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Transcriptome analysis of an ochratoxin-A biodegrading bacteria

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Abstract: Fighting against and decreasing the effect of mycotoxins is an emerging problem. Among post-harvest methods are physical, chemical, and biological ones. This study is focusing on the biological tools for minimalizing the harmful effect of the ochratoxin-A (OTA) occurring on crops and fodders. The bacteria *Cupriavidus basilensis* ÖR16 strain has very good ability to detoxify ochratoxin-A to phenylalanine and ochratoxin-alfa. In previous studies the degradation rate of the ÖR16 bacteria was over 98%. The whole genome sequencing was also performed by our group in 2012. During this research, the enzymes, and genes responsible for the OTA degradation were characterized via transcriptome analyses. 15 genes were identified, which could play role in the degradation of OTA. Testing and investigating these nominated genes and enzymes could lead for a prepared fodder additive, which can help in the elimination of the negative effects of OTA in the future.

Keywords: OTA, genes, biodegradation

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Introduction

Ochratoxin-A (OTA) is one of the most important mycotoxins, which is produced as toxic metabolites by genera belonging to *Aspergillus* and *Penicillium* species, mainly *Aspergillus niger*, *A. ochraceus*, *A. carbonarius*, and *Penicillium verrucosum* (De Bellis et al., 2015). The chemical structure of OTA is (N-[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl-L-phenylalanine) includes a b-phenylalanine-dihydro isocoumarine derivative, which is very constant at intense temperature and resistant to hydrolysis. The currently used managing methods of raw materials in feed and food production does not reduce the OTA, therefore the toxin persists in the final food and feed products (Ferenczi et al., 2014). OTA is considered to be one of the important contaminants which targets cereal grains and crop products, peanuts, coffee beans, red wine and pork products (Bragulat et al., 2008). The occurrence of OTA can be detected in all regions due to inappropriate storage of human foods and animal derived products and weather conditions. The International Agency for Research on Cancer (IARC) has been classified OTA as group 2B - possible carcinogenic to humans (IARC, 1993). OTA mainly target the kidney in humans as well as to its ability to induce porcine nephropathy (Duarte et al., 2012).

There are several strategies focusing on the reduction or elimination of OTA concentration in human food and animal feeds. The physical adsorbents procedure, besides, has various disadvantages despite that its widely used method, such as nonspecific bindings of some important nutrients (vitamins, minerals, and therapeutic agents), high cost and limited efficiency. According to the literature, the biological methods are the most effective and promising methodology for controlling the contamination of OTA in food and animal feeds, thus diminish the risk on animals and human health. More than ten species of bacteria, including our selected bacteria, have the ability to degrade OTA: *Acinetobacter calcoaceticus* (De Bellis et al., 2015), *Acinetobacter sp.* (Liuzzi et al., 2017), *Alcaligenes faecalis* (Zhang et al., 2017), *Bacillus licheniformis* (Petchkongkaew et al., 2008), *Bacillus amyloliquefaciens* (Chang et al.,

2015), *Brevibacterium spp.* (Rodríguez et al., 2011), *Lactobacillus acidophilus* (Fuchs et al., 2008), *Pediococcus parvulus* (Abrunhosa et al. 2014), *Lactobacillus spp.* (Luz et al., 2017).

In this study the *Cupriavidus basilensis* ÖR16 strain OTA degradation was investigated in the level of RNA expression. The genus *Cupriavidus* was identified in 2004 (Coenye et al., 2003). Members of this genus are Gram-negative, chemoorganotrophic and facultative chemolithotrophic bacteria that can be found in diverse habitats such as soil, root nodules and aquatic environment. The genus *Cupriavidus* belongs to the family Burkholderiaceae and the class β-proteobacteria. The genus consists of nineteen type strains. Remarkable heavy metal tolerance of environmental isolates has been confirmed (Goris et al., 2001). According to the literature in the case of 7 strains different xenobiotic biodegradation was observed. For example chlorinated aromatic chemicals; halo benzoate and nitrophenols were degraded by *Cupraividus necator* CCUG 52238T (Makkar and Casida, 1987) and some xenobiotic genes and enzymes such as benzoate1,2-dioxygenase and chlorocatechol-degradative for this strain were reported (Ogawa and Miyashita, 1999). *Cupriavidus basilensis* RK1 DSM 11853T strain was originally isolated as a 2,6-dichlorophenol degrading strain (Steinle et al., 1998). Other isolates of the species are also capable for degradation of various xenobiotics such as furfural, 5-hydroxymethyl furfural, bisphenol-A, chlorophenols and atrazine (Stamper et al. 2003, Koopman et al., 2010).

In the case of *Cupriavidus basilensis* ÖR16 one study in mice demonstrated the effectiveness of the strain's detoxification ability (Ferenczi et al., 2014). There was efficient degradation for the OTA by the 5th day of the experiment by the strain. The by-product group which arrived from OTA degradation process of the *C. basilensis* ÖR16 was not toxic on the mice kidney cells. In 2012 the total genome project was preceded of the *C. basilensis* ÖR16 strain for making a foundation of future studies (Cserháti et al., 2012).

Materials and Methods

The experiment reagents

Ochratoxin-A mycotoxin (OTA) (Sigma-Aldrich Co., USA). Luria-Bertani medium (LB) (100%: 10 g tryptone, 5 g yeast extract, 9 g sodium-chloride). Minimal buffer medium (3.1 g of K_2HPO_4, 1.7 g of $NaH_2PO_4 \cdot 2H_20$, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 20 mg of EDTA, 4 mg of $ZnSO_4 \cdot 7H_20$, 2 mg of $CaCl_2 \cdot 2H_20$, 10 mg of $FeSO_4 \cdot 7H_20$, 0.4 mg of $Na_2MoO_4 \cdot 2H_20$, 0.4 mg of $CuSO_4 \cdot 5H_2O$, 0.8 mg of $CoCl_2 \cdot 6H_2O$, 2 mg of $MnCI_2 \cdot 2H_2O$).

Bacterial strain and culture conditions in the biodegradation experiment

The strain *Cupriavidus basilensis* ÖR16, was isolated from a Hungarian pristine soil sample. It was deposited in the National Collection of Agricultural and Industrial Microorganisms (NCAIM BO2487). It was grown on LB agar plates and incubated at 28 °C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 °C for 72 h. After resuspension, the optical density (OD600) of the culture was measured at 600 nm (OD 600) (IMPLEN SpectroPhotometer, GENESIS 10S, Thermo Fischer Scientific) and adjusted to 0.6 (OD600 = 0.6) to prepare bacterial inoculum, from this 10 ml was added to 45 ml minimal buffer, which contained 7 mg/l OTA in final concentration.

OTA degradation experiment for getting the RNA to transcriptome analysis

The *C. basilensis* ÖR16 strain was cultured in LB media for growing and getting the exact cell number. The OTA degradation was

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carried out in a minimal buffer, *C. basilensis* ÖR16 was grown on LB agar plates and incubated at 28 °C for 72 h. Single colonies were inoculated into 50 ml liquid LB medium and incubated at 170 rpm at 28 °C for 72 h. Cultures then centrifuged and cleaned from LB media via minimal buffer.

10 ml of the ÖR16 was added to 45 ml minimal buffer and 45 ml 2% fructose (200 ml Demineralized Water, 4.0 g D-Fructose), only with fructose as carbon source, to activate just those genes, which are responsible or act in the presents of OTA or OTA degradation and incubated for 11 hours (till reaching the log phase of ÖR16).

For control *E. coli* TOP10 was used in LB and in minimal buffer media, incubated in the same circumstance as ÖR16. After 11 hours incubation, OD was measured to reach 0.4-0.8 (to be suitable with the requirement of the RNA isolation kit). 7 ppm of OTA was added to the target groups (ÖR16 + OTA). Samples were set in duplicates.

Remaining OTA concentrations in the supernatant and pellet were analysed by High Performance Liquid Chromatography (Szent Istvan University, Advanced Chemistry Department) and by Neogen Accuscan Gold ELISA equipment (Szent Istvan University, Environmental Protection and Safety Department).

RNA extraction, RNA quality test

In order to obtain good quality RNA, 100 ml of the matrix (45 ml of 2% fructose + 45 ml minimal buffer + 10 ml of culture of ÖR16 + 7 mg/l of OTA) was used for the biodegradation experiment for the transcriptome analysis. Samples were centrifuged at 4600 rpm at 4 °C for 30 minutes after reaching the log phase (11 h). Total RNA was extracted from the pellets using the Trizol Plus RNA Purification Kit (Thermo Fisher Scientific Co., USA) at SZIU, Gödöllő, according to the manufacturer's instructions. The quality and the quantity or RIN (RNA integrity number) of the RNA sample were analysed by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Hungary) (Table 1.).

Transcriptome analysis

Whole transcriptome sequencing was performed using TrueSeq RNA Library Preparation Kit v2 (Illumina Co., USA) according to the manufacturer's instructions. Briefly, RNA quality and quantity measurements were performed using RNA ScreenTape and Reagents on TapeStation (all from Agilent Co., USA) and Qubit (Thermo Fisher Scientific Co., USA); only high quality (RIN 7 and 8) total RNA samples were processed. Next, 1 μg of RNA was treated with DNaseI (Thermo Fisher Scientific Co., USA), the ribosomal RNA depleted using RiboZero Magnetic Kit for Gram-negative bacteria (Epicentre Co., USA) and the leftover was ethanol precipitated. The success of rRNA removal was determined by measurement on TapeStation using high-sense RNA ScreenTape and Reagents (Agilent Co., USA).

RNA was purified and fragmented; first strand cDNA synthesis was performed using SuperScript II (Thermo Fisher Scientific Co., USA) followed by second strand cDNA synthesis, end repair, 3′-end adenylation, adapter ligation, and PCR amplification. All the purification steps were performed using AmPureXP Beads (Beckman Coulter Co., USA). Final libraries were quality checked using D1000 ScreenTape and Reagents on TapeStation (Agilent Co., USA). The concentration of each library was determined using the KAPA Library Quantification Kit for Illumina (KAPA Biosystems Co., USA). RNA quality control, RNA preparation and sequencing were performed by Seqomics, Ltd, Hungary on an Illumina NextSeq instrument using the NextSeq 500/550 High Output Kit v2 (300 cycles; Illumina Co., USA) generating ~10 million clusters for each sample.

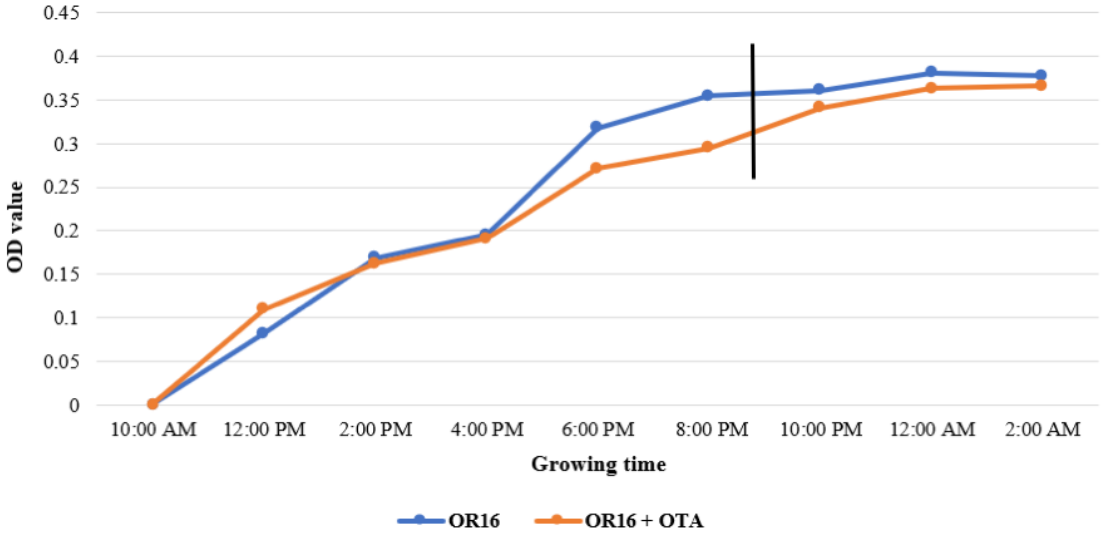

Figure 1. The log phase age of *C. basilensis* ÖR16 in the minimal buffer, black line means the end of the log phase.

Bioinformatics analysis of RNA-sequencing data

After sequencing, paired-end Illumina reads were quality trimmed in CLC Genomics Workbench Tool (v.11.0, Qiagen Bioinformatics Co., Denmark) applying an error probability threshold of 0.01. No ambiguous nucleotide was allowed in trimmed reads. For filtering, reads were mapped on CLC with a length fraction of 0.9 and a sequence identity threshold of 0.95. RNA-Seq analysis package from CLC was then used to map filtered reads on a custom-masked *C. basilensis* ÖR16 genome version. Only those reads were considered that displayed an alignment longer than 80% of the read length while showing at least 95% sequence identity against the reference genome. Next "Total gene read" RNA-Seq count data was imported from CLC into R 3.3.2 for data normalization and differential gene expression analysis. Function "calcNormFactors" from package "edgeR" v.3.12.1 was applied to perform data normalization based on the "trimmed mean of M-values" (TMM) method. Genes displaying at least one -fold gene expression change with an FDR (false discovery rate) value below 0.05 were considered as significant (Seqomics Ltd, Hungary).

Results

Log phase identification of Cupriavidus basilensis ÖR16 strain

Estimating the log phase of *Cupriavidus basilensis* ÖR16 was important, to find the correct time for extracting the best quality RNA from the inoculum. During the pre-experiments, the RNA extraction was according the peak of the OTA degradation process on the 3rd day, but at that time the RNA was already broken, not useful for transcriptome analysis. The 11th hour was the proper time for making the RNA extraction, getting good quality of RNA, which can be used for the analysis (Figure 1).

Results of the RNA isolation

The biodegradation in minimal buffer was stopped at the 11th hour, according the log phase peak for getting good quality RNA. There were two parallel settings from each

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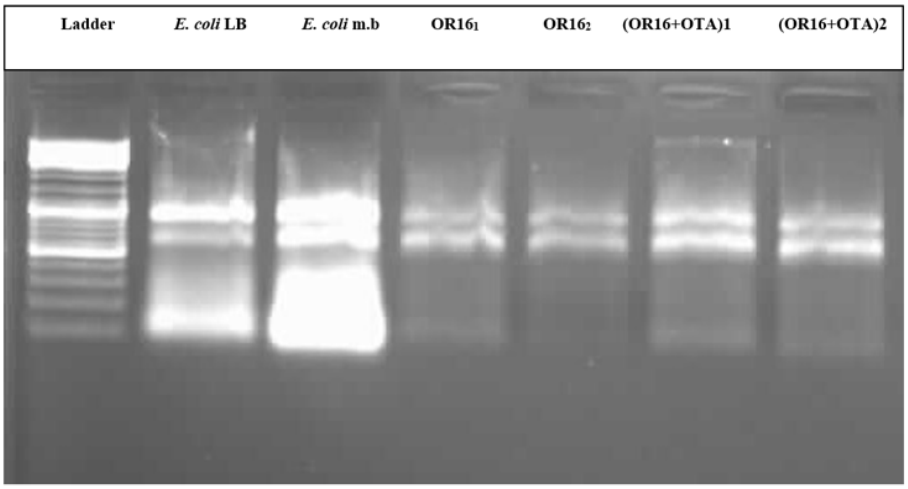

Figure 2. RNA bands of *Cupriavidus basilensis* ÖR16 with and without OTA from the OTA degradation experiment conducted in minimal buffer for transcriptome analysis, sampled after 11 hour of incubation.

Table 1. RNA quality results of the different setting from the OTA degradation matrix in minimal buffer from the Agilent 2200 Technologies (Seqomics Ltd, Mórahalom, Hungary), RIN =RNA integrity number.

Sample description	23S/16S (Area)	Conc. [ng/μl]	RIN
Electronic Ladder	-	84.9	-
OR 16_1	0.8	99.2	8.2
OR 16_2	0.7	57.8	8.2
OTA OR 16_1	1.3	92.3	7.0
OTA OR 16_2	0.5	70.1	7.6

set: OTA+ÖR16 strain; ÖR16 strain; *E.coli* LB (in LB media) and *E. coli* MB (in minimal buffer media). RNA was extracted and the RNA integrity was confirmed in 1% agarose gel electrophoresis (Figure 2). RNA quality was tested by Agilent 2200 Technologies and using TapeStation software (Seqomics Ltd, Mórahalom, Hungary) (Table 1).

Results of the transcriptome analysis

During bioinformatics analysis it turned out that 3500 genes were up regulated in the ÖR16 strain in the presents of OTA. A decision system had to be developed for the identification of the potential genes and enzymes, which could play a role in the OTA degradation. The decision system concluded the following circumstances:

- At least 2-fold expression
- Playing role in any aromatic ring opening
- Should be a protease
- Could be connected to the hypothetical degradation pathways in literature
- There is any literature about the role in degradation of xenobiotics or OTA

The ideal case was when all the circumstances were standing. Of course there were exceptions like the low fold expression genes. Out of the 3500 gene, only 15 genes

could be enrolled into the criteria of the decision system (Table 2).

Discussion

In the literature there are two hypothetical microbiological degradation pathways involved in the OTA degradation as illustrated in Figure 3. The first pathway (a) is the hydrolysis occurred in the amide bond, which links the L-β-phenylalanine molecule to ochratoxin-alpha (OTα) moiety both of them are non-toxic (Abrunhosa et al., 2010). The second pathway (b), the lactone ring hydrolysis can be considered a more hypothetical process in the OTA degradation and detoxification (Bruinink et al., 1998; Abrunhosa et al., 2010).

According to the literature, there are several enzymes which might be involved in the biodegradation of OTA. There are two types of carboxypeptidases among microbes, which may be involved in the OTA-biodegradation (Chang et al., 2015; Liuzzi et al., 2017). The first one is carboxypeptidase-A (CPA), where the "A" refers to aromatic compound, carboxypeptidases that have a stronger preference for those amino acids containing aromatic or branched hydrocarbon chains. The CPY is the second enzyme, where the "Y" refers to yeast origin. A CPY was isolated from *Saccharomyces cerevisiae*, it could degrade 52% of OTA and converted it to OTA-α after five days of incubation with pH 5.6 at 37 °C (Abrunhosa et al., 2010).

There are different enzymes besides the carboxypeptidases, which can degrade OTA. *Aspergilus niger* strains have a few enzymatic tool for OTA degradation: a lipase enzyme can hydrolyse OTA through the amide bond (Stander et al., 2000) and Protease-A have been reported to degrade around 87.3% of 1μg OTA respectively with pH 7.5 in 25 hour-incubation period (Abrunhosa et al., 2010). At last, amidase 2, which is encoded by open reading frame (ORF) of *Aspergillus niger* has the hydrolytic activity to degrade 83% of 50 µg/ mL of OTA (Loi et al., 2017).

Among the 15 identified genes, there are interesting members, which can degrade different chemicals according to the literature. For example, *dienelactone hydrolase* can degrade protoanemonin, which is a toxic metabolite, which may be formed during the degradation of some chloroaromatic compounds, such as polychlorinated biphenyls (Brückman et al. 1998). The *Aromatic Ring hydroxylase* can convert closed-ring structures to non-aromatic cis-diols (Neidle et al., 1991) The *Predicted metal-dependent hydrolase* are acting on carbon – nitrogen bonds and *2-keto-4-pentenoate hydratase* participates in 9 metabolic pathways: phenylalanine metabolism, benzoate degradation via hydroxylation, biphenyl degradation, toluene and xylene degradation, 1,4-dichlorobenzene degradation, fluorene degradation, carbazole degradation, ethylbenzene degradation and styrene degradation (Zhen et al., 2006). In 2017 Luizzi and colleagues cloned and investigated CPA genes of the *Acinetobacter* sp. *neg1* strain responsible for OTA biodegradation. In our transcriptome result the ÖR16 CPAs showed weak fold expression, not matching by the criteria of the decision system. Even so the future investigation of these CPAs is still important.

According to the results of the transcriptome analyses there is a chance to identify the enzymes via cloning and expression. Testing the expressed proteins in OTA degradation system the OTA degraders can be identified. The following genes should be investigated in the future for their OTA-biodegradation and detoxification potential: OR16_12645 coded membrane CPA (penicillin-binding protein), OR16_24100 coded membrane proteins related to metalloendopeptidases, OR16_31869 coded membrane CPA (penicillin-binding proteins PbpC), OR16_31894 coded phenylalanine-4-hydroxylase and OR16_16257 coded aro-

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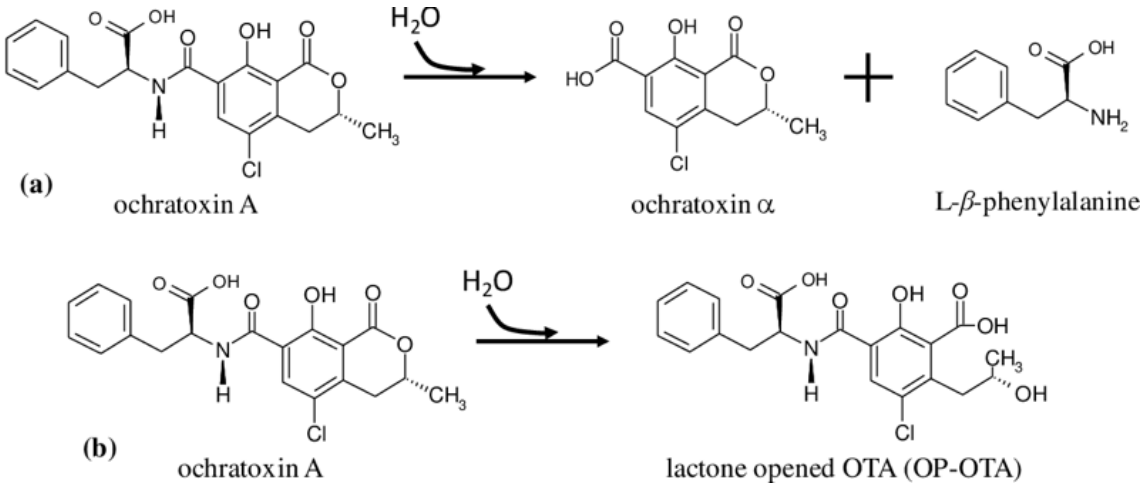

Figure 3. The biodegradation pathways of OTA adapted from Abrunhosa et al., 2010).

matic ring hydroxylase.

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Table 2. Fifteen nominated genes out of 3500 according the transcriptome analyses of the ÖR16 strain in present of OTA in minimal buffer.

Nominated genes	Chromosome	Gene name	Region	Fold expression	Size in bp	Contig number
Aromatic Ring hydroxylase	AHJE01000038	OR16_16257	Complement (106897..108471)	8.1	1575	38
Shikimate 5-dehydrogenase	AHJE01000021	OR16_09609	Complement (8185..8595)	7.6	411	212
Predicted metal-dependent hydrolase	AHJE01000108	OR16_35215	Complement (47971..48636)	6.5	666	108
Dienelactone hydrolase and related enzymes	AHJE01000063	OR16_25377	Complement (58185..59420)	6	1236	63
Amidases related to nicotinamidases	AHJE01000045	OR16_19156	Complement (94947..95645)	4.8	699	45
Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases	AHJE01000019	OR16_08912	Complement (70900..71211)	4.3	312	19
Membrane proteins related to metalloendopeptidases	AHJE01000060	OR16_24100	Complement (30086..30808)	3.8	727	60
2-keto-4-pentenoate hydratase	AHJE01000003	OR16_01040	Complement (14846..17032)	3.2	2187	3
Metal-dependent amidase/aminoacylase/carboxypeptidase	AHJE01000017	OR16_07981	Complement (138451..139662)	2.5	1212	17
Phenylalanine-4-hydroxylase	AHJE01000094	OR16_31894	Complement (17995..18927)	2.3	927	94
Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	AHJE01000003	OR16_01015	Complement (11210..11980)	2.1	771	3
Membrane carboxypeptidase/penicillin-binding protein PbpC	AHJE01000094	OR16_31869	Complement (8236..10443)	1.9	2208	94
Membrane carboxypeptidase (penicillin-binding protein)	AHJE01000029	OR16_12645	Complement (43999..46188)	1.8	2190	29
D-alanyl-D-alanine carboxypeptidase	AHJE01000058	OR16_23878	Complement (72768..73976)	1.3	1209	58
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 4)	AHJE01000027	OR16_12223	Complement (91672..93207)	1.1	1536	27

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