outer membrane lipoprotein Blc	3.9E-05	−4.2	A0A0K8NWJ9	Extracytoplasmic solute receptor protein
	6.7E-05	4.6	A0A0K8P6Z8	Hypothetical membrane-anchored protein	2.6E-07	−4.2	A0A0K8NYG2	Aspartate–tRNA(Asp/Asn) ligase[IV]
					2.5E-05	−5.7	A0A0K8NVV3	Chaperone protein HtpG
					4.1E-05	−5.6	A0A0K8NT97	30S ribosomal protein S1
EGL	1.3E-03	1.9	A0A0K8NXZ4	Type IV pilus biogenesis and competence protein PilQ	2.2E-06	−5.5	A0A0K8NYT9	Chaperone protein ClpB
					0.016	−4.3	A0A0K8P729	Elongation factor G[IV]
					2.8E-03	−4.2	A0A0K8NTT5	Trigger factor[IV]

Table 2
Differentially expressed secreted proteins of *I. sakaiensis* strain 201-F6 upon exposure to polyethylene terephthalate (PET). The roman number in superscript refers to Fig. 2.

Increased abundance				Decreased abundance			
p-value	Difference	Protein ID	Protein name or description	p-value	Difference	Protein ID	Protein name or description
1.5E-04	8.6	A0A0K8P8E7	Mono(2-hydroxyethyl) terephthalate hydrolase (MHETase)[V]	5.4E-04	−0.96	A0A0K8P727	Putative membrane protein
5.8E-04	6.5	A0A0K8P8F3	4-hydroxythreonine-4-phosphate dehydrogenase				
3.2E-06	6.1	A0A0K8P8D2	Putative exported protein	5.4E-04	−0.91	A0A0K8NXQ1	Capsular polysaccharide export system inner membrane protein KpsE
2.7E-04	1.0	A0A0K8NZY0	Putative transmembrane protein				

chaperonin GroES (A0A0K8NX31), Trigger factor (A0A0K8NTT5), and Peptidyl-prolyl cis-trans isomerase (A0A0K8NZG1), p-value $\leq$ 0.05 and $\log_2 \leq$ -1.6) and TCA cycle (Dihydrolipoyllysine-residue succinyl transferase component of 2-oxoglutarate dehydrogenase complex (A0A0K8P8B3), Oxoglutarate dehydrogenase (succinyl-transferring) (A0A0K8P744), and Succinate dehydrogenase iron-sulfur subunit (A0A0K8P1T7), p-value $\leq$ 0.08 and $\log_2 \leq$ -2.3) (Table 1, for full list of differentially expressed proteins, see Table S1).

When *I. sakaiensis* was exposed to SGM, it was observed that the following proteins related to the outer membrane (Ferrichrome-iron receptor (A0A0K8NXT4), Peptidoglycan-associated protein (A0A0K8P207), and TonB-dependent hemin, ferrichrome receptor (A0A0K8P5R6), p-value ≤ 0.0009 and $\log_2 \geq 2.8$) and protein transport (MotA/TolQ/ExbB proton channel family protein (A0A0K8NTE8), type IV pilus biogenesis and competence protein PilQ (A0A0K8NXZ4), and biopolymer transport protein ExbD/TolR (A0A0K8P839), p-value ≤ 0.008 and $\log_2 \geq 1.8$) were significantly higher expressed (Table 1, for full list of differentially expressed proteins, see Table S1). Proteins with a decreased expression when exposed to SGM belonged to the categories of protein biosynthesis (Proline–tRNA ligase (A0A0K8NX46), Aspartate–tRNA(Asp/Asn) ligase (A0A0K8NYG2), and Threonine–tRNA ligase (A0A0K8P3T2), p-value ≤ 0.08 and $\log_2 \leq -1.9$) and redox-active center (Dihydrolipoyl dehydrogenase (A0A0K8P7E1), Alkyl hydroperoxide reductase C (A0A0K8NX37), Thioredoxin domain protein EC-YbbN (A0A0K8P5H0), p-value ≤ 0.03 and $\log_2 \leq -2.1$) (Table 1, for full list of differentially expressed proteins, see Table S1).

Growth in the presence of EGL resulted in one protein related to protein transport which was found to have a significantly increased expression, the type IV pilus biogenesis and competence protein PilQ (A0A0K8NXZ4) p-value $= 0.001$ and $\log_2 = 1.9$) (Table 1). The proteins with decreased abundance for this monomer can be grouped into transcription (DNA-directed RNA polymerase subunit alpha (A0A0K8P186), DNA-directed RNA polymerase subunit beta (A0A0K8P723), and DNA-directed RNA polymerase subunit beta' (A0A0K8P7Z7)), translation (Transcription elongation factor GreA (A0A0K8NWK2), Elongation factor P (A0A0K8P313), and Elongation factor Ts (A0A0K8P705), p-

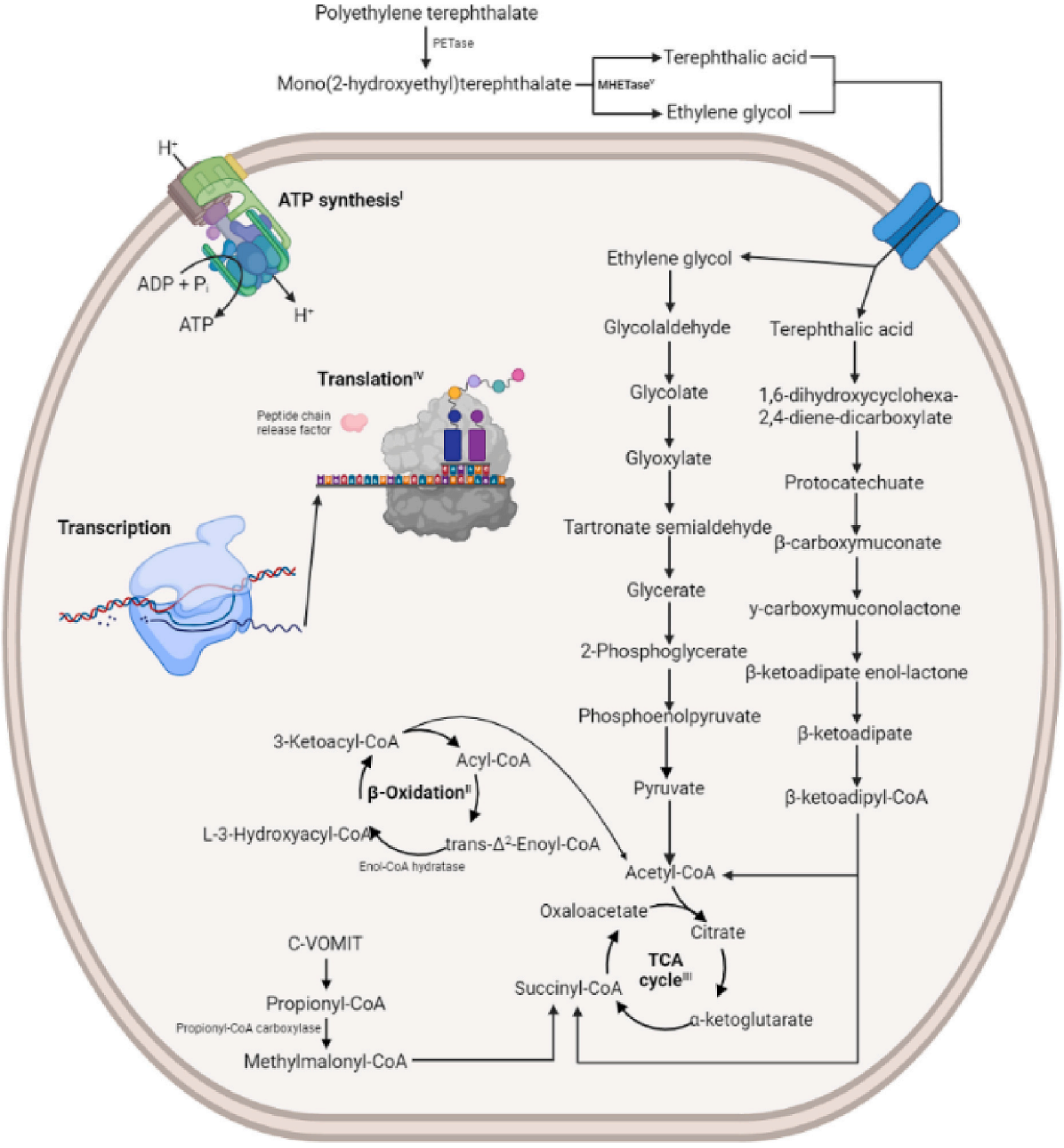

Fig. 2. Proposed degradation pathway of polyethylene terephthalate and differentially expressed pathways/proteins upon exposure to polyethylene terephthalate, ethylene glycol, sodium glyoxylate monohydrate, terephthalic acid, and ethyl glyoxylate. MHETase: Mono(2-hydroxyethyl)terephthalate hydrolase, C-VOMIT: cholesterol, valine, odd chain fatty acids, methionine, isoleucine, and threonine and TCA cycle: tricarboxylic acid cycle. The roman number in superscript refers to Table 1.

value $\leq$ 0.02 and $\log_2 \leq$ -2.3), and TCA cycle (Citrate synthase (A0A0K8P1S1), Succinate dehydrogenase iron-sulfur subunit (A0A0K8P1T7), and Malate dehydrogenase (A0A0K8P1U3), *p*-value $\leq$ 0.01 and $\log_2 \leq$ -2.3) (Table 1, for full list of differentially expressed proteins, see Table S1).

4. Discussions

In the present study, the physiological responses to the presence of PET and its monomers by *I. sakaiensis* were investigated. The approach, analysis of differential protein expression, was chosen to provide insight into the enzymes with increased expression when exposed to the polymer and its monomers and thereby a better understanding of the mechanistic degradation of PET and its monomeric components.

Growth was observed both in the presence and absence of both PET (Fig. 1) and its monomers (data not shown), and without any significant inhibition. Among the inventory proteins, only a very limited number of these are considered to be stress-related proteins. This indicates a proper experimental set-up, execution, and collection of reliable results. Furthermore, several doubling times during the incubation allowed for sufficient time to register the physiological response upon the exposure to PET and its monomers.

4.1. Differentially expressed proteins when exposed to polyethylene terephthalate

For the incubations with PET, it was seen that only a very limited number of proteins were induced upon the exposure. This could be due to multiple reasons, one being that the PET is not able to migrate across the membrane and thus directly induce the expression of specific genes. Another reason for this could be that the proteins are constitutively expressed, hence no difference is seen upon exposure to PET.

Overall, the differentially increased proteins are part of energy-producing pathways. However, the observed increased level of enoyl-CoA hydratase was interesting, since it has been previously reported to be linked to being responsible for the second step in the physiologically important β-oxidation pathway of fatty acid metabolism [26] (Fig. 2). More specifically, this protein facilitates the *syn*-addition of a water molecule across the double bond of a trans-2-enoyl-CoA thioester, resulting in the formation of a β-hydroxyacyl-CoA thioester. Apart from this, the enoyl-CoA hydratase has also been connected to polyhydroxyalkanoate (PHA) formation in PET degradation [27]. Trans-2-decenoyl-CoA is hydrated by the R-specific enoyl-CoA hydratase to form R-3-hydroxydecanoyl-CoA, which then acts as the direct lipid precursor. PHAs have attracted significant interest as they open the doors to a sustainable polymer with interesting properties for the future regarding food packaging and the biomedical fields [28], due to their biodegradability and degradation products being non-toxic.

The two proteins with decreased expressions, identified when exposed to PET, were either directly or indirectly linked to the TCA cycle. The primary function of Propionyl-CoA carboxylase is to catalyse the carboxylation of propionyl-CoA to produce methylmalonyl-CoA, which will ultimately end up in the TCA cycle as succinyl-CoA [29]. However, the downregulation of this protein could be hypothesized to be the result of an excess of acetyl-CoA from PET degradation, thereby reducing the need for propionyl-CoA carboxylase contributing with TCA intermediates.

The other protein with decreased expressions, fumarase, is conserved and present in any organism with an aerobic metabolism [30,31] and is involved in the TCA cycle and part of the DNA damage response [32]. Such a decreased expression would result in an energy-poor state, or it could simply be that the need for reducing powers has been met from other pathways, e.g., β-oxidation. However, multiple proteins related to ATP synthesis were observed to have an increased expression (Fig. 2).

Also, for the secreted proteins only a limited number of proteins were induced upon exposure to PET. However, one of the two main enzymes enabling *I. sakaiensis* to metabolise PET as a carbon source, was found to be induced upon exposure to PET. This enzyme is well known as a key player in the PET degradation, since MHET inhibits the efficiency of depolymerisation catalysed by various PET hydrolases [9,33]. This is where the MHETase from *I. sakaiensis* plays its role, to hydrolyse MHET into TPA and EG to be further metabolised by *I. sakaiensis* and facilitate growth.

The PETase was not found to be differentially expressed in the intracellular proteome nor among the secreted proteins. This is in contradiction to a previous study based on transcriptomics in which it was observed that the presence of PET induced the expression of PETase [1], however, in this study no difference in the PETase expression was observed between the control and the ones exposed to PET. This might be due to the difference in the two methods used to detect the expression levels of the enzyme, since regulation can happen both at transcript and translational level, and the soluble expression levels of PETase is low [34]. Therefore, even with the induced expression the detection of the secreted proteins in the supernatant from the incubations would not have significant difference in the levels of PETase. Resulting in detection of the PETase, but not differentially expressed. The results from Yoshida and colleagues are generally consistent with the results presented here, e.g., expression of the putative exported protein (A0A0K8P8D2) was found to be induced in the presence of PET by both the proteomic approach and the transcriptomic approach [1].

4.2. Differentially expressed proteins when exposed to monomers of polyethylene terephthalate

In relation to degradation of PET, some of the proteins with significantly increased abundance, when exposed to EG, fit into the previously proposed degradation pathway [27] (Fig. 2). Some of the proteins with increased abundance are affiliated with the TCA cycle (succinate–CoA ligase [ADP-forming] subunit beta and phosphoenolpyruvate carboxykinase [GTP]), indicating that the EG monomer of PET has been degraded to compounds entering the TCA cycle (Fig. 2). Phosphoenolpyruvate carboxykinase plays an important role in removing citric acid cycle anions, acting as a cataplerotic enzyme [35]. This enzyme can use the oxaloacetate from the TCA cycle as initial substrate for four pathways: gluconeogenesis, glyceroneogenesis, synthesis of serine, and conversion of carbon skeletons of amino acids to phosphoenolpyruvate and then to pyruvate for subsequently entering the TCA cycle again.

One of the proteins with increased abundance was the electron-transferring flavoprotein (ETF). This protein functions as a hub by transferring electrons from various flavoenzymes and feeding them into the electron transport chain [36]. The ETF thereby mediates a major flow of reducing power for the electron transport chain and eventually the ATP synthetase. The oxidation of fatty acids or other similar compounds, such as PET and its monomers, can through enzymes related to β-oxidation pathway act as a major supplier of reducing power. The ability to donate electrons to dehydrogenases and act as electron acceptor from ferredoxin and NADH has been described elsewhere [36,37]. This hypothesis is well supported by the findings of two proteins with increased expression related to the putative iron-sulfur cluster insertion protein ErpA and a Ferredoxin–NADP(+) reductase. These two proteins act directly or indirectly in the electron transport system, providing electrons for the ETF. Furthermore, iron-sulfur proteins have been proven to be crucial for the cell metabolism of both prokaryotic and eukaryotic cells [38].

The protein with the highest decrease in expression when exposed to TPA was a protein with the essential role of assisting folding of other proteins, GroEL, and its co-chaperonin, GroES, was also seen with a significant decrease in expression. A decreased expression of the GroEL-GroES complex results in improper folding of complicated larger proteins [39], thereby a less functioning cell. From the proteins with decreased abundance, it also seems as if *I. sakaiensis* was in an energy-poor state when exposed to TPA, since multiple proteins related to the

TCA cycle showed decreased abundance (Fig. 2).

Exposure to TPA resulted in an increased expression of the core biological process, protein biosynthesis. More specifically, the proteins that were found to have increased expression are part of the elongation and termination of the translation step (Fig. 2). This could be speculated to be connected to the cell trying to overcome the decreased expression of chaperonins and therefore, by chance, resulting in properly folded new proteins.

When *I. sakaiensis* was exposed to SGM, expression of proteins related to the protein biosynthesis decreased, thereby decreasing the cells' ability to generate the building blocks for new biomass and maintain themselves. As a response to this inhibition, it seems that the cells increased the expression of proteins promoting cell growth in various ways, e.g., ferrichrome binding and transport and organelle biogenesis [40,41].

Exposure to EGL appears to inhibit the cells proliferation, as only one protein is found to have an increased expression, and the proteins with decreased expression are either related to energy-generating pathways or core biological processes such as transcription and translation (Fig. 2). However, the one protein with increased expression has been proven to act as a pilus channel or pore in the terminal steps of organelle biogenesis [41].

As mentioned, a significant part of the enzymatic degradation of PET originates from homology studies, but the present study gives in-depth information on the degradation of PET. The exposure of PET and its monomers resulted in differential expression of proteins directly or indirectly reflecting the physiological responses related to the TCA cycle, indicating that PET and its monomers were degraded into compounds entering the TCA cycle. Another major pathway that was observed to be affected by the exposure was the β-oxidation pathway, since many of the conditions resulted in an increased expression of proteins directly or indirectly involved, underlying the importance of this pathway in the degradation of PET by *I. sakaiensis*.

Knowledge to the pathways used by *I. sakaiensis* during degradation of PET, and more specifically, which proteins/enzymes from *I. sakaiensis* are responsible for the degradation, can be used to stop the degradation at the monomer level and use these for resynthesizing new polymers. Blocking the expression of specific enzymes makes it possible to favour specific monomers of interest.

Significance statement

This study provides deeper insight into the physiological changes during degradation of PET. The biochemical pathways and enzymes involved are determined and lead to a better understanding of the degradation mechanism. This experimentally based study provides novel insights into the biodegradation of PET and represents an alternative to previous bioinformatic studies based on homology.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifiers PXD035497 (10.6019/PXD035497) and PXD037565 (10.6019/PXD037565).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2023.104888.

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