Polyvinyl Chloride Biodegradation Fuels Survival of Invasive Insect Larva and Intestinal Degrading Strain of *Klebsiella*

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Abstract

Microbial degradation of polyvinyl chloride (PVC) is eco-friendly and economically attractive, but extremely challenging due to the lack of mechanistic understanding on the degrading strains and enzymes. Motivated by an accidental discovery that the larva of an agricultural invasive pest, *Spodoptera frugiperda*, effectively survived solely on PVC film, we profiled the intestinal microbiota of *S. frugiperda* and screened for PVC-degrading strains. The results showed PVC film feeding significantly changed the larvae intestinal microbiota through selective enrichment of *Enterococcus*, *Ochrobactrum* and *Klebsiella*. From the larva intestines, we isolated and named a biofilm-forming strain EMBL-1, and experimentally verified it as the first *Klebsiella* bacterium that can actively degrade and utilize PVC based on various classic physicochemical and morphological analyses. We further used multi-omics analyses that complementarily integrate whole genomic, transcriptomic, proteomic, and metabolic insights to identify enzyme-coding genes responsible for PVC degradation and proposed a putative biodegradation pathway by the bacterial strain. All in all, both *S. frugiperda* and its intestinal strain EMBL-1 are discovered to effectively survive on PVC film by exploiting its polymer as a sole energy source. Moreover, this work exemplifying PVC biodegradation provides reference for discovering more degrading microbes and enzymatic resources of other recalcitrant plastics.

Keywords: *Spodoptera frugiperda*; intestinal microorganisms; polyvinyl chloride (PVC); *Klebsiella*; degrading enzymes; multi-omics

Introduction

The globally increasing accumulation and pollution of wasted plastics is a serious eco-environmental and socio-economic problem [1]. Polyvinyl chloride (PVC) is one of the six widely used plastic polymers, such as polyethylene (PE), polystyrene (PS), polypropylene (PP), polyurethane (PUR) and polyethylene terephthalate (PET). Its market share (10.0%) is second only to PE (29.7%) and PP (19.3%) based on the European polymer demand[2], producing tremendous plastic wastes. Landfill and incineration processes are commonly used for the treatment and final disposal of plastic wastes. However, such energy-intensive technologies and industries, unfortunately, treat plastics solely as useless wastes rather than recyclable resources. Worse still, numerous harmful secondary pollutants (e.g., chloride and dioxins) and green-house gases are largely and continuously released into our water, soil and air environment[3], making these economically unattractive technologies eco-environmentally risky and unsustainable. Unfortunately, no green or sustainable approach is practically available for cost-effective and environmentally friendly disposal of PVC wastes, calling for methodological and technological innovations in the treatment and recycling of such man-made plastics.

Biological treatment and recycling of organic wastes is a promising approach for the future development of circular green economy [4,5]. PVC is considered very difficult to be biodegraded because of its recalcitrant nature [6]. It has the same linear structure as PE except for one of its hydrogens replaced with a chloride atom. However, current research progress on the biodegradation of PVC polymers far lags behind that of PE [7-12] and other plastics (e.g., PET[13-15]). Until now, although limited research reported the biodegradation of PVC materials including plasticizers by microbial consortia [3,16,17], little is, however, known about the responsible PVC-degrading microbes or biodegradation pathways (e.g., enzymes and products). Other research empirically speculated PVC biodegradation by several fungal (i.e., Basidiomycotina, Semi-bacterial, Ascomycota) or bacterial (i.e., *Pseudomonas*, *Mycobacterium*, *Bacillus* and *Acinetobacter*) groups based on solely morphological and physicochemical changes (e.g., surface damage and molecular weight loss) that visually

signify plastic degradation [18-22]. While these prior studies may generally provide preliminary evidence for microbial PVC degradation, the biodegradation mechanisms remain unexplored. In particular, there is no report on microbial or bacterial genes and enzymes in the degradation of PVC [23], excluding Nazia Khatoon's documentation of PVC-degrading activity of an extracellular lignin peroxidase of fungal *Phanerochaete chrysosporium* [24]. Therefore, global efforts are urgently needed to discover PVC-degrading microbes and enzymatic resources, and untangle the underlying biodegradation mechanisms to develop oriented engineering tools and biotechnology for prospering future industry of PVC wastes treatment and recovery.

During laboratory cultivation of an agriculturally invasive insect *Spodoptera frugiperda*, we accidentally discovered that the insect larva actively bit and fed on PVC film (Figure 1a). Motivated by curiosity and recent reports that some insect species (particular the larvae of wax moths and meal moths[25]) can consume plastic polymers of PE or PS [26-29], we specifically designed triplicate cultivation experiments to check whether the larva can survive solely on PVC film and whether their intestinal microbiota may play a role in the film digestion, leading to our first discovery of intestinal microbiota-dependent ability of *S. frugiperda* larva to degrade PVC for effective survival. Further, using PVC film as the sole energy and organic carbon source, we successfully isolated the first PVC-degrading *Klebsiella* strain (named EMBL-1) from the intestinal microbiota of the larvae. We explored the biodegradation enzymes and genes, and metabolic products of PVC film and proposed the first metabolic pathway for bacterial PVC biodegradation based on multi-omics approaches (e.g., genomics, transcriptomics and proteomics). This work systematically exemplifies an integrated use of multi-omics approaches universal for comprehensively mining biodegradation strains, genes and enzymes of plastics other than, providing a theoretical basis for future biodegradation-based enzymatic recycling of plastic wastes, as has been recently demonstrated for PET [13-15].

Results

Discovery and verification of *S. frugiperda* larva survival fueled by PVC biodegradation

To verify our discovery that larva of *S. frugiperda* can consume PVC film for effective

survival, laboratory cultivation experiments were specifically designed and conducted in triplicates to compare the key physiological indexes (i.e., survival rate, body weight and body length) and intestinal microbiota between the larva under starvation (Starvation group, n = 15), feeding solely on PVC film (PVC group, n = 50), and feeding normally on corn leaves (Corn group, n = 30), respectively (Figure 1a). Overall, the survival rate of the larvae in the PVC film group (70%) after 5-day cultivation was significantly higher than that of the Starvation group (25%), although lower than that of the Corn group (100%, $P < 0.0001$) (Figure 1b). This result consisted with the significantly increase ($P < 0.0001$) in the body weight (Figure 1c) and length (Figure S1a) of the larva groups in the following order: Starvation < PVC < Corn, indicating that the PVC film can provide energy for and maintain the survival of the larva, although the growth efficiency of this specialized feeding with PVC is lower than that with the normal corn leaves, as also morphologically manifested by the contrasting excreted feces (Figure S1b). Moreover, scanning electron microscopy (SEM) analysis of the PVC fragments recovered from excreted feces showed strong surface damage (Figure S1c). These results together verify our discovery that the gut PVC biodegradation fuels the survival of *S. frugiperda* larva.

Interconnections between intestinal microbiota and PVC biodegradation

The lack of evidence to date for plastic degradation by germ-free invertebrate larvae generally supports that intestinal microbiota are key drivers of plastic degradation[25]. Supporting our hypothesis that gut microbiota is essential for PVC degradation by *S. frugiperda* larva, the survival rate of the larva after 5 days was significantly reduced because of the gentamicin inhibition of intestinal microbiota. Accordingly, the body weight (Figure 1c) and body length (Figure S1a) of PVC-fed larva group treated by gentamicin were significantly lower than that without gentamicin treatment. The microbial biomass in the larvae intestinal microbiota of the Corn group kept stable during the experiment (from 3.90±0.58 to $3.70 \pm 0.63 \times 10^6$ CFUs/piece), while the antibiotic-treated group showed an over 99% reduction in microbial biomass. These results suggest the dependence of PVC biodegradation on the larva intestinal microbiota.

The PVC film degradation by intestinal microbiota can release transformation products, which we hypothesize should create new ecological niches for microbiome selection through cross feeding. Consistent with the assumption, 16S rRNA gene amplicon sequencing analysis of intestinal microbiota showed that PVC film degradation triggered a dramatic compositional shift from Proteobacteria-dominated (87.5±8.0% to 49.5±16.0%) to Firmicutes-abundant (11.9±7.2% to 44.2±17.0%,) microbiota (Figure 1d). Further cross-group comparisons down to the levels of genus and amplicon sequence variants (ASVs) showed that compared with the normal feeding with corn leaves, PVC feeding and biodegradation dramatically increased the alpha diversity of ASVs in the larvae gut microbiota (i.e., Shannon's H index increased from ~0.7 to 2.0 and observed species from 30.6 to 70.0) and largely favored the selective enrichment of an unclassified *Enterococcus* (4.7±3.7% to 37.0±19.8%), *Ochrobactrum* (0.1±0.2% to 3.4±0.7%) and *Klebsiella* (1.4±0.6% to 1.7±0.4%) (Figure 1e), revealing close interconnection between intestinal microbiota and PVC biodegradation.

Identification of strain EBML-1 as the first PVC-degrading *Klebsiella* bacterium

Because the larvae intestinal microbiota of *S. frugiperda* are found to associate with PVC film degradation, we then assumed that the larvae intestine should represent an important reservoir of PVC-degrading strains and promiscuous enzymatic resources. During laboratory screening (Figure S2a), a gram-negative strain (Figure S2b-2c), named as EMBL-1, formed a visible biofilm on the surface of the PVC film after 10-day incubation, causing cracks on the surface of the PVC film (Figure 2a), accompanying a dramatic increase in biomass concentration, i.e., OD600 from 0.20 to 0.94 (Figure 2b). The cracks formed during initial film degradation could facilitate further plastic degradation [30]. The strain was further identified as a new *Klebsiella* bacterium most closely related to *Klebsiella variicola* and *Klebsiella pneumoniae* based on PCR cloning, sequencing, and phylogenetic analysis of 16S rRNA gene (Figure S2d-2e).

The surface of the PVC film already formed a compact biofilm after 90-day incubation, and after removing the biofilm it showed more pits and cracks. The results of contact angle

(Figure S3a) and tensile strength tests of PVC films (Figure 2c) indicated the surface hydrophobicity and tensile strength of PVC film cultured with strain EMBL-1 had changed significantly. These results together suggested that EMBL-1 did damage the physical integrity of the PVC film. During the experiment, the weight loss of the PVC film inoculated with strain EMBL-1 continued to significantly increase over 90 days, and the final average weight loss of the PVC film reached 19.57% (Figure 2d). The results of Advanced Polymer Chromatography (APC, Waters, China) showed that compared with the control group, the molecular weight measures, i.e., Mn and Mw, of the PVC film in EMBL-1 strain group were decreased by 12.4% and 15.0%, respectively (Figure 2e), indicating that the long-chain structure of PVC was depolymerized, producing lower molecular weight fragments. Moreover, Thermogravimetric Analysis (TGA/DSC 3+/1600 HT, Mettler-Toledo, Switzerland) results showed that the T_{max} and T_{onset} of the PVC film in the EMBL-1 group markedly dropped from 316°C to 279°C, and 273°C to 253°C (Figure 2f), respectively, while those metrics showed limited change (T_{max}: 316°C to 310°C and T_{onset}: 273°C to 265°C) in the control group (Figure S3b-3c), suggesting that the strain EMBL-1 had attacked the PVC polymer chain and reduced the chemical stability of the PVC film.

Strain EBML-1 degrades PVC polymer but not plasticizers in the film

Once PVC is demonstrated to be depolymerized by EMBL-1, we further questioned on the diversity of transformation products by the strain. Semiquantitative FTIR MICRO SPECTROMETER (ThermoFisher, Nicolet iS50,China) analysis showed that the infrared spectrum of the surface of the PVC film inoculated with strain EMBL-1 gradually differed from that of the control group over time (Figure 2f), forming new functional groups such as hydroxyl (3500-3300 cm^{-1}) and carbonyl (1550-1650 cm^{-1}), indicating microbial oxidation of the PVC film. Moreover, the peaks intensity of C-Cl stretch[3] (690 cm^{-1}) showed a decreasing trend over time, indicating that the PVC film had a dechlorination reaction under the action of the EMBL-1 strain (Figure 2g). Further, we used GC-MS to quantitatively profile the degradation products of PVC films within 90 days. By comparative inspection of the peaks with significant differences between the EMBL-1 group and control group, we identified six

degradation products (Compounds 1 to 6) (Figure 2h), which were sequentially identified as "2-ethylhexanol" (1), " 2-nonanol adipic acid" (2), "adipic acid, methy octyl ester" (3), "octyl myristate" (4), "dodecanoic acid, isooctyl ester" (5) and "hexadecenoic acid, 1-methylheptyl ester (6)" according to high match score (> 800) of each compound in the NIST library (Figure S4). In contrast, the GC-MS results of the liquid culture did not show inspectable soluble degradation products of PVC (data not shown).

PVC film is composed of both polymer and plasticizers, the latter usually accounting for 40% to 65% in amounts [31]. As expected, three main plastic additives, i.e., dioctyl adipate (DOA), dioctyl terephthalate (DOTP) and erucylamide (Figure S5a-5d and Table S1), were detected in the PVC film by Pyrolysis-GC-MS. Recently, PVC film degradation by enriched anaerobic marine consortia is implicated to start from the degradation of plasticizers and then extend to the degradation and destruction of the PVC polymer[17]. However, whether strain EMBL-1 effectively degrade the main plasticizers detected in the PVC film is unknown. Our triplicate degradation experiments showed no or negligible growth of strain EMBL-1 over 30-day incubation using each plasticizer as a sole energy source (Figure S5f), revealing that the strain could not degrade examined plasticizers under tested conditions.

Genome-level taxonomy and functional profiles of strain EMBL-1

To explore the biodegradation mechanisms of PVC film, the complete genome of strain EMBL-1 was constructed based on co-assembly of short reads (150 bps × 2) and long reads (average 27628 bps) derived from Illumina next-generation sequencing and Nanopore third-generation sequencing, respectively. The result showed that the genome contains a 5,662,860 bps circular chromosome with a G-C content of 57.31% and 5646 open reading frames (ORF) predicted as protein-coding genes. Interactive Tree of Life (iTOL) analysis with whole-genome strain information of *Klebsiella* extracted from the GTDB database (Figure 3a) showed that the genome sequence of strain EMBL-1 showed 99.01% average nucleotide identity (ANI) to it closest relative *Klebsiella variicola* (RS_GCF_000828055.2).

Functional annotation of strain EMBL-1 genome (Figure 3b) was performed by homology-based search against four databases, i.e., NR, GO, KEGG and CAZy. Through KEGG metabolic pathways and network analysis, strain EMBL-1 genome was found to encode 87 genes involving in the metabolism and biodegradation of xenobiotics such as phenylacetic acids and 4-hydroxyphenylacetate. Carbohydrate-Active Enzyme (CAZy) analysis showed the genome encoded 74 glycoside hydrolases (GHs), which should contribute to the strong digestion of lignocellulose-containing biomass (e.g., corn leaf) and absorption capacity of carbohydrates by the larvae of *S. frugiperda*. To facilitate identification of PVC-degrading enzymes, we systematically summarized reported genera and enzymes related to PE, PVC, and PET degradation, and found that strain EMBL-1 had a total of 11 candidate plastic-degrading genes annotated as laccase, alkane monooxygenase, lipase, esterase, peroxidase and carboxylesterase in the KEGG database (Table 1 and Dataset S1), which provide firsthand genomic evidence for our finding that the EMBL-1 strain has the ability to degrade PVC polymer.

Proteomic analysis of PVC degradation by strain EMBL-1

To further verify PVC-degrading enzymatic activities, strain EMBL-1 was regrown with PVC film for 30 days before harvesting cells for intracellular (IN) or extracellular (OTU) protein extraction and expression activity tests. The control group supplied with 1% glucose (glu) was set up to better differentiate metabolic activities of PVC degradation from those of common carbohydrates. The result showed no significant difference in the weight loss of PVC film between the experimental and control groups (Figure 4a), revealing that additional organic carbon source did not improve the PVC degradation efficiency of the strain. Instead, glucose addition only increased the glucose metabolism of the EMBL-1 strain (as evidenced by the following proteomic analysis), which in turn increased the protein (a) and biomass (b) contents of the strain (Figure S6). Moreover, the *in vitro* activity measurement showed PVC-degrading activity of the four protein extracts in the following decreasing order: OUT (13.5%) > OUTglu (10.4%) > IN (5.2%) > INglu (5.0%) (Figure 4b), suggesting that the strain exhibited stronger extracellular than intracellular activities for PVC degradation.

To comprehensively explore metabolic functions underlying PVC degradation by strain EMBL-1, we used LC-MS/MS quantitative proteomics to identify a total of 29 proteins jointly expressed in all four experimental and control groups (Figure 4c), plus 10 proteins jointly expressed in only the two PVC-degrading experimental groups (Dataset S2). By inspecting the differential expression activities of the 39 key proteins between intracellular (IN) and extracellular (OUT) proteomes, we nominated two main categories of proteins associated with PVC degradation. Firstly, we focused on the five most extracellularly (OTU) expressed proteins (Figure 4d), including i) catalase-peroxidase, enolase, and aldehyde dehydrogenase which are most likely responsible for the biodegradation of PVC or depolymerized byproducts, and ii) highly-conserved and universal elongation factor Tu and chaperone protein closely related to microbial translation and protective cell responses to nutrient starvation [46] and heat shock (or other co-defensed harmful conditions such as alcohols, inhibitors of energy metabolism, and heavy metal) [47], respectively. Among them, catalase-peroxidase has strong redox capacity and polymer depolymerization ability, which has also been reported to degrade lignin [48], while enolase with lyase activity and aldehyde dehydrogenase with redox activity on aldehyde groups are considered the putative PVC-degradation proteins. Moreover, we identified another five proteins strongly upregulated (defined here as $Log_2(OUT/IN) \geq 3$) extracellularly (OTU) than intracellularly (IN) (Figure 4d), such as i) dihydroxy-acid dehydratase which can degrade depolymerized products through cleavage of carbon-oxygen bonds (Figure 5), ii) entericidin EcnA/B family protein that can manifest the strain's stress responses to toxic substances (e.g., PVC plasticizers), iii) porin OmpC and outer membrane proteins that can transport some small molecule metabolites, and iv) glutamate synthase large subunit known to involve in ammonia assimilation [49].

Transcriptomic analysis of PVC degradation by strain EMBL-1

To identify enzyme-coding genes associated with PVC film degradation, strain EMBL-1 was first grown on the PVC film in triplicate for 10 days. The weight loss of the film reached about 7% (Figure 4a), accounting for ~ 3 times increase in the strain biomass (OD_{600} increase

from 0.20 to 0.61, Figure 4e). We then performed whole transcriptomic analysis of strain EMBL-1 to screen out 77 out of 96 differentially expressed genes that were significantly (FDR-adjusted $P \leq 0.05$) up-regulated ($\log_2(FC) > 0.5$) or downregulated ($\log_2(FC) < -0.5$) in the PVC film group (see red spheres, Figure 4f), compared with the control group (Dataset S3). Most of the gene expression activities were ascribed to cell growth and death (e.g., elongation factor G, 50S ribosomal protein, and DNA-directed RNA polymerase), followed by transport and catabolism (e.g., MFS transporter, amino acid ABC transporter, and TonB-dependent siderophore receptor) (Figure 4g). Among them, three transcriptionally active genes were also highly represented in the extracellular (OUT) and/or intracellular (IN) proteomes of the strain (see proteins marked in red, Figure 4d). Notably, biodegradation-related genes, such as MBL fold metallo-hydrolase, phenylacetic acid degradation protein, and alkyl hydroperoxide reductase, showed active upregulated expression during the strain's PVC-dependent growth (Figure 4g), revealing their involvement in the PVC degradation.

Multi-omics analysis of PVC degradation pathway by strain EMBL-1

Based on the above multi-omics approaches that incorporates complementary results of genome, transcriptome, proteome and metabolites analyses, strain EMBL-1 was found to encode peroxidase, alkane monooxygenase, laccase, lipase, esterase, and carboxylesterase known to degrade PE and/or PET (Table 1 and Dataset S1) [48,50-53]. These enzymes probably participate in the biodegradation of PVC and its byproducts through a putative pathway which includes the abiotic effect, extracellular enzymatic depolymerization, and the intracellular metabolism of degradation by products (Figure 5).

First, abiotic factors including light and oxygen are widely considered to induce plastic degradation reactions which are initiated via C-C and C-H scission[12,50,52,54]. These factors are most likely to attack and modify PVC polymer via hydroxyl dichlorination and carbonylation, as supported by FTIR diagram (Figure 2f). Then, catalase-peroxidase, known to degrade polymers (e.g., lignin [48]) and found by our study as the 4th most extracellularly expressed protein during the PVC-dependent growth of strain EMBL-1 (Figure 4d), depolymerizes the

modified polymer and converts it into C24~C5 byproducts, as detected by GC-MS/MS analysis (Figure 2g). The multi-omics profiles of other metabolites and degradation enzymes support further stepwise transformation of the long-chain products eventually into shorter ones (importable by strain EMBL-1) through a series of enzymatic reactions catalyzed by laccase[34], monooxygenase[55], dioxygenase, aldehyde dehydrogenase, esterase and dihydroxy-acid dehydratase (Figure 5), among which dioxygenase has been reported to modify and degrade plastic polymer via oxidation of C=C functional group[56]. Although laccase, alkane monooxygenase, dioxygenase, esterase, and lipase are below detection by proteomic analysis of EMBL-1, the strain possesses genes encoding these enzymes (Table 1). It is likely that the binding substrate intermediates of these enzymes are promptly consumed by the strain during PVC-dependent growth. Last but not the least, many genes encoding transport and catabolic proteins are found to be highly represented in the proteomes (d) and transcriptomes (g) of strain EMBL-1 (Figure 4). They are responsible for the transportation of small organic molecules and fatty acids to support intracellular catabolism to support the strain's growth using PVC as sole energy source.

Discussion

Microbial and enzymatic degradation of PVC is a grand global challenge. The limited number of reported PVC-degrading strains are originated from natural and non-host environments such as soil[22], landfills[16], and marine environments[17,21,57], and the underlying biodegradation mechanisms remain unexplored. This study is the first report on PVC-degrading microbiota, strain, enzymes, and mechanism in the larvae intestines of an agriculturally invasive insect. We discover that the larva of *S. frugiperda* can survive solely on the energy derived from PVC degradation by intestinal microbiota, and further successfully isolate and experimentally verify strain EMBL-1 as the first PVC-degrading *Klebsiella* bacterium. The isolate is taxonomically identified to belong to *Klebsiella variicola* (ANI > 99%). This species is regarded as an emerging pathogen of human and other animals[58]. It also harbors plant-associated isolates[59] that can fix nitrogen (to enable its co-existence with plants)[60], and degrade xenobiotic pollutants (e.g., atrazine[61]) and natural polymers (e.g., lignin and

cellulose[62,63]). Most importantly, our discovery of strain EMBL-1 and multi-omics exploration of its PVC biodegradation mechanisms (as further discussed below) provide promising methodology framework and research direction to guide future mechanistic investigations on other PVC-degrading microbes as well as microbial degradation of other plastics.

Besides the discovery of PVC-degrading strain EMBL-1, we also demonstrate an innovative use of multi-omics approaches that complementarily integrate DNA, mRNA, protein, and metabolite analyses for mining and elucidating microbial PVC biodegradation. Our multi-omics results show that PVC polymers and the monomers are effectively utilized by the strain to derive energy for growth. The *Klebsiella* strain is found to utilize combined actions of extracellular and intracellular enzymes to achieve a powerful function in depolymerizing and decomposing monomers of PVC (Figure 5). It also exhibits strong adhesion ability and easily forms a biofilm on the PVC film (Figure 2a), which should largely facilitate its initial destruction of the hydrophobic surface structure of the PVC film by secreting extracellular proteins (as shown by proteome analysis, Figure 4b-4d), thus paving the road for its alternative lifestyle that utilizes PVC for growth[64]. During PVC degradation, 39 proteins are differentially co-expressed by the strain, both extracellularly and intracellularly. They are responsible for either direct degradation of PVC and its depolymerized byproducts, or fundamental metabolism related with genetic or environmental information processing and cellular processes that supports PVC-dependent growth of the strain (Figure 4d). In particular, comparative transcriptomics showed significant upregulation of functional genes involved in the xenobiotics biodegradation and metabolism, cell growth and death, and transport and catabolism during cell growth (Figure 4g), revealing the strain's strong growth ability and molecule transport ability closely linked with extracellular PVC polymerization, further conversion, and eventual intracellular utilization.

Until now, except for the only report on the PVC-degrading activities of fungal lignin peroxidase in *Phanerocheate chrysosporium* [24], little is known about PVC-degrading pathway, genes and enzymes in bacteria. PVC and PE have similar stable chain structures. Oxidase is a class of enzymes with better activity in the PE-degradation enzymes, which can add oxygen

to the long carbon chain [65], then it generates free radicals to form carboxyl groups, alcohols, ketones and aldehydes[31], such as monooxygenase and dioxygenase, etc.[66] The oxidation and cleavage of PE make the polymer more hydrophilic, thus facilitate its contact with other extracellular enzymes (e.g., lipase and esterase) after carboxyl group formation, or endopeptidase of amide group[31]. In addition, Eyheraguibel et al. reported that the Facilitor superfamily (MFS) or a vector containing an ATP binding cassette (ABC) can integrate small molecular weight PE oligomers into cells to achieve PE degradation[67]. The results of genomic, transcriptomic, and proteomic of the EMBL-1 strain together lead to our proposal of its microbial PVC biodegradation pathway (Figure 5), advancing current mechanistic understanding of bacterial PVC degradation.

In summary, we discover and fully verify EMBL-1 as the first PVC-degrading bacterium belong to *Klebsiella*, and further demonstrate the use of multi-omics approaches to achieve first in-depth insights into and systematic knowledge on its degradation mechanism of PVC. In addition, our discovery of the *S. frugiperda* larva survival fueled by microbial biodegradation of polyvinyl chloride lays the foundation for a follow-up study to elucidate the biodegradation mechanisms of PVC polymers in the larvae intestinal microbiota of this agricultural invasive insect and to mine other hidden plastic-degrading strains and enzymatic resources therein.

Conclusion

Microbial biodegradation-based recycling of plastic wastes is promising but extremely challenging, especially when biodegradation mechanism is unknown. In this study, we started from the discovery of *S. frugiperda* larvae feeding on PVC film, explored the intestinal microbiota changes underlying effective larvae survival, and finally isolated PVC-degrading *Klebsiella* strain EMBL-1 which exhibited PVC-dependent growth. Through multi-omics analyses integrating first genome, transcriptome, protein and metabolite-level insights into in the strain, we identified a group of functional genes, enzymes, and metabolic pathways closely related to PVC degradation and proposed a hypothetical biodegradation pathway via

dechlorination, oxidation, depolymerization, and further degradation and mineralization. This study not only opens the gate to PVC-degrading microbiota, microbe, and enzymatic resources in the larvae intestine of an invasive agricultural insect, but also provide a multi-omics framework and an intriguing scenario that inspire future studies to exploit microbial biodegradation of other recalcitrant plastics or xenobiotic contaminants (e.g., pesticides). More importantly, when today microbial and enzymatic biodegradation is demonstrated as an eco-environmentally sustainable and commercially promising biotechnology for the recycling of wasted PET [13-15], our study striving to systematically decipher the mechanisms underlying bacterial PVC biodegradation should lay a foundation for a follow-up study to explore and eventually realize sustainable treatment and recycling of PVC plastics enabled by the iterative cycle of "Design-Build-Test-Learn (DBTL)" for DNA, strain and enzymatic engineering.

Methods

Field sampling and laboratory cultivation experiments

The larva of *S. frugiperda* were collected from corn fields in Jiangcheng County, Yunnan Province, China. They were then cultured indoors by feeding corn leaves (artificial insect breeding room with temperature of 25°C and humidity of 50%-60%). To validate the larva's ability to live on PVC film, 120 pieces of 4_{th}-instar larva with the same growth status were divided into three groups: 1) Control group (starvation, 15 pcs), 2) Corn group (fed with corn leaves, 40 pcs), and 3) PVC group (fed with PVC film, 60 pcs). Other experimental conditions, such as specific breeding conditions and PVC film cleaning, were kept the same, as detailly described in Method S1. The weight and body length of all numbered larva were measured after 24-h starvation and after the 5-day experiment. By the end of the experiment, the excreted feces of each experimental group were collected and the number of survivals was counted. Subsequently, the surviving larva in the Corn group and PVC group were dissected under aseptic conditions to obtain intestinal samples (see details in Method S2), which were labeled and temporarily stored at 4 □ until further operation. The intestinal feces (IF) samples collected from the PVC and Corn groups were labeled as PVC_IF and Corn_IF, respectively.

In addition, residual PVC fragments were also recovered from the excreted feces of the PVC group to characterize their surface morphological changes by Hitachi Field Emission Scanning Electron Microscope (Regulus 8230, Japan).

We hypothesized that intestinal microbiota of *S. frugiperda* larva should play an essential role in digesting PVC film to enable its observed and experimentally verified survival on the PVC film. To test the hypothesis, cultivation experiments were conducted to compare the key physiological indexes (i.e., body length, weight and survival rate) of PVC-fed larva with and without gentamicin pretreatment of their intestinal microbiota. The number of culturable cells in the intestinal microbiota were counted after ending the experiment.

16S rRNA gene amplicon sequencing analysis of intestinal microbiota

Molecular experiments and sequencing Total DNA was extracted from intestinal feces samples collected from the experimental groups using QIAamp Fast DNA Stool Mini Kit following the manufacturer's recommendations (QIAGEN GmbH, Germany). Then, hypervariable V4-V5 regions of prokaryotic 16S rRNA gene were amplified using 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'). The amplicon products of each sample were evenly mixed and sequenced using a paired-end sequencing strategy (PE250) on the Illumina HiSeq2500. More details on the PCR conditions and experimental procedures were available in the Method S3.

Bioinformatics and statistical analyses For the 16S rRNA gene amplicon data analysis, FastQC (*v*0.11.9) and cutadapt (*v*1.18) were first used to check the quality of the raw data and excise double-ended primers (fastaq files). Dada2 (*v*1.14) was then used to cluster the input sequence with default parameter settings and further denoise after importing double-ended data through Quantitative Insight into Microbial Ecology (QIIME2-2020.6) and input-format setting parameters. The next step was to select high-quality areas based on FastQC's report results. The taxonomic classification was conducted using the qiime2 built-in package, the feature-classifier classify-sklearn machine learning method was used for taxonomic

annotation using the SILVA 138 SSU as the reference database. The generated files were imported into R studio Version 1.1.414 (R version 4.0.3), and phyloseq (*v*1.32.0) was used for data statistical analysis and visualization. In addition, survival (*v*3.2.7) and survminer packages were used to calculate and draw the survival curve, while ggplot2 (*v*3.3.3) was used to draw box plots of weight and body length. The ANOVA test was used to check the significance of differences between experimental groups.

Enrichment, isolation, and identification of PVC-degrading strain EMBL-1

To isolate PVC-degrading microbe, intestine materials of ten larva were suspended in 10-mL PBS solution and vortexed for 5 min. Then, the intestinal mucosa was removed from the mixed solution. The remaining suspension was used as a microbial inoculum and transferred to a 250-mL flask containing 0.1-g PVC film and 100-mL MSM liquid medium. Then it was cultivated on a shaker (150 rpm/min) at 30 □, transferred every 15 days. After 45 days, the culture medium was first diluted and then spread onto MSM agar medium plate with PVC film as the sole carbon source to cultivate and enrich for degrading strains. The enrichment degrading strain was further sub-cultured until a pure colony of isolate was obtained. Depending on whether the isolates were grown in the PVC film-amended liquid MSM, the surface changes of the PVC film were inspected by SEM, until a PVC-degrading strain (named EMBL-1) was successfully obtained. To identify the strain, near full-length 16S rRNA gene sequence was PCR amplified using the universal primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). The 16S rRNA gene amplicon sequence obtained from Sanger sequencing were deposited in the National Center for Biotechnology Information (NCBI accession no.: MZ475068) and annotated using the NCBI's online Basic Local Alignment Search Tool (BLAST) in August 2021.

Biodegradation experiments of PVC film by strain EMBL-1

To further explore the ability of strain EMBL-1 to degrade PVC film, three experimental

groups were designed: 1) MSM liquid medium (20 mL) + EMBL-1 strain (OD600=0.2); 2) MSM liquid medium (20 mL) + EMBL-1 strain (OD600=0.2) + PVC film (weighed); 3) MSM liquid medium (20 mL) + PVC film (weighed). Each experimental group was prepared in 27 replicates and immediately started upon inoculation of the strain cells. The following operational procedures were repeated every 10 days: i) take out the PVC films from three replicates of each group, ii) SEM microscopically inspect degradation of the PVC films and weigh them, and iii) replace the half of the MSM liquid medium. The entire experiment was lasted for 90 days, during which PVC films on 10 d and 90 d were collected and stored. The post-treatment methods of PVC film and downstream morphological and physicochemical characterization methods were described with details in the Method S4 and Method S5, respectively.

To ensure the biodegradation ability of strain EMBL-1 to PVC polymer, Gas Chromatography Mass Spectrometry (GC-MS, Trace1300-ISQ7000, ThermoFisher, Singapore) method was established to analyze the composition of additives in the film (Method S6). Further, culture experiments were set up in triplicates and operated for 30 days to check whether the EMBL-1 strain is able to degrade and utilize the three major additives pre-identified from the PVC film (Method S7).

Characterization of PVC film damage and biodegradation products

PVC film damage To validate and follow PVC film biodegradation progress by strain EMBL-1, multiple classic physicochemical methods were co-used to analyze the temporal changes in the morphological, compositional, and other physiochemical properties over 90 days. First, the colonization of the strain was morphologically characterized by SEM after cell fixation. Meanwhile, the degradation efficiency of the strain was directly measured based on the weight loss of the PVC film on a 10-day basis (Method S5). Further, changes in the physical properties of the PVC film were detected by contact angle and tensile strength tests (Method S5). The depolymerization of the plastic materials was also recorded by the change of molecular weight. FTIR MICRO SPECTROMETER (FTIR, ThermoFisher, Nicolet iS50,

China) was used to analyze and detect the changes in the surface chemical composition and functional groups of the PVC film. Thermogravimetric Analysis (TGA/DSC 3+/1600 HT, Mettler-Toledo, Switzerland) was used to compare the initial degradation temperature and the maximum degradation temperature of the PVC film, and obtain the composition and heat stability and thermal decomposition of the PVC film and the possible intermediate products. The Advanced Polymer Chromatography (APC, Waters China) was additionally used to determine the molecular weight of different groups of PVC films. The specific processing steps and testing conditions of FTIR, TGA and APC analyses were recorded in Method S5.

Biodegradation products

To master more biodegradation evidence of the PVC film by EMBL-1 strain, we detected potential biodegradation products of PVC in the PVC films and cultural mediums by using GC-MS. Degraded PVC film weighing 0.3 g were cut into pieces and mixed with 10 mL tetrahydrofuran, the mixture was ultrasonicated for 30 min at room temperature. The extract was concentrated to 0.5 ml by nitrogen blowing treatment and it was mixed with 1 mL N-hexane to obtain some possible products by vortex an ultrasonic for 10 min. The samples filtered using 0.22 um PTFE syringe filter for further step[68]. The liquid culture (150 mL) was centrifuged at 10,000 rpm for 15 min, and the supernatant was extracted with the same volume of n-hexane. The extract was concentrated to 1 mL by nitrogen blowing treatment. The soluble daughter products in the filtrate were analyzed using GC-MS.

GC-MS analysis of PVC-degradation products The sample was injected at an initial temperature of 40 □ (hold 4 min) which was progressively increased at 10°C per minute and held at 280°C (hold 5 min). Moreover, the detector conditions such as transfer line temperature, ion source temperature, ionization mode electron impact and scan time were maintained at 250°C,280°C, 70 eV and 0.3 s respectively.

Whole-genome sequencing analysis of PVC-degrading strain EMBL-1

To further explore the biodegradation mechanism of PVC film by strain EMBL-1 and

discover the responsible PVC-degrading genes or enzymes, TIANamp Bacteria DNA Kit was used to extract the genomic DNA of the strain and the genomic DNAs of the EMBL-1 strain was split into two fetches and sequenced using on both Illumina next-generation sequencing (PE150) and Oxford Nanopore (PromethION). The experimental procedures including sample quality testing, library construction, library quality testing, and library sequencing were performed in accordance with the standard protocol provided by sequencer providers. Bioinformatics analysis includes five major steps: raw data quality control, genome assembly, genome component analysis, functional annotation, and genome visualization. In brief, the quality control of raw short reads from Illumina sequencing and raw long reads from Nanopore sequencing were performed in Fastp 0.19.5 and Mecat 2, respectively. Then, the clean short and long reads were co-assembled to reconstruct compete genome using Unicycle (https://github.com/rrwick/Unicycler) to generate complete. Predictive coding sequence (CDS) was predicted using Glimmer version 3.02[69]). Databases such as KEGG, COG, GO, and CAZy are used for function annotation. In addition, the MUMmer software (*v*3.23) was used to compare the target genome with the reference genome to determine the collinearity between the genomes.

Proteomic analysis of PVC film degradation by strain EMBL-1

To mine enzymatic activities and metabolic pathways related to PVC degradation, biodegradation experiments of PVC film by the EMBL-1 strain were conducted with and without additional supply of 1% (*w*/*v*) glucose (to resolve its proteomic signals from that of PVC film). Then, both intracellular and extracellular proteins were separately extracted from the cells harvested after 30 days based on the acetone precipitation method. The ability of the protein solutions to degrade PVC film was further tested *in vitro* (Method S8). Meanwhile, proteins were resolved with the Thermo Ultimate 3000 integrated nano-HPLC system which is directly interfaced with the Thermo orbitrap fusion lumos mass spectrometer (LC-MS/MS) to explore some related PVC degradation proteins. Details on the experimental setups and procedures, protein extraction methods, protein activity tests, and proteomic analysis were described in Method S9.

Transcriptomic analysis of PVC film degradation by strain EMBL-1

Experimental design To mine genes related to PVC degradation, a degradation experiment of PVC film by the EMBL-1 strain were conducted for 10 d. a) MSM medium + EMBL-1 (OD600=0.2), b) MSM medium +EMBL-1 (OD600=0.2) + PVC film (weighed), three repeats per group. All treatments were cultured in a shaker (30℃, 150 rpm). By the end of the experiment, the liquid culture was centrifuged in 4℃ to harvest the cells. The total RNA of each treatment was extracted, and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies). Sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) and sequenced on an Illumina Hiseq 2500 platform. Sequencing was performed at Beijing Novogene Bioinformatics Technology Co., Ltd. The raw data of transcriptomic of 6 samples from two groups were uploaded in the CNGB database (Sub 022027).

Bioinformatics analysis Raw reads were first filtered using fastp to remove the reads that contain 10 low-quality bases (base quality score less than 20) or length shorter than 36bp. Then, the resulting high-quality (HQ) reads were aligned to *Klebsiella variicola* reference genome (*Klebsiella variicola* strain FH-1) using hisat2. After alignment the read counts for each gene were extracted using htseq-count. The gene expression profiles of triplicate transcriptomes in two groups were compared with PCoA, which inspected one outlier dataset in each group (due to unexpected experimental errors) that was discarded from downstream analysis. Differential expression (DE) at a gene level in our two groups (group a) and group b)) was evaluated using edgeR version 3.30.3, implemented in R 4.0.3. The p-values presented are adjusted for multiple-testing with the procedure of Benjamini and Hochberg to control the type I error rate, and a cut off of $p \leq 0.05$ was used as a threshold to define differential expression. Kraken2 was used to check the contamination in RNA-seq.

Multi-omics-based prediction of degradation pathway of PVC film

To further explore the degradation mechanism of PVC film by strain EMBL-1, multi-omics results from genome, transcriptome, proteome and metabolite analyses were co-used to propose a putative pathway of PVC degradation. In brief, the potential plastic-degrading genes encoded in the EMBL-1 genome (Table 1 and Datasets S1) and the metabolites detected by GC-MS were co-used to build a PVC degradation pathway. Further, 39 proteins jointly expressed during PVC-dependent growth of strain EMBL-1 (Figure 4d and Dataset S2) were aligned against the 96 differentially expressed genes revealed by transcriptomic analysis (Figure 4f and Dataset S3) using NCBI's BLAST+ 2.9.0 at an e-value cut off of 0.01, generating a list of gene expression and proteomic activities ascribed to the PVC-dependent metabolism of the strain.

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Figure and Table Legends

Figure 1 Laboratory cultivation and intestinal microbiota composition of *Spodoptera frugiperda* larva feeding on corn leaf and PVC film. a, laboratory feeding of *S. frugiperda* larva with corn leaf (Corn group) and PVC film (PVC group). b-c, cumulative survival and body weight loss of larva in the Corn group, PVC group, Starvation group (no feeding) and Antibiotic group (gentamicin pretreatment of intestinal microbiota before PVC feeding). d-e, intestinal microbiota composition at the phylum (d) and genus (e) levels.

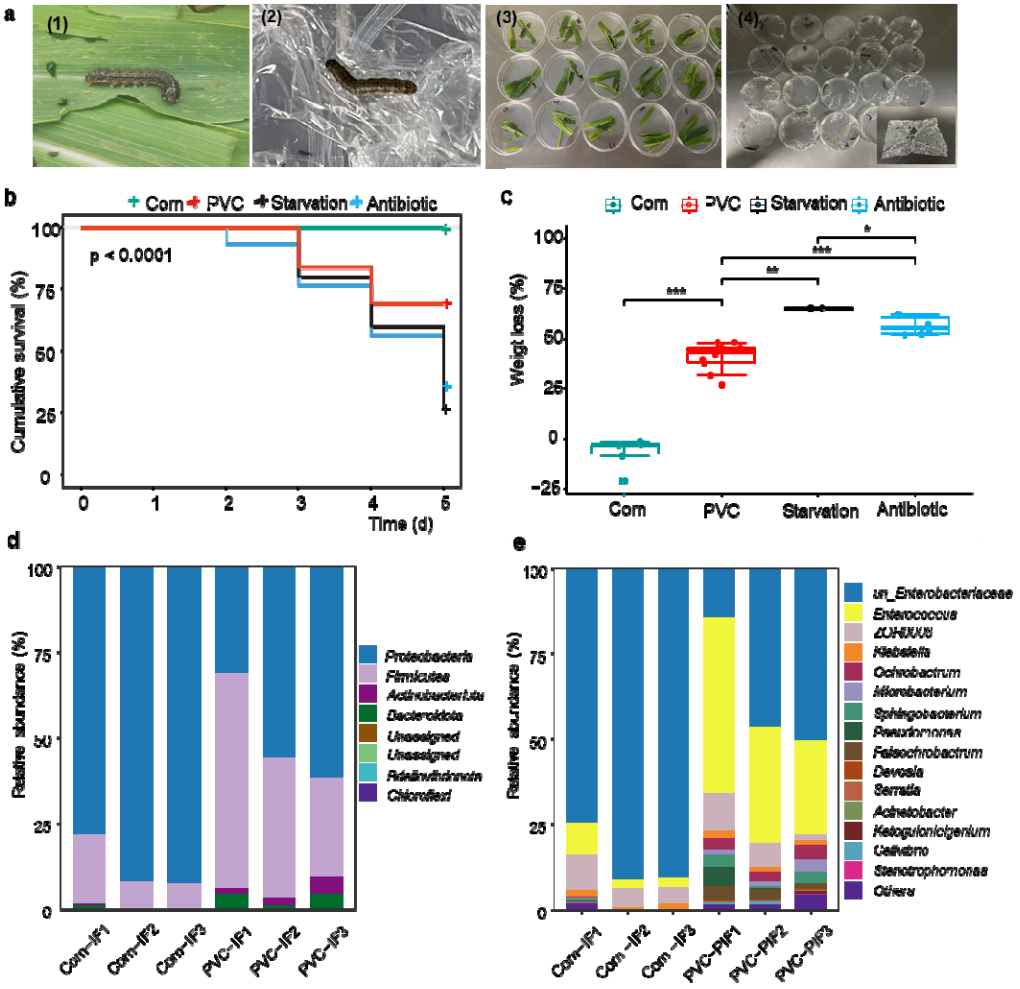

Figure 2 Physicochemical characterization of PVC film degradation by strain EMBL-1. a-b, the SEM image (a) and the growth curve (b) of strain EMBL-1 after co-culturing with PVC film for 10 days. c & e, the tensile strength (c) and the molecular weight (e) of PVC film in the Control group and the EMBL-1 group after 90 days. d, f & g, the temporal change in the weight loss (d) and the thermogravimetric analysis TGA (f) and FTIR (g) diagrams of PVC film in the EMBL-1 group over 90 days. h, the total ion chromatogram (TIC) depicting PVC degradation products.

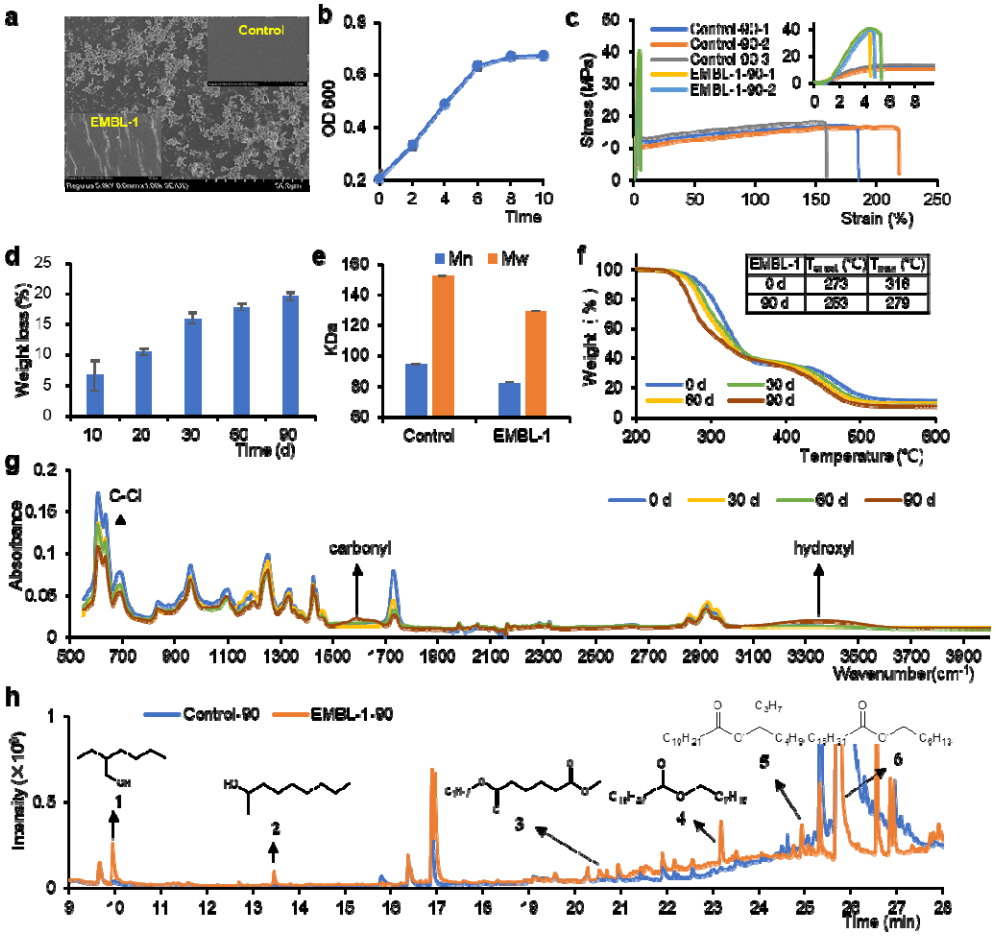

Figure 3 Genome-level phylogeny and functional annotation of strain EMBL-1. a, the phylogenetic tree of EMBL-1 strain built with ITOL. b, the whole genome map of strain EMBL-1. Rings from the outermost to the center: 1) scale marks of the genome, 2) protein-coding genes on the forward strain, 3) protein-coding genes on the reverse strand, 4) tRNA (black) and rRNA (red) genes on the forward strand, 5) tRNA (black) and rRNA (red) genes on the reverse strand, 6) GC content, 7) GC skew. Protein-coding genes are color coded according to their COG categories.

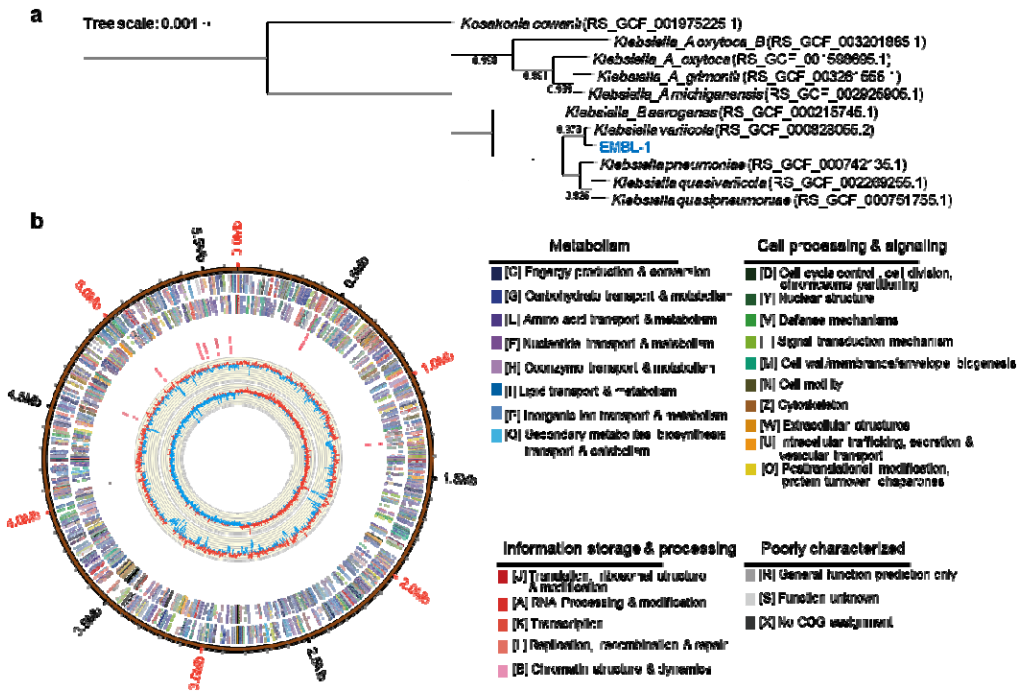

Figure 4 Proteomic and transcriptomic analyses of PVC-degrading metabolic functions of strain EMBL-1. a-b, the weight loss of PVC film under the actions of strain EMBL-1 without and with glucose (glu) as supplementary carbon source (a), and four intracellular (IN) or extracellular (OUT) protein extracts under *in vitro* conditions (b), respectively. c, venn diagram showing shared and unique proteins in the protein extracts; d, 39 proteins involved in intracellular and extracellular metabolism of PVC film. The five most abundant proteins in the OUT extracts were marked in bold, while those were more upregulated in OUT extracts than IN extracts (OUT/IN≥8) were marked in blue. The proteins marked in red were detected in both proteomes and transcriptomes. e, strain EMBL-1 cell culture concentration (as OD_{600}) using PVC film as sole carbon source; f, volcano map showing protein-coding genes (read spheres) significantly upregulated in the PVC group than control group. g, functional annotation of up-regulated genes with those marked in red also detected in the proteome.

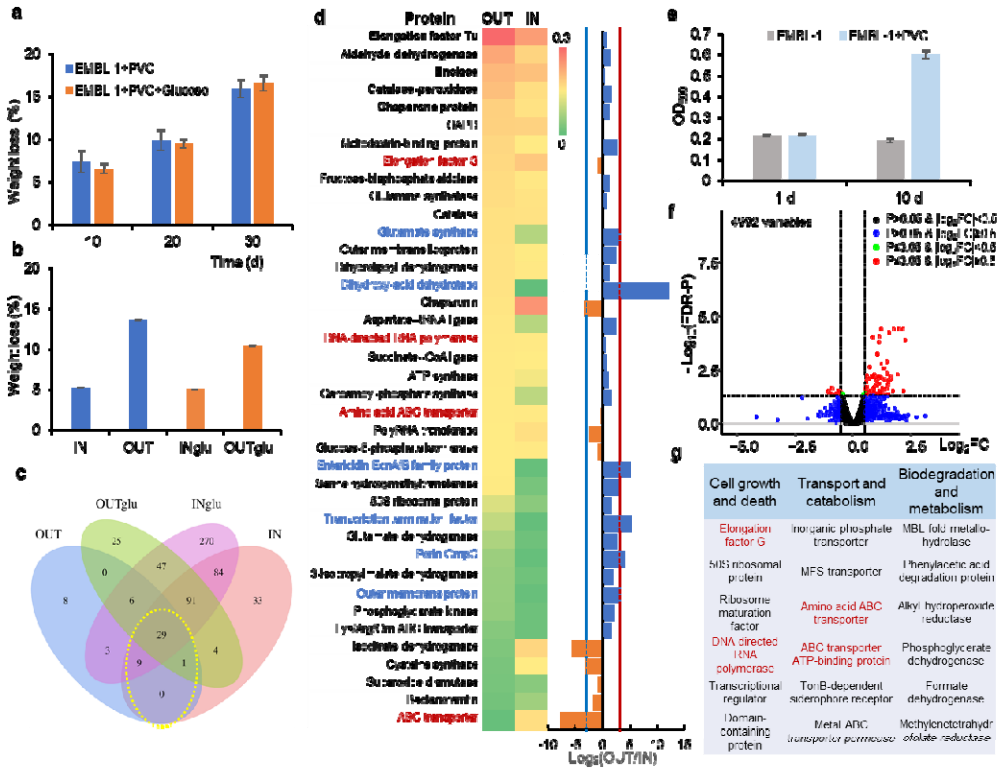

Figure 5 Schematic diagram of the putative PVC degradation pathway by strain EMBL-1. The pathway was proposed based on the multi-omics analyses that integrate genomic, proteomic, transcriptomic and metabolic results of the strain during growth on PVC film.

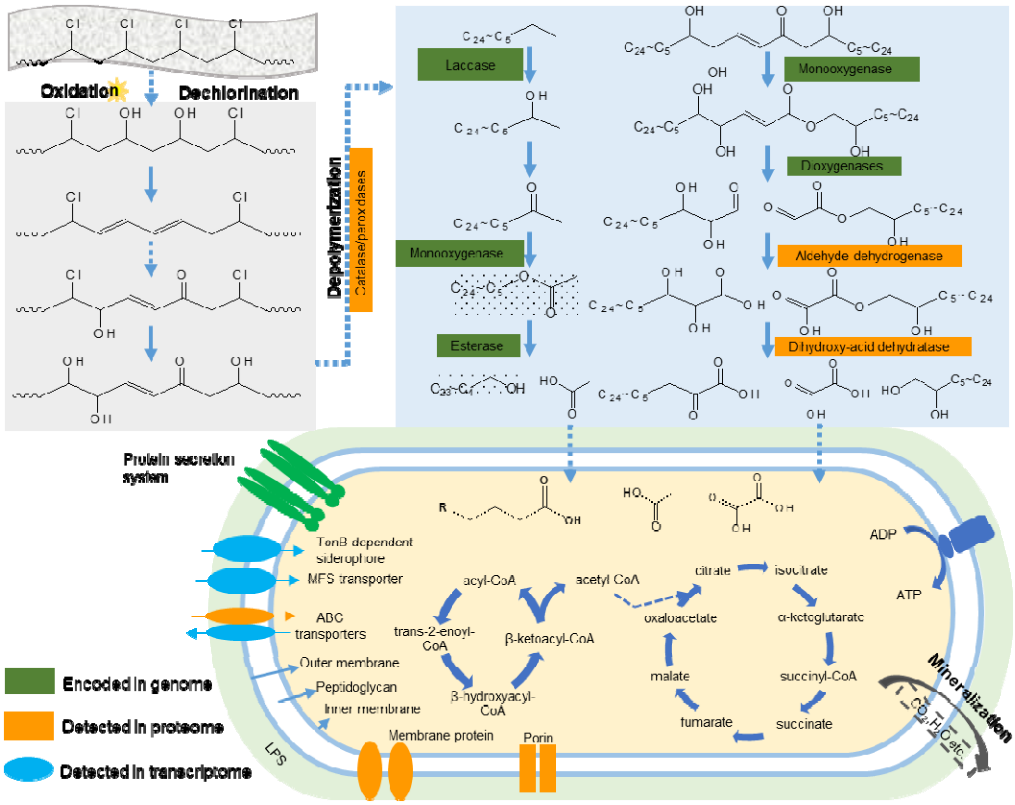

Table 1 Literature review showing limited understanding on degrading microbial strains and degrading enzymes of PVC relative to PE and PET. Strain EMBL-1 is the first experimentally verified PVC-degrading Klebsiella isolate by this study.

	Enzyme name	Source	Functional validation	Protein-coding gene ID in the genome of strain EMBL-1
PE	alkane hydroxylase[1]	*Pseudomonas sp. E4*	Recombinant strains (E coli)	--
	laccases[2,3]	*Rhodococcus ruber*	Crude culture supernatant	orf01799, orf03107
	lignin peroxidases[4]	*Phanerochaete chrysosporium*	Partially purified enzyme used	--
	alkane monooxygenase[5]	*Pseudomonas aeruginosa*	Recombinant strains (E coli)	orf03472
	rubredoxin reductase[5]	*Pseudomonas putida*	Recombinant strains (E coli)	--
	Manganese peroxidase[6,7]	*Phanerochaete chrysosporium*	Partially purified enzyme used	orf03592
	soybean peroxidase[8]	Commercial enzyme	Purified enzyme used	
PET	PETase[9-11]	*Ideonella sakaiensis*	Purified enzyme used	--
	lipases[12]	*Ideonella sakaiensis*	Purified enzyme used	orf03181
	esterase[12,13]	*Ideonella sakaiensis*	Purified enzyme used	orf00935,orf01938,orf03042,orf06956
	cutinases[12,14]	*Ideonella sakaiensis , Humilica insolens, Pseudomonas mendocina, Fusarium solani*	Purified enzyme used	--
	carboxylesterases[15,16]	*Thermobifida fusca, Bacillus licheniformis, Bacillus subtilis , Thermobifida fusca*	Purified enzyme used	orf00475, orf03403
PVC	lignin peroxidase[17]	*Phanerocheate chrysosporium*	Partially purified enzyme used	--