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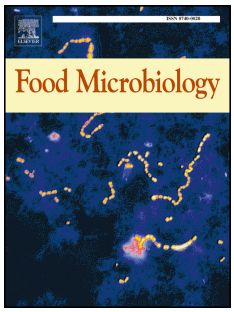


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# Proteomic analysis of the food spoiler *Pseudomonas fluorescens* ITEM 17298 reveals the antibiofilm activity of the pepsin-digested bovine lactoferrin.

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**KEYWORDS**:

food spoilers, pigments, temperature adaptation, antimicrobial peptides, genomics, GeLC-MS/MS.

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**LIST OF ABBREVIATIONS:**

AMPs: antimicrobial peptides

BLF: bovine lactoferrin

BLFPs: bovine lactoferrin-derived peptides

LB: Luria Bertani

HLF: bovine lactoferrin hydrolysate

1D-SDS PAGE: one-dimensional sodium dodecyl sulphate poly acrylamide gel electrophoresis

CFU: colony-forming unit

GeLC-MS/MS: in-gel tryptic digestion followed by liquid liquid chromatography-tandem mass spectrometry

MBIC: minimum biofilm inhibitory concentration

MIC: minimal inhibitory concentration

TE: Tris-Ethylenediaminetetraacetic acid

FDR: protein false discovery rate

GO: Gene Ontology

NCBI: National Center for Biotechnology Information

NSAF: normalized spectrum abundance factor

SpC/L: spectral counts/protein length

KEGG: Kyoto Encyclopedia of Genes and Genomes

ANOVA: Analysis of Variance

QS: *quorum sensing*

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## ABSTRACT

*Pseudomonas fluorescens* is implicated in food spoilage especially under cold storage. Due to its ability to form biofilm *P. fluorescens* resists to common disinfection strategies increasing its persistance especially across fresh food chain. Biofilm formation is promoted by several environmental stimuli, but gene expression and protein changes involved in this lifestyle are poorly investigated in this species.

In this work a comparative proteomic analysis was performed to investigate metabolic pathways of underlying biofilm formation of the blue cheese pigmenting *P. fluorescens* ITEM 17298 after incubation at 15 and 30°C; the same methodology was also applied to reveal the effects of the bovine lactoferrin hydrolysate (HLF) used as antibiofilm agent.

At 15°C biofilm biomass and motility increased, putatively sustained by the induction of regulators (PleD, AlgB, CsrA/RsmA) involved in these phenotypic traits. In addition, for the first time, TycC and GbrS, correlated to indigoidine synthesis (blue pigment), were detected and identified. An increase of virulence factors amounts (leukotoxin and PROKKA_04561) were instead found at 30°C. HLF caused a significant reduction in biofilm biomass; indeed, at 15°C HLF repressed PleD, TycC and GbrS and induced the negative regulators of alginate biosynthesis; at both temperatures induced the cyclic-di-GMP-binding biofilm dispersal mediator (PROKKA_02061).

In conclusion, in this work protein determinats of biofilm formation were revelead in ITEM 17298 under the low temperature; the synthesis of these latter were inhibited by HLF confirming its possible exploitation as antibiofilm agent for biotechnological applications in cold stored foods.

## 1. INTRODUCTION

*Pseudomonas fluorescens* are widespread psychrotrophic Gram-negative bacteria implicated in food spoilage, especially under cold storage, causing the reduction of shelf-life and loss of foodstuffs (Baruzzi et al., 2012; Caldera et al., 2016). *Pseudomonas* spp. contaminations in food chain are mostly derived from water and pipe surfaces where these bacteria grow as biofilms (Srey et al., 2013). During biofilm formation, the transition from planktonic (free living) cells to the attached aggregated form is triggered by *de-novo* expression of transcriptional regulators and key genes responsible for surface-cell and intracellular interactions, metabolic pathways, virulence and resistance mechanisms (Waite et al., 2005). Moreover, the formation of a biofilm is considered a strategy to counteract microbial competition (Oliveira et al., 2015).

Biofilm formation can be influenced and promoted by different factors, such as nutrients, kind of surfaces, stress response (Monds and O'Toole, 2009). Recently, a positive correlation between low temperatures and biofilm production by foodborne *P. fluorescens* was found by Rossi et al. (2018) reporting that the number of biofilm-forming strains at 15°C was higher than that at 30°C. Likewise, Chierici et al. (2016) and Caputo et al. (2015) reported that low temperatures (4 and 15°C) induced pigment production for this bacterial species. In *P. aeruginosa* the role of pigments in biofilm formation (Mavrodi et al., 2013; Park et al., 2014), as well as other genes and factors involved in the transition to aggregated cells and biofilm maintenance has been studied for a long time. By contrast, to the best of our knowledge, no metabolic pathways have been deeply investigated to explain *P. fluorescens* responses to environmental conditions. After all, the human risks correlated with the spread of this species had been underestimated. It is only recently that some studies identified *P. fluorescens* in clinical environment (Dickinson et al., 2014; Nishimura et al., 2017) and correlated them to human diseases (Madi et al., 2010; Nishimura et al., 2017). In addition to this, *P. fluorescens* harbors an enormous pool of antibiotic and biocide resistance genes

92 that can be trasmitted to human and animals via horizontal gene transfer through contaminated
93 foods (Donnarumma et al., 2010; Naghmouchi, et al., 2012). It is clear that these results highlighted
94 the urgent need for further researches to better characterize and counteract the spread of this
95 microorganism.
96 In this regard, several strategies preventing biofilm formation have been investigated and also
97 identified from diverse natural sources, such as plant-derived compounds (Hentzer et al., 2003;
98 Caputo et al., 2018). In this context, the application of natural cationic peptides was reported as a
99 promising antibiofilm strategy against different species (Rajput and Kumar, 2018; Pletzeret al.,
100 2016); however, biophysical properties required for anti-biofilm activity and its mechanism are not
101 fully known.
102 In our previous works, we investigated the antimicrobial efficacy of bovine lactoferrin-derived
103 peptides (BLFPs) in counteracting the growth of foodborne pseudomonads (Quintieri et al., 2012,
104 2013a); the antimicrobial efficacy of these peptides was demonstrated *in vitro*, in cold stored foods
105 and on functionalized coatings (Baruzzi etal., 2015; Quintieri et al., 2013b; 2015); BLFPs were also
106 able to block the blue discoloration of Mozzarella cheese contaminated by the pigmenting *P.*
107 *fluorescens* ITEM 17298 (Caputo et al., 2015). Studies by other authors showed that peptides
108 derived from human-lactoferrin significantly inhibited these phenotypic traits also in other
109 microorganisms (Morici et al., 2016; Xu et al., 2010; Sánchez-Gómez et al., 2015); however, no
110 results revealed how these compounds act.
111 Therefore, in this work we firstly investigated the ability of the pigmenting *P. fluorescens* ITEM
112 17298 to form biofilm under two temperatures (15°C and 30°C); then, we present a comparative
113 proteomic analysis of *P. fluorescens* ITEM 17298 planktonic cells, grown under the assayed
114 temperatures in order to reveal metabolic pathways and physiological changes that characterize
115 strain adaptation to these conditions. In addition to this, the same methodology was applied on the

planktonic cells treated with bovine lactoferrin hydrolysate (HLF) acting as “anti-biofilm agent” at its sub-lethal concentration. The results of this study reveal some protein targets and metabolic pathways involved in the expression of biofilm phenotype at the assayed temperatures and affected by peptide treatment.

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## 2. MATERIAL AND METHODS

### *2.1 Bacterial strain,growth conditions and genome analysis*

The foodborne *Pseudomonas fluorescens* ITEM 17298 (previously named as 84095) from the ISPA-CNR microbial collection (http://server.ispa.cnr.it/ITEM/Collection/; Fanelli et al., 2017) was freshly streaked onto Luria Bertani agar (LB broth: 10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of NaCl per liter added with 16 g/L of technical agar, Sigma-Aldrich, Milan, Italy) and grown overnight at 30°C. After incubation a single colony was inoculated into LB broth (5 mL) and incubated overnight (30°C, 150 rpm) in order to be used for the subsequent experiments.

Draft genome sequence was performed as reported by Fanelli et al. (2017) and it was deposited in Genbank under the accession number NPKB00000000. Contigs were annotated using Prokka pipeline implemented in the Galaxy platform (Seemannet al., 2014). UniProtKB AC/ID identifiers retrieved by PFAM annotator tools were mapped against the PSEUDOCAP database and used to categorize genes in functional classes (Winsor et al., 2010).

### *2.2 Static biofilm formation and motility assays under two temperatures*

Biofilm formation was assayed in 96-well microtiter plates (Corning®, NY, USA) and quantified as described by O'Toole (2011). Briefly, overnight cultures of *P. fluorescens* ITEM 17298 were diluted 1:100 into fresh LB (100 µL; 8 biologicalreplicates for each timepoint sampling) and incubated at 15 and 30°C for 48 hours. Not inoculated LB was used as negative control. At 24 and 48 h, planktonic cell growth was determined by measuring optical density (OD) at 600 nm with a microplate reader (Varioskan Flash, Thermo Fisher, Milan, Italy); then, planktonic cells were carefully removed and wells were washed twice with distilled water; biofilm cells adhering to the bottom and side of each well were stained with crystal violet (CV; 0.1%, w/v). After a second washing step, biofilm-associated crystal violet was solubilized with 30% acetic acid (v/v) and its

144 optical density was measured at 570 nm.

145 Swarming and swimming motility assays were performed in Petri dishes (polystyrene, diameter of
146 50 mm) containing 10 mL of LB (Khan et al., 2009) solidified with 0.5 and 0.3% (w/v) of agar,
147 respectively. Swim and swarm plates were inoculated with 2.5 µL of bacterial broth culture
148 representing approximately 1 $\times 10^8$ CFU/mL (corresponding to 0.3 $OD_{600nm}$; Caputo et al., 2015).
149 The swarming assay was carried out placing this inoculum volume on the agar surface at the center
150 of the plate. Instead, for the swimming assay, the inoculum was placed directly in the center of the
151 thickness of the agar. All plates were incubated at 15 and 30°C. The diameters of the swarming and
152 swimming motility zones were measured at 24, 48 and 72 h of incubation. By contrast, twitching
153 motility was evaluated on LB medium supplemented with 1% agar (w/v) (Deziel et al., 2001).
154 Bacterial cells were inoculated at the bottom of the agar-dish interface. The plates were incubated at
155 15 and 30°C. At selected times (24, 48, 72 h), the agar layer was carefully removed, and the plates
156 were stained with 0.1% of CV (w/v). After washing step biofilm was solubilized and quantified as
157 described above.

158

159 ***2.3 Effect of HLF treatment on motility and biofilm formation: determination of the minimum***
160 ***biofilm inhibitory concentration (MBIC)***

161 Freeze-dried HLF was obtained by hydrolysis of BLF with pepsin according to Quintieri et al.
162 (2012). Then, overnight cultures of *P. fluorescens* ITEM 17298 were inoculated in triplicate at a
163 final concentration of *ca.* 3 log CFU/mL, in sterile Falcon$^{(R)}$ 6 wells polystyrene microplates (BD
164 Biosciences, Erembodegem, Belgium) filled with 5 mL of LB (control) and LB with increasing
165 concentration of HLF (1.5, 3, 6, 12 mg/mL). Microplates were incubated at 15 and 30°C for 48h.
166 Microbial counts were determined at 7, 24, 32 and 48 h by plating serial 10-fold dilutions on LB
167 agar (LB amended with 16 g/L of technical agar). Subsequently, sub-lethal HLF concentrations

which did not cause any significant changes in viable cell count, were assayed for the inhibition of biofilm development in 6 wells polystyrene microplates (O'Toole, 2011). The Minimum Biofilm Inhibitory Concentration (MBIC) was determined as the HLF concentration needed to reduce biofilm biomass by more than 50% (HLF-MBIC). At the end of incubation (48h), planktonic cells, grown in the presence or not of HLF-MBIC were removed from wells and stored at -20°C for proteomic analysis.

The effects of HLF on bacterial motility were also determined in Petri dishes (polystyrene, diameter of 50 mm) containing 10 mL of LB added with increasing HLF concentrations, as above described.

### *2.4 GeLC-MS/MS analysis of proteins from planktonic cells*

Proteome changes were determined in planktonic cells grown in 6 wells polystyrene microplates containing 5 mL of LB added or not with HLF-MBIC for 48 h at 15 and 30°C. Three biological replicates for each sample were performed. After incubation planktonic cells were harvested by centrifugation at 7500 x *g* for 10 min at 4°C. Cell pellets were washed twice with 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and re-suspended in 700 µL of TE buffer containing 1% Triton X-100 (v/v; Sigma-Aldrich, Milan, Italy). Cell suspension was then transferred in a 2 mL screw cap micro tube containing 500 µL of glass beads with a diameter of 0.1 mm (Sigma-Aldrich). Mechanical disruption of the cells was achieved using a FastPrep®-24 homogenizer (MP Biomedicals Life Sciences) for 30 sec at 6.5 m/s (3 cycles). Cell debris and glass beads were separated from the proteins by two centrifugation-steps (20600 x *g*, 30 min at 4°C). Soluble proteins were then precipitated overnight at -20 °C by adding 6 volumes of ice-cold acetone, and re-suspended in 8 M urea/2 M thiourea buffer. After measuring protein concentration by Roti-Nanoquant (Carl Roth, GmbH, Germany), 25 µg of proteins from each sample were separated by 1D-SDS-PAGE using Criterion TGX Precast Gels (BioRad Laboratories, Hercules, CA, USA) for 1

192 h at 150 V. Each lane was cut in ten equidistant pieces and these were subsequently subjected to
193 trypsin in-gel digestion as described by Grube et al. (2015). The peptide mixtures were desalted by
194 Zip-Tip µC18 pipette tips (Millipore, USA).
195 LC-MS/MS analyses were done using an EASY-nLC coupled to a LTQ Orbitrap Velos mass
196 spectrometer (Thermo Fisher Scientific, Waltham, USA). Peptide mixtures were separated by
197 Reverse Phase (RP) chromatography with a non-linear 75 min gradient from 5 to 75% buffer (0.1%
198 acetic acid in acetonitrile) and a flow rate of 300 nL/min. All samples were measured in parallel
199 mode. Survey scans were recorded in the Orbitrap with a resolution of 30,000 in a m/z range from
200 300-2000. The 20 most intense peaks were selected for collision-induced fragmentation in the LTQ,
201 excluding ions with unknown charge state and singly-charged ions. Dynamic exclusion of precursor
202 ions was enabled after 20 sec. Internal calibration was used (lock-mass 445,120025).
203
204 ***2.5 Protein identification***
205 For protein identification, spectra were searched against the annotated protein sequences from the
206 respective *P. fluorescens* ITEM 17298 genome (Fanelli et al., 2017), including reverse sequences
207 and common laboratory contaminants (11,526 entries). Database searches were performed using
208 Sorcerer SEQUEST (Lundgrenet al., 2009; Version v. 27 rev. 11, Thermo Scientific) and Scaffold
209 4.0.5 (Proteome Software, Portland, OR, USA) with the following search parameters: parent ion
210 tolerance: 10 ppm, up to two missed cleavages were allowed and methionine was set as variable
211 modification (López-Mondéjar et al., 2016). Protein quantification was based on the normalized
212 spectrum abundance factor (NSAF; Zybailovet al., 2016). Functional classification of proteins was
213 done using Prophane 2.0 (www.prophane.de) and is based on TIGRFAMs annotations. Voronoi
214 treemaps were generated using Paver (Decodon, Greifswald, Germany; http://www.decodon.com/).
215 An analysis of KEGG pathways was also carried out; KO identifiers were extrapolated by Uniprot

database through Uniprot accession numbers available in genome file.

The raw mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2016) with the dataset identifier PXD010477 (user: reviewer49185@ebi.ac.uk,password: hnkNIhfw).

### *2.6 Experimental design and statistical rationale*

All experiments were conducted in three independent biological replicates; only static biofilm assay was performed with 8 biological replicates. Homegeneity of variances was assessed by Levene's test ($P < 0.05$) before conducting a two-way ANOVA with SPSS 20.0 (IBM, Armonk, NY, USA) to examine the effects of time, temperature levels on planktonic cell optical density, related biofilm biomass, and colony diameters in swarming and swimming assays. The two-way ANOVA was also carried out in order to examine the effects of the sub-lethal HLF concentrations on *P. fluorescens* ITEM 17298 counts and biofilm biomass in relation to incubation time at each incubation temperature. Multicomparison analyses were performed by Tukey's HSD post hoc-test ($P < 0.05$) in order to evaluate differences among the means of each assay.

Proteins detected in two out of three biological replicates were considered for statistical analysis using MeV v4.8.1 (Saeed et al., 2003). Each group of samples was compared by Student's t-test with a *P*-value of 0.01. Only proteins showing at least 2 fold changes in addition to statistical significance were considered for further analysis. So-called 'off/on' proteins needed to be detected or absent in at least two replicates of one experimental condition.

## RESULTS

### *3.1 Genomic features of P. fluorescens* ITEM 17298

The draft genome sequencing resulted in 18 MB of 125 bp paired-end reads and indicated a genomic size of 6,318,747 bp with a GC content of 59%. The evaluation of the raw data quality performed by FastQC software indicated that more that 95% of reads per sample showed an average quality score higher than 30. Reads were assembled into 247 contigs > 200 bp (Fanelli et al., 2017). Analysis of protein domains categorized 48 of the predicted proteins as involved in antibiotic and cationic antimicrobial peptides resistance, 176 in biosynthesis of antibiotics, 4 in putrescine biosynthesis and 56 in virulence.

### *3.2 Phenotypic changes of P. fluorescens* ITEM 17298 *in response to the temperature*

In the first 24h of incubation, the growth of *P. fluorescens* was lower at 15°C than at 30°C (absorbance values $OD_{600nm}$ of 0.44 ± 0.008 and 1.16 ± 0.12, respectively). This difference was leveled out after additional 24 h of incubation (1.9 ± 0.05 and 1.7 ± 0.14, respectively). By contrast, at both sampling times, biofilm biomass registered at 15°C was higher than that determined at 30°C (Figure 1). Two-way ANOVA confirmed the statistically significant interaction between the effects of time and temperature on biofilm formation ($F(2, 30) = 17.420$, $p = 9.531 \times 10^{-6}$). Simple main effects analysis showed that at 15°C the biofilm yields were significantly ($p < 0.000002$) higher than those shown at 30°C throughout the entire incubation period.

At both incubation temperatures *P. fluorescens* was able to undertake all three types of motility (Fig. 2).

The results showed a statistically significant ($P < 0.05$) interaction between experimental parameters (temperature and time of incubation) on the analyzed variable. Simple main effects analysis showed that the values of swarming and swimming motility of the strain grown at 30°C were significantly higher than those found at 15°C ( $p < 2.2 \times 10^{-4}$ ) at each analyzed time point. A time-dependent increase was also found for both parameters.

However, the swimming assay performed at 15°C showed that the strain formed tendrils migrating outwards from the point of bacterial inoculation, with continued branching as the bacteria moved farther from the center (Fig. 2B). As concerns twitching motility, significant differences in biofilm biomass quantified at the bottom of the plate, were registered at 72h of incubation; at this time of sampling, the absorbance values showed increased twitching motility at 15°C (Fig. 2C).

### *3.3 Evaluation of the HLF treatment: MBIC determination and motility assay.*

In order to establish the lowest amount able to inhibit biofilm formation without affecting bacterial growth, HLF was preliminarily assayed at different concentrations by monitoring *P. fluorescens* ITEM 17298 counts at both experimental temperatures. The results showed that no growth was registered using 12 mg/mL of HLF; by contrast, a significant ( $p = 1.109 \times 10^{-7}$) reduction in cell counts by average of 3 log CFU/mL was observed in cultures treated with 6 mg/mL of HLF compared to the untreated control sample at each experimental temperature throughout the incubation period (data not shown). In addition, and concerning the two lowest HLF concentrations (1.5 and 3 mg/mL) together with the untreated control sample, two-way ANOVA results revealed that the growth of the tested strain at each incubation temperature was statistically affected only by time ($P < 0.05$) up to 24 h regardless the applied HLF concentrations (Fig. S1). In fact, no significant differences ($P > 0.0.5$) were found among treated and control samples at each incubation time suggesting that HLF concentrations lesser or equal than 3 mg/mL did not counteract the growth of the strain (Fig. S1). These concentrations were thus selected to perform the subsequent biofilm inhibition assays.

Results from biofilm biomass determination showed that the HLF concentration of 3 mg/mL was able to reduce the biofilm biomass by an average of *ca.* 74% and 54% at 15 and 30°C, respectively, over the entire period of incubation (Fig. 3). By contrast, the lowest HLF concentration (1.5

mg/mL) led to a slight reduction (*ca.* 25%, on average). Based on these results HLF-MBIC value was established at 3 mg/mL.

HLF concentrations (ranging from 12 to 1.5 mg/mL) were also tested in motility assays at both temperatures. The treatment with 12 mg/mL caused a reduction of colony diameter in swarming motility at both temperatures (Fig. S2, panel A); similar results were registered also in twitching assay (Fig. S2, panel C). By contrast, in swimming motility the supplementation of HLF caused significant differences only at 15°C; indeed, under this temperature of incubation, a significant reduction of colony diameter was registered for the culture incubated in the presence of 12 mg/mL.HLF concentrations of 3 mg/mL inhibited tendrils development for 48 h; this effect persisted for 72 h with 6 mg/mL of HLF (Fig. S2, panel B). No changes were instead registered for the lowest HLF concentration (1.5 mg/mL; Fig. S2, panel B).

### *3.4 Effect of incubation temperature on the proteome of P. fluorescens* ITEM 17298 *planktonic cells in the absence of HLF*

The comparative proteomic analyses of cell cultures, grown at 15 and 30°C, allowed to identify 1143 proteins in at least two biological replicates (Fig. 4, Supplementary Table S1). Among these, 871 proteins were identified in both growth conditions; 186 were exclusively induced at 15°C, whilst 86 were found only at 30°C. Moreover, based on normalized spectral counts 298 proteins were found to be differentially expressed ($P < 0.01$, at least two-fold change).

As shown in Figures 4 and Supplementary S3, proteins of all functional classes altered their amount at 15°C in comparison to 30°C. In order to decipher main differences, a deep analysis of metabolic pathways complemented by genome sequencing were performed.

Genomic analysis showed that 6-phophofructokinase was absent in *P. fluorescens* ITEM 17298; thus, it could be supposed that glucose was metabolized through the Entner-Doudoroff pathway.

312 Moreover, at 15°C the levels of the enzymes involved in this metabolic pathway (PROKKA_04144
313 and PROKKA_05654) clearly increased; high amounts of enzymes correlated to ribose metabolism
314 and pentose phosphate pathway (PROKKA_03487, PROKKA_01588, PROKKA_02784) and
315 releasing glyceraldehyde-3-phosphate (G3P) intermediate were also found; then, G3P was
316 converted into pyruvate by the 15°C-induced PROKKA_02238, PROKKA_04749,
317 PROKKA_04632, PROKKA_02035, and PROKKA_02420 (Table 2).
318 The pyruvate produced was putatively metabolized both for aminoacids (valine and leucine,
319 through the up-regulated PROKKA_05528 and PROKKA_05530) and fatty acid biosynthesis, also
320 strongly stimulated at 15°C (Fig. 4, Supplementary Table S1).
321 As regards aminoacid metabolism, at 15°C increased concentrations were observed for enzymes
322 involved in tryptophan and tyrosine (*via* shikimate pathway), serine, glutamate and aspartate
323 biosynthesis (Figs. 4, Tables 2 and Supplementary S1). In addition, glutamate was also generated
324 both by the proline oxidation (PROKKA_03112) and from oxoglutarate (PROKKA_01879), in turn
325 released from enzymes (PROKKA_02649; PROKKA_03275, PROKKA_02416 and
326 PROKKA_04321, Table 2) involved in the synthesis of polyamines (putrescine and spermidine).
327 Signal transduction pathways, regulatory functions as well as transcriptional processes also changed
328 indicating adaptation processes to different temperatures (Figs. 4 and Supplementary S3, and Table
329 S1). Among regulators, positively affected by the low temperature, we found two members of GnrT
330 family (PROKKA_01026 and PROKKA_02008), AlgB (PROKKA_00527), PleD
331 (PROKKA_00530), the hydrogen peroxide-inducible genes activator (PROKKA_03320), the GTP-
332 binding protein TypA/BipA (PROKKA_03008) involved in cold stress response. In addition, at
333 15°C, we also exclusively detected the carbon storage regulator CsrA/RsmA (PROKKA_02793),
334 implicated in changes in energy metabolism.
335 The level of purine and pyrimidine biosynthesis enzymes increased at 15°C (Table 2). Likewise,

protein synthesis was strongly stimulated (Figs. 4, S2 and Table 2). However, 18 proteins involved in proteolysis also changed their levels (e.g. PROKKA_00281, PROKKA_02544, PROKKA_02545, and PROKKA_01048).

Induced proteins were also grouped in detoxification processes (PROKKA_05169) or adaptation to atypical condition, such as oxidative stress (PROKKA_03674, PROKKA_04934).

Cold stress adaptation also led to the increase of enzymes belonging to the lipopolysaccharide (LPS), peptidoglycan and polyketides biosynthetic pathways (Tables 2 and Supplementary Table S1). Among these we found the cellulose synthase 1 (PROKKA_04779), and Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase precursor (PgA, PROKKA_04558) involved in biofilm formation. Conversely, filamentous hemagglutinins (PROKKA_04562; PROKKA_05581) increased at 30°C.

Notably, 127 proteins with significant changes (Supplementary Table S1 and Fig. S3) does not belong to a specific functional classification or was with unknown function. At 15°C, among unclassified proteins we found the gramicidin S synthase 2 (GbrS; PROKKA_02721) and tyrocidine synthase (TycC; PROKKA_02721), sharing 50% identity with IndC of the plant pathogen *Dickeya dadantii* 3937 (Table 2).

The list of unclassified proteins included also the hemolysin transporter protein ShlB precursor (PROKKA_04561) which increased at 30°C. In addition to this, other changed proteins were found involved in pathogenesis: the virulence factor Mce family protein (PROKKA_00520), leukotoxin (PROKKA_02401) and the chitinase ChiD (PROKKA_01272) differently synthesized at the two temperatures (Tables 2 and 3).

### *3.5 Effect of HLF-MBIC on the proteome of P. fluorescens* ITEM 17298 *planktonic cells.*

As depicted in Figures 5 and Supplementary S4 significant changes were registered under HLF

treatment at each temperature of incubation. Most repressed pathways included cellular processes, transport and binding, and fatty acids metabolism. Conversely, HLF treatment led to the increased amount of proteins classified in cell envelope, purines, pyrimidines, nucleosides, nucleotides and protein synthesis, and regulatory functions. A relevant percentage of varied proteins were without a functional classification or of unknown function. The deep analysis of metabolic pathways allowed to reveal main differences induced by HLF treatment.

Under treatment and regardless growth temperature no clear effect was highlighted for enzymes correlated with energy metabolisms, such as Entner-Doudoroff, pentose phosphate pathways and gluconeogenesis (Tables 3 and 4, and Supplementary S2 and S3).

By contrast, the synthesis of amino acids was differently affected depending on the incubation temperature. Indeed, at 15°C HLF-treatment favored the production of glutamate, arginine citrulline (PROKKA_03077, PROKKA_03335, PROKKA_03449, PROKKA_05608), and histidine (PROKKA_02989, PROKKA_02986, PROKKA_02987), whereas it inhibited the synthesis of aromatic aminoacids from chorismate (PROKKA_00905, PROKKA_01326, PROKKA_00904, PROKKA_00895). Conversely the biosynthesis of BCAA and proline increased at 30°C as well as those of arginine and glutamate; sulphurated amino acids and tryptophan (*via* shikimate) synthesis were repressed or completely inhibited.

Regarding the fatty acid metabolism, synthesis and catabolism were differently affected under treatment at the two temperatures of incubation (Supplementary **Table S2**). However, the cyclic-di-GMP-binding biofilm dispersal mediator protein, an 3-oxoacyl-[acyl-carrier-protein] reductase, catalogued in fatty acid biosynthesis was induced in treated samples under both incubation temperatures (PROKKA_02061; Table 3).

Our results suggested that under HLF treatment some modifications in the bacterial cell wall occurred; regardless of the temperature of incubation, most of ABC transporters (e.g involved in

384 proline, histidine BCCA, phosphate and nickel uptake), TonB-dependent receptors lowered their
385 levels or were repressed whilst, some multidrug resistance proteins were exclusively detected in
386 treated samples (Tables 3 and 4). Interestingly, the synthesis of PROKKA_04557 and
387 PROKKA_04558, involved respectively in the synthesis and the transport of the biofilm adhesin
388 polysaccharide poly-beta-1,6-N-acetyl-D-glucosamine (PGA), were blocked.
389 Most proteins involved in regulatory functions and transcription factors underlying physiological
390 behaviour were down regulated by the HLF-MBIC treatment. Among these, at 15°C we found the
391 transcriptional regulators: PROKKA_03320, the nitrogen regulator NtcA (PROKKA_01987),
392 PROKKA_05493, PROKKA_01744, and PleD. The anti-anti-sigma factor (PROKKA_01761),
393 PROKKA_00712, PROKKA_01744, and PROKKA_02036 were instead negatively HLF-affected
394 at 30°C. The synthesis of LutR and Sigma 54 modulation protein (PROKKA_01641), Glucitol
395 operon repressor (PROKKA_00634), and the negative regulators of alginate biosynthesis in biofilm
396 (MucA and MucB: PROKKA_02349, PROKKA_02350) were induced at 15°C; MucA levels
397 increased also under higher temperature, as well as the transcriptional regulator YdfH. At 30°C, the
398 two-component system BarA (PROKKA_02681), involved biofilm formation via the CsrA/CsrB
399 regulation, was repressed.
400 Peptidases, metalloproteases, and oxidoreductases, enzymes involved in repair processes
401 (chaperones), degradation of misfolded proteins were found with increased levels at both
402 temperatures in the treated samples.
403 HLF treatment also affected chemotaxis and flagellar assembly. In particular, at 30°C, proteins
404 involved in transmission of sensory signals from the chemoreceptors to the flagellar motors
405 (PROKKA_01735, PROKKA_01742, PROKKA_05548, and PROKKA_03270) decreased their
406 levels or they were repressed in presence of HLF. Similar response was found at 15°C
407 (PROKKA_05548; PROKKA_02393; PROKKA_04405, PROKKA_05339; PROKKA_01744).

Finally, a high number of uncharacterized or unclassified proteins varied under HLF treatment at each temperature (115 and 103, at 15 and 30°C, respectively; supplementary Table S3). Among these, proteins with lower amount after treatment included proteins involved in the synthesis of indigoidine pigment (PROKKA_02721 and PROKKA_02722).

## 3. DISCUSSION

*P. fluorescens* exhibits a broad temperature adaptability affecting its spoilage activity mainly in cold stored foods. This behavior causes an evident competitive microbial advantage that is also favoured by biofilm formation and the ability to tackle to environmental changes. In this context, the mechanisms underlying physiological and spoilage traits of this microorganisms have been poorly studied. To this purpose, we firstly investigated strain phenotypic traits (biofilm biomass produced and formation of motility appendages) at 15 and 30°C. These temperature values were chosen according both the optimal growth condition of this species (30°C) and the ability of this strain to survive under cold stress, also exhibiting specific behavior, such as pigment production and biofilm formation (15°C; Caputo et al., 2015; Chierici et al., 2016).

In this study *P. fluorescens* ITEM 17298 increased nearly twice the biofilm biomass at 15°C, compared to that produced under higher temperature; in addition, twitching was induced in the same conditions as well as the appearance of tendrils in swimming motility. In accordance with other studies (Chierici et al., 2016; Cabrita et al., 2015), these results suggested that the low temperature favored the coordinated expression of genes and proteins involved in the lifestyle changes of this bacterium. It has been reported that flagellar motility and biofilm formation is affected by high level of c-di-GMP (Muriel et al., 2018), in turn regulated by a diguanylate cyclases with a GGDEF domain (Fazli et al., 2014). In our work the induction of the response regulator PleD with a GGDEF domain was found at 15°C. Thus, the role of PleD in the appearance of appendices

in *P. fluorescens* swimming phenotype could not be excluded. In addition, in ITEM 17298 strain the alginate biosynthesis transcriptional regulator (AlgB) coding gene was found in the genomic locus containing PleD regulator. PleD locus also showed genetic content and organization similar to what reported for *P. aeruginosa* PAO1 and *P. fluorescens* Pf0-1 (http://www.pseudomonas.com/), thus suggesting a similar transcriptional regulation (Stover et al., 2000; Silby et al., 2009). Interestingly, at 15°C the amount of AlgB increased by 2.8 fold compared to that found at 30°C. The hypothesis that the low temperature promoted strain colonization was further supported by the increase of cellulose synthase 1, involved in cellulose biosynthesis.

Cellulose, alginate and poly-N-acetylglucosamine (PGA), extracellular polysaccharides of the bacterial biofilm matrix are likely synthesized and secreted by a conserved mechanism, activates by C-di-GMP levels (Morgan et al., 2015). This mechanism putatively included the carbon storage regulator (CsrA), exclusively detected in ITEM 17298 grown at 15°C. In *E. coli* the complex protein cascade caused by CsrA culminated with the repression of the enzyme required for the synthesis of the adhesin PGA; however, in cold-adapted ITEM 17298 cells, PROKKA_04558, involved in the N-deacetylation needed for surface adhesion, was induced; thus, a complex mechanism based on the interaction among CrsA and the cold-induced RsmE, RsmD, RsmH regulators could not be excluded for this food spoiler (Kulkarni, et al., 2014; Reimmannet al., 2005).

In *P. aeruginosa* CrsA also regulates the expression of LysR-type regulator (Fazli et al., 2014), required for the transcription of the *pqs*ABCDE and *phn*AB operons and the biosynthesis of signaling molecule of (PQS)-mediated *quorum sensing* (QS) (Kulkarni, et al., 2014). Even though, no PQS-related genes were found in the genome of ITEM 17298, high amount of the LysR family transcriptional regulator, the unclassified PhnA (PROKKA_04927) and the enzymes linked to the QS regulation of anthranilate metabolism (PROKKA_03906, PROKKA_04397, PROKKA_03985,

PROKKA_04707; PROKKA_04900) were detected at 15°C.

Protein regulators also included the HTH-type transcriptional regulators, LutR and YdfH, belonging to the GntR family, that were exclusively detected or upregulated at 15°C; these proteins were previously associated to biofilm formation and antibiotic biosynthesis (İrigül-Sönmezet al., 2014). Inspection of the *P. fluorescens* LutR C-terminal domain showed a high homology with FadR-like proteins, a transcription factor that regulates the expression of genes encoding fatty acid biosynthesis; thus, LutR could be implicated in the upregulation of enzymes related to the fatty acid biosynthesis, as registered at 15°C. The modulation of fatty acid composition is expected in order to maintain the proteins function in presence of a altered membrane fluidity under cold incubation.

In addition, in other bacteria *gntR* family transcriptional regulator was reported together with *luxR, luxI* genes as forming a QS regulated operon (Hao et al., 2010; Sakihama, et al., 2012).

Likewise, genomic analysis showed genetic determinants of the QS *las, lux, rhl*, and cyclic-di-GMP systems as well as proteomic results reveal differentially expressed QS-regulated proteins (PROKKA_00428, PROKKA_04707, PROKKA_05356 at 15°C, and PROKKA_04762, PROKKA_01619, PROKKA_00073 at 30°C). This cell-to cell communication could be at the basis of the bacterial spoilage (proteolysis, lipolysis) of some food products (Bai et al., 2011); thus, understanding bacterial QS or the regulated phenotypic traits (biofilm) can help in deciphering population dynamics in cold stored foods and in controlling the growth of undesirable food-related bacteria.

During food storage, spoilage bacteria can release polyamines, considered markers of spoilage degree, and harmful to human health at high concentrations (Shalaby, 1996). In bacterial cells polyamines are organic polycationic molecules playing a crucial role both in modulate biofilm formation (Karatan and Watnick, 2009) and in DNA metabolism (Venancio-Marques, 2014). Interestingly, in ITEM 17298 under low temperature, the arginine metabolism was favored to

produce polyamines and glutamate; the high amount of enzymes involved in polyamine synthesis could be correlated with the induced proteins involved in DNA replication, transcription and translation, and protein synthesis; these latter pathways probably sustained the cold adaptive bacterial response, as previously reported (Iost et al., 2013).

Mechanisms of adaptation to low temperature also involved the iron uptake;indeed, in ITEM 17298 at 15°C only proteins responsible for iron recovery and storage were exclusively detected upregulated; these data suggested that the storing of this nutrient occurred in response to a higher demand for metabolic energy (Dhungana et al., 2003) or to counteract oxidative damage (Ma et al., 1999); this latter condition was sustained by the increase in the levels of proteins responsible for repair and defense mechanisms (PROKKA_03320, PROKKA_03674, PROKKA_05169, PROKKA_03672, PROKKA_04041, PROKKA_03426).

In light of these results, it can be suposed that cellular mechanisms, here for the first time investigated, could be responsible for strain adaptation and persistence under the low temperature, also making it difficult to control their spread in the food chain.

Recently, antimicrobial peptides (AMPs) have shown good antibiofilm activity at the point of being considered as promising therapeutic agents in human infection (Batoni et al., 2016). In this study, the sub-lethal concentration of pepsin digested bovine lactoferrin (HLF; *ca.* 17-fold lower than that used for its antimicrobial activity in cold-stored cheese; Caputo et al., 2015), significantly reduced biofilm formation at the assayed temperatures; swimming and twitching motility were mostly affected at 15°C and tendrils were inhibited in a dose-dependent manner. Thus, in accordance with other studies (Ho et al., 2012), these results sustained the hypothesis that BLF-derived peptides penetrated the cell membrane and affected intracellular targets.

Indeed, proteomic analysis revealed that the PleD regulator was absent under HLF treatment at 15°C, whilst the negative AlgB regulators (MucA and MucB) were induced in the treated samples

504 at both temperatures; the synthesis of these transcriptional factors inhibited the conversion from a
505 non-mucoid to a mucoid phenotype of *P. fluorescens* and *P. aeruginosa* (Ahmed, 2007). Likewise,
506 HLF treatment inhibited the 30°C-induced adhesion factor filamentous hemagglutinin in
507 accordance with previous results (Di Biase, et al., 2004). The cyclic-di-GMP-binding biofilm
508 dispersal mediator protein (PROKKA_02061) was also detected in all treated samples; as reported
509 for other species, this protein reduced c-di-GMP causing biofilm dispersal (Ma et al., 2011).
510 The low temperature favored the synthesis of proteins involved in the response to oxidative stress in
511 the untreated samples. Interestingly, these protein (PROKKA_03320, PROKKA_03674,
512 PROKKA_04041) were repressed in all treated-HLF samples. Similar effects were registered for
513 the TonB-dependent receptors and PvdQ, involved in the synthesis of the siderophore pyoverdine
514 and degradation of QS molecules (3-oxo-$C_{12}$-homoserine lactone); by contrast, proteins responsible
515 for iron storage were up-regulated. Recently, modulators of oxidative stress response and iron
516 acquisition have been proposed as a suitable strategy to reduce *P. aeruginosa* virulence and
517 persistence (Sethupathy et al. 2016; Wurst, et al., 2014) and therefore could be also exploited to
518 counteract *P. fluorescens* spread in the refrigerated food and environments.
519 In our previous study, we reported the finding of the pigment leuco-indigoidine in cold- stored
520 mozzarella cheese inoculated with ITEM 17298; this compound is the reduced form of the reactive
521 blue pigment indigoidine (Caputo et al., 2015). Our research demonstrated that the treatment with
522 HLF inhibited pigment release throughout the entire refrigerated period. Although Andreani et al.
523 (2015) suggested that the blue pigment was not indigoidine, in this study PROKKA_02721 and
524 PROKKA_02722 proteins correlated with the synthesis of this pigment were found at 15°C. These
525 proteins are non-ribosomal peptide synthetases subdivided into domains responsible for substrate
526 adenylation, thiolation and condensation that culminated in pigment biosynthesis. A conserved core
527 motif (DNFFELGGHSL) similar to that found in the thiolation (T) domain of *S. chromofuscus*

528 (DDFFELGGNSL; Yu et al., 2013) was also shown. In this last species the stability of the modular
529 indigoidine synthase Sc-IndC and the product indigoidine was attributed to the optimal temperature
530 of 18°C. In addition, in *D. dadantii* 3937, IndC synthesizes the blue pigment indigoidine together
531 with the pantetheine-phosphate adenylyltransferase (CoaD; Reverchon et al. 2002), also cold-
532 induced in our target strain (PROKKA_00418). Even though the biosynthetic pathway of
533 indigoidine has been proposed for other microorganisms, the specific role of this pigment and its
534 regulation, including *luxRI* quorum sensing regulators, have been just suggested (Yu et al., 2013;
535 Cude et al., 2015).

536

537 **5 CONCLUSION**

538 For the first time a proteome profile of a blue pigmenting and biofilm forming *P. fluorescens* was
539 presented in this work. Proteomic results were consistent with microbiological ones favoring at the
540 low temperature both the highest biofilm biomass and an increase of different protein determinants
541 related with biofilm formation, cell motility, and adhesion. Conversely, at 30°C some virulence
542 factors such as leukotoxin were detected, highlighting the need to further investigate this strain.
543 Notably, a high percentage of proteins with relevant changes in amount was without a specific
544 functional classification or of unknown function; among these latter, for the first time, we identified
545 enzymes related to the blue pigment indigoidine that was produced at low temperature.
546 The work also proposes a strategy based on the application of milk protein-derived peptides to
547 hamper biofilm formation by this food spoiler. Indeed, by using a sublethal HLF concentration,
548 proteins involved in biofilm regulation and exopolysaccharide synthesis were repressed at 15°C,
549 whilst the cyclic-di-GMP-binding biofilm dispersal mediator was instead detected at both
550 temperatures. In addition, HLF treatment inhibited indigoidine synthesis related enzymes involved
551 in blue cheese discoloration and reduction of shelf life of cold stored cheeses.

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## FOOTNOTES

Author contributions: LQ, KR designed research; FF, VCL performed genomic analysis; LQ, DZ performed proteomic analysis; DB and CH performed mass spectrometry analyses; LQ and LC performed and analyzed microbiological data; LQ and DZ analyzed proteomic data; LQ and DZ wrote the paper; LQ, DZ, AFL, LC, FF and KR revised the manuscript.

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781 # FIGURE LEGENDS

782 **Fig. 1**. Biofilm biomass produced by *P. fluorescens* ITEM 17298, grown at two temperatures (15°C
783 and 30°C) measured at 24 and 48 hours. Values were determined by measuring the absorbance of
784 crystal violet (CV) at 570 nm (O'Toole, 2011). Bars represent the average ± the standard deviation
785 (n = 8). Similar values (*P*> 0.05) of CV are annoted with the same superscript letters according to
786 post hoc HSD Tukey's test.

787

788 **Fig. 2**. Motility assays performed at 15 and 30°C for 72h**.** Swarming (**A**) and swimming motility
789 (**B**) of *P. fluorescens* ITEM 17298 in LB agar. Values represent the mean diameter of
790 corresponding motility zones. Twitching motility (**C**) of *P. fluorescens* ITEM 17298 in LB; these
791 values were determined by measuring the absorbance of crystal violet (CV) at 570 nm. Bars
792 represent the average ± the standard deviation (n = 3). Similar values ( $P > 0.05$) for each motility
793 parameter are annoted with the same superscript letters according to HSD Tukey's test.

794

795 **Fig.3**. Biofilm biomass produced by *P. fluorescens* ITEM 17298 treated with 1.5 and 3 mg/mL of
796 HLF at 15°C and 30°C for 48 hours. Values were determined by measuring the absorbance of
797 Crystal Violet at 570 nm. Bars represent the average ± the standard deviation (n = 3). Different
798 superscript letters represent values statistically different ($P < 0.05$) within the same incubation
799 temperature and according to to HSD Tukey's test.

800

801 **Fig. 4.** Voronoi treemap visualization of *P. fluorescens* ITEM 17298 protein pattern cultivated in
802 LB medium at 15°C and 30°C. Proteins are depicted as single cells and grouped according to their
803 functional classification. Classification was achieved using *Prophane 2.0* software and is based on
804 TIGRFAMs. Large treemap: Proteins with higher amounts at 30°C are shown in blue; proteins with

higher amounts at 15°C are shown in red. Grey cells represent proteins that were not identified in the respective condition. Small treemap represents higher level of functional classification (main role), whereas large treemap shows subrole level.

**Fig. 5**. Voronoi treemap visualization of *P. fluorescens* protein pattern after treatment with HLF. Proteins are depicted as single cells and grouped according to their functional classification. Classification was achieved using *Prophane 2.0* software and is based on TIGRFAMs. Depicted is the classification level *main role*. Large treemaps: Proteins with higher amounts in treated cells are shown in red; proteins with higher amounts in untreated cells are shown in blue. Grey cells represent proteins that were not identified in the respective condition (panel **A**: 15°C; panel **B**: 30°C). Small treemap represents higher level of functional classification (main role), whereas large treemaps show subrole level.

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819 **Supplementary Figures**

820 **Fig. S1.** Bacterial counts (expressed as Log CFU/mL) of *P. fluorescens* ITEM 17298 in LB broth in
821 the absence (UT) or presence (T) of HLF (1.5 or 3 mg/mL) at 15 and 30°C up to 48 h of incubation.
822 Values represent the average ± the standard deviation (n = 3). No statistically differences ($P > 0.05$)
823 were found among values at each incubation time for both assayed temperatures according to HSD
824 Tukey's test.

825

826 **Fig. S2**. Effect of HLF on *P. fluorescens* ITEM 17298 motility evaluated 15 and 30°C for
827 72h**.**Swarming (**A**) and swimming motility (**B**) and twitching motility (**C**) in LB agar supplemented
828 or not with HLF (1.5 -12 mg/mL). Bars represent the average ± the standard deviation (n = 3) of
829 swimming and swarming motility, whereas twitching motility values were measured by absorbance
830 units of crystal violet (CV) at 570 nm. Different superscript letters represent significant different
831 values ($P < 0.05$) according to HSD Tukey's test. Photograph in panel B showed the effect of 3
832 mg/mL and 6mg/mL of HLF on tendrils formation at 15°C for for 48 h and 72 h.

833

834 **Fig. S3.** Impact of growth temperature on proteome pattern of *P. fluorescens*. The percentage of
835 proteins with significantly changed amount at 15°C compared to 30°C in relation to all identified
836 proteins is depicted.

837

838 **Fig. S4**. Impact of HLF on proteome pattern of *P. fluorescens*. The percentage of proteins with
839 significantly changed amount after HFL treatment at 15°C and 30°C is depicted in relation to all
840 identified proteins.

841 **Table 1.**Proteins induced at 15 °C in comparison to 30 °C.

| Identifier | Function[1] | Fold change[2] UT15/UT30 |
|---|---|---|
| ***Amino acid biosynthesis*** | | |
| PROKKA_00444 | Pyrroline-5-carboxylate reductase | on |
| PROKKA_00484 | 5,10-methylenetetrahydrofolate reductase | on |
| PROKKA_01073 | Histidinol-phosphate aminotransferase | on |
| PROKKA_02680 | D-lactate dehydrogenase | on |
| PROKKA_02989 | 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase | on |
| PROKKA_03376 | Diaminopimelate epimerase | on |
| PROKKA_03460 | Gamma-glutamylputrescine synthetase PuuA | on |
| PROKKA_03906 | Phospho-2-dehydro-3-deoxyheptonate aldolase | on |
| PROKKA_03985 | 3-phosphoshikimate 1-carboxyvinyltransferase | on |
| PROKKA_04062 | 2-hydroxy-3-keto-5-methylthiopentenyl-1-phosphate phosphatase | on |
| PROKKA_04321 | Carboxynorspermidine synthase | on |
| PROKKA_04397 | Shikimate 5-dehydrogenase-like protein | on |
| PROKKA_05528 | 3-isopropylmalate dehydratase small subunit 1 | on |
| PROKKA_05530 | 3-isopropylmalate dehydrogenase | on |
| PROKKA_04034 | Argininosuccinate synthase | 10.90 |
| PROKKA_04490 | Phosphoserine aminotransferase | 7.72 |
| PROKKA_00579 | Tryptophan synthase alpha chain | 4.27 |
| PROKKA_01890 | Aspartokinase | 4.22 |
| PROKKA_04932 | Glutamate 5-kinase | 3.63 |
| PROKKA_05654 | 2-dehydro-3-deoxy-phosphogluconate aldolase | 3.12 |
| PROKKA_04081 | Glutamate synthase [NADPH] large chain | 3.00 |
| PROKKA_04990 | Homoserine dehydrogenase | 2.98 |
| PROKKA_05356 | Phospho-2-dehydro-3-deoxyheptonate aldolase, Tyr-sensitive | 2.70 |
| PROKKA_03160 | Phosphoserine phosphatase | 2.63 |
| PROKKA_00905 | Tryptophan synthase alpha chain | 2.43 |
| PROKKA_04707 | Anthranilate synthase component 1 | 2.11 |
| ***Biosynthesis of cofactors, prosthetic groups, and carriers*** | | |
| PROKKA_00136 | ATPase family associated with various cellular activities (AAA) | on |
| PROKKA_00405 | Dihydrofolate reductase type 3 | on |
| PROKKA_01482 | NifU-like protein | on |
| PROKKA_01888 | Low specificity L-threonine aldolase | on |
| PROKKA_02355 | tRNA-modifying protein YgfZ | on |
| PROKKA_02541 | Bifunctional protein FolD protein | on |
| PROKKA_03079 | Uroporphyrinogen decarboxylase | on |
| PROKKA_03546 | Riboflavin biosynthesis protein RibBA | on |

| | | |
|---|---|---|
| PROKKA_03551 | GTP cyclohydrolase-2 | on |
| PROKKA_03555 | 1-deoxy-D-xylulose-5-phosphate synthase | on |
| PROKKA_03878 | 4-hydroxy-3-methylbut-2-enyl diphosphate reductase | on |
| PROKKA_04184 | Sulfite reductase [ferredoxin] | on |
| PROKKA_04334 | DNA nickase | on |
| PROKKA_00427 | Protease 3 precursor | 9.68 |
| PROKKA_03618 | Glutamate-1-semialdehyde 2,1-aminomutase | 5.13 |
| PROKKA_00418 | Phosphopantetheine adenylyltransferase | 4.41 |
| PROKKA_01483 | Cysteine desulfurase | 2.65 |
| PROKKA_03437 | 2-octaprenylphenol hydroxylase | 2.19 |
| PROKKA_04743 | 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase | 2.02 |
| | | |
| ***Cell envelope*** | | |
| PROKKA_01070 | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | on |
| PROKKA_01121 | D-alanine--D-alanine ligase | on |
| PROKKA_01908 | Membrane bound L-sorbosone dehydrogenase | on |
| PROKKA_03735 | Phosphoglucosamine mutase | on |
| PROKKA_04504 | UDP-glucose 4-epimerase | on |
| PROKKA_04558 | Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase precursor | on |
| PROKKA_04759 | UDP-3-O-acylglucosamine N-acyltransferase | on |
| PROKKA_04779 | Cellulose synthase 1 | on |
| PROKKA_01120 | UDP-N-acetylmuramate--L-alanine ligase | 13.04 |
| PROKKA_02849 | Glucose-1-phosphate thymidylyltransferase 2 | 5.84 |
| PROKKA_01125 | UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase | 5.39 |
| PROKKA_03355 | Alanine racemase | 5.34 |
| PROKKA_03138 | Bifunctional protein HldE | 4.69 |
| PROKKA_01039 | Rod shape-determining protein MreB | 3.34 |
| PROKKA_02911 | D-methionine-binding lipoprotein MetQ precursor | 3.12 |
| PROKKA_01060 | Lipopolysaccharide export system protein LptA precursor | 2.82 |
| PROKKA_02851 | dTDP-4-dehydrorhamnose reductase | 2.69 |
| PROKKA_00635 | Glutamine--fructose-6-phosphate aminotransferase [isomerizing] | 2.28 |
| PROKKA_02850 | dTDP-4-dehydrorhamnose 3,5-epimerase | 2.27 |
| PROKKA_03604 | Membrane-bound lytic murein transglycosylase B precursor | 2.17 |
| PROKKA_01117 | UDP-N-acetylmuramoylalanine--D-glutamate ligase | 2.12 |
| PROKKA_03033 | Glucans biosynthesis protein G precursor | 2.05 |
| PROKKA_01062 | 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC | 2.04 |
| | | |
| ***Cellular processes*** | | |
| PROKKA_01123 | Cell division protein FtsA | on |

| | | |
|---|---|---|
| PROKKA_01309 | General stress protein 14 | on |
| PROKKA_01755 | Flagellar motor switch protein FliN | on |
| PROKKA_02616 | NADP-dependent glyceraldehyde-3-phosphate dehydrogenase | on |
| PROKKA_03063 | cell division protein FtsN | on |
| PROKKA_03674 | Catalase precursor | on |
| PROKKA_04028 | Septum site-determining protein MinD | on |
| PROKKA_04126 | Septum site-determining protein MinD | on |
| PROKKA_04934 | Hydroperoxy fatty acid reductase gpx2 | on |
| PROKKA_01124 | Cell division protein FtsZ | 6.09 |
| PROKKA_03612 | Magnesium and cobalt efflux protein CorC | 4.78 |
| PROKKA_01272 | Chitinase D precursor | 4.58 |
| PROKKA_03681 | Serine hydroxymethyltransferase 2 | 3.05 |
| PROKKA_05169 | Alkyl hydroperoxide reductase subunit C | 2.43 |
| PROKKA_03387 | Sensory transduction protein LytR | 2.02 |
| ***Central intermediary metabolism*** | | |
| PROKKA_02416 | S-adenosylmethionine synthase | on |
| PROKKA_02855 | 3'(2'),5'-bisphosphate nucleotidase CysQ | on |
| PROKKA_03275 | Biosynthetic arginine decarboxylase | on |
| PROKKA_05514 | (R)-stereoselective amidase | on |
| PROKKA_04344 | Peptidyl-prolyl cis-trans isomerase C | 4.40 |
| PROKKA_04698 | Chaperone SurA precursor | 2.24 |
| ***DNA metabolism*** | | |
| PROKKA_01794 | ATP-dependent DNA helicase RecQ | on |
| PROKKA_03707 | Type I restriction enzyme EcoKI M protein | on |
| PROKKA_04026 | DNA mismatch repair protein MutS | on |
| PROKKA_04576 | Type I restriction enzyme EcoR124II R protein | on |
| PROKKA_04579 | putative type I restriction enzymeP M protein | on |
| PROKKA_05553 | 8-oxo-dGTP diphosphatase | on |
| PROKKA_00259 | Recombination-associated protein RdgC | 3.39 |
| PROKKA_00612 | DNA polymerase III subunit beta | 3.20 |
| PROKKA_03903 | ATP-dependent RNA helicase DeaD | 2.85 |
| PROKKA_04737 | Cold shock protein CapB | 2.81 |
| PROKKA_01628 | DNA-binding protein HRm | 2.70 |
| PROKKA_00481 | ATP-dependent RNA helicase RhlE | 2.43 |
| PROKKA_04882 | recombinase A | 2.37 |
| PROKKA_02589 | Exodeoxyribonuclease III | 2.33 |
| PROKKA_00610 | DNA gyrase subunit B | 2.06 |
| ***Energy metabolism*** | | |
| PROKKA_00293 | 3-oxoadipate enol-lactonase 2 | on |
| PROKKA_00606 | D-glycero-beta-D-manno-heptose-1,7-bisphosphate 7-phosphatase | on |

| | | |
|---|---|---|
| PROKKA_00980 | Tagatose-6-phosphate kinase | on |
| PROKKA_01297 | Fructose dehydrogenase large subunit | on |
| PROKKA_01306 | NADH oxidase | on |
| PROKKA_01396 | Levansucrase | on |
| PROKKA_01588 | Transketolase | on |
| PROKKA_01831 | 8-oxoguanine deaminase | on |
| PROKKA_01879 | Succinylornithine transaminase/acetylornithine aminotransferase | on |
| PROKKA_01882 | Arginine N-succinyltransferase subunit beta | on |
| PROKKA_02631 | NADH-quinone oxidoreductase subunit I | on |
| PROKKA_02783 | D-ribose pyranase | on |
| PROKKA_02784 | Ribokinase | on |
| PROKKA_02793 | hypothetical protein | on |
| PROKKA_03016 | 8-oxoguanine deaminase | on |
| PROKKA_03017 | HTH-type transcriptional repressor YvoA | on |
| PROKKA_03440 | Aminomethyltransferase | on |
| PROKKA_03487 | Ribose-5-phosphate isomerase A | on |
| PROKKA_03492 | Phosphoenolpyruvate-protein phosphotransferase | on |
| PROKKA_03672 | Ferredoxin--NADP reductase | on |
| PROKKA_04041 | Glutaredoxin-4 | on |
| PROKKA_04187 | Pyruvate dehydrogenase [ubiquinone] | on |
| PROKKA_04385 | Disulfide-bond oxidoreductase YfcG | on |
| PROKKA_04900 | Tryptophan 2,3-dioxygenase | on |
| PROKKA_04909 | Catechol 1,2-dioxygenase | on |
| PROKKA_04958 | Trehalose-6-phosphate hydrolase | on |
| PROKKA_04960 | Phosphoenolpyruvate-protein phosphotransferase | on |
| PROKKA_05120 | hypothetical protein | on |
| PROKKA_02420 | Phosphoglycerate kinase | 12.53 |
| PROKKA_02649 | Isocitrate dehydrogenase [NADP] | 7.27 |
| PROKKA_02092 | Electron transfer flavoprotein subunit alpha | 6.46 |
| PROKKA_02093 | Electron transfer flavoprotein subunit beta | 5.29 |
| PROKKA_02634 | NADH-quinone oxidoreductase subunit F | 4.50 |
| PROKKA_02836 | UDP-glucose 4-epimerase | 4.17 |
| PROKKA_05658 | (R)-specific enoyl-CoA hydratase | 4.01 |
| PROKKA_02035 | Phosphoenolpyruvate synthase | 3.78 |
| PROKKA_03112 | Bifunctional protein PutA | 3.55 |
| PROKKA_04632 | Pyruvate kinase II | 3.48 |
| PROKKA_02633 | NADH-quinone oxidoreductase subunit G | 3.40 |
| PROKKA_05607 | Carbamate kinase 2 | 3.28 |
| PROKKA_02423 | Fructose-bisphosphate aldolase | 3.23 |
| PROKKA_01450 | Non-heme chloroperoxidase | 3.05 |
| PROKKA_04938 | Glucosaminate ammonia-lyase | 2.88 |
| PROKKA_01079 | Malate:quinone oxidoreductase | 2.54 |
| PROKKA_04460 | Malate:quinone oxidoreductase | 2.51 |

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|---|---|---|
| PROKKA_04749 | Enolase | 2.44 |
| PROKKA_02238 | Glyceraldehyde-3-phosphate dehydrogenase | 2.40 |
| PROKKA_05696 | Glycerophosphoryl diester phosphodiesterase | 2.29 |
| PROKKA_02510 | 2-oxoisovalerate dehydrogenase subunit alpha | 2.05 |
| PROKKA_01884 | N-succinylarginine dihydrolase | 2.03 |
| | | |
| ***Fatty acid and phospholipid metabolism*** | | |
| PROKKA_01305 | Cyclopentanol dehydrogenase | on |
| PROKKA_01776 | 3-oxoacyl-[acyl-carrier-protein] synthase 3 | on |
| PROKKA_01961 | 3-oxoacyl-[acyl-carrier-protein] synthase 3 | on |
| PROKKA_05080 | Malonyl CoA-acyl carrier protein transacylase | on |
| PROKKA_05542 | Glucose 1-dehydrogenase 1 | on |
| PROKKA_02045 | Aconitate hydratase 1 | 7.64 |
| PROKKA_00314 | 3-oxoacyl-[acyl-carrier-protein] synthase 1 | 6.39 |
| PROKKA_05079 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | 4.36 |
| PROKKA_04753 | Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha | 4.19 |
| PROKKA_03267 | Biotin carboxylase | 3.91 |
| PROKKA_05078 | Acyl carrier protein | 2.58 |
| PROKKA_00502 | Acyl-CoA dehydrogenase | 2.27 |
| PROKKA_05221 | Acyl-CoA dehydrogenase | 2.16 |
| | | |
| ***No classification*** | | |
| PROKKA_02270 | Kinase A inhibitor | on |
| PROKKA_00123 | hypothetical protein | on |
| PROKKA_00238 | Protein of unknown function, DUF | on |
| PROKKA_00297 | Putative NADP-dependent oxidoreductase YfmJ | on |
| PROKKA_00557 | hypothetical protein | on |
| PROKKA_00573 | Limonene 1,2-monooxygenase | on |
| PROKKA_00623 | putative chromosome-partitioning protein ParB | on |
| PROKKA_00653 | hypothetical protein | on |
| PROKKA_00705 | Putative glucose-6-phosphate 1-epimerase | on |
| PROKKA_00846 | hypothetical protein | on |
| PROKKA_00859 | Decarbamoylnovobiocin carbamoyltransferase | on |
| PROKKA_00901 | putative oxidoreductase YjmC | on |
| PROKKA_00963 | hypothetical protein | on |
| PROKKA_01044 | hypothetical protein | on |
| PROKKA_01047 | hypothetical protein | on |
| PROKKA_01240 | putative rhodanese-related sulfurtransferase | on |
| PROKKA_01298 | hypothetical protein | on |
| PROKKA_01299 | hypothetical protein | on |
| PROKKA_01517 | hypothetical protein | on |
| PROKKA_01700 | hypothetical protein | on |
| PROKKA_01921 | hypothetical protein | on |

| | | |
|---|---|---|
| PROKKA_02407 | hypothetical protein | on |
| PROKKA_02569 | putative chaperone protein EcpD | on |
| PROKKA_02721 | Gramicidin S synthase 2 | on |
| PROKKA_02722 | Tyrocidine synthase 3 | on |
| PROKKA_02831 | hypothetical protein | on |
| PROKKA_02980 | hypothetical protein | on |
| PROKKA_03018 | hypothetical protein | on |
| PROKKA_03311 | PP2C-family Ser/Thr phosphatase | on |
| PROKKA_03324 | NAD dependent epimerase/dehydratase family protein | on |
| PROKKA_03372 | hypothetical protein | on |
| PROKKA_03375 | hypothetical protein | on |
| PROKKA_03383 | Phosphate-starvation-inducible E | on |
| PROKKA_03426 | Quinone oxidoreductase 1 | on |
| PROKKA_03433 | hypothetical protein | on |
| PROKKA_03476 | hypothetical protein | on |
| PROKKA_03713 | hypothetical protein | on |
| PROKKA_03836 | Ribosome-binding ATPase YchF | on |
| PROKKA_04331 | hypothetical protein | on |
| PROKKA_04378 | SCP-2 sterol transfer family protein | on |
| PROKKA_04402 | putative protease YhbU precursor | on |
| PROKKA_04444 | D-inositol-3-phosphate glycosyltransferase | on |
| PROKKA_04575 | hypothetical protein | on |
| PROKKA_04639 | Flavin reductase like domain protein | on |
| PROKKA_04700 | Phosphotransferase enzyme family protein | on |
| PROKKA_04719 | Nitronate monooxygenase | on |
| PROKKA_04786 | hypothetical protein | on |
| PROKKA_04927 | hypothetical protein | on |
| PROKKA_04989 | Thiol:disulfide interchange protein DsbC precursor | on |
| PROKKA_05083 | hypothetical protein | on |
| PROKKA_05381 | Xylose isomerase-like TIM barrel | on |
| PROKKA_05473 | hypothetical protein | on |
| PROKKA_05492 | hypothetical protein | on |
| PROKKA_05549 | AAA-like domain protein | on |
| PROKKA_05752 | hypothetical protein | on |
| PROKKA_04144 | 6-phosphogluconolactonase | 5.46 |
| PROKKA_02387 | hypothetical protein | 4.25 |
| PROKKA_01604 | Quinoprotein glucose dehydrogenase | 3.82 |
| PROKKA_04468 | Putative reductase/y4119/YP_4011 | 3.71 |
| PROKKA_01655 | hypothetical protein | 3.22 |
| PROKKA_03582 | hypothetical protein | 3.10 |
| PROKKA_01238 | hypothetical protein | 3.00 |
| PROKKA_01727 | hypothetical protein | 2.48 |
| PROKKA_04711 | Indole-3-glycerol phosphate synthase | 2.44 |
| PROKKA_00243 | Carboxymuconolactone decarboxylase family protein | 2.40 |

| | | |
|---|---|---|
| PROKKA_02865 | Tetratricopeptide repeat protein | 2.34 |
| PROKKA_02240 | hypothetical protein | 2.11 |
| PROKKA_02623 | hypothetical protein | 0.27 |
| | | |
| ***Protein fate*** | | |
| PROKKA_00428 | Signal recognition particle receptor FtsY | on |
| PROKKA_01479 | Chaperone protein HscA | on |
| PROKKA_02233 | Lipoprotein-releasing system transmembrane protein LolE | on |
| PROKKA_02543 | ATP-dependent Clp protease proteolytic subunit | on |
| PROKKA_02658 | Outer-membrane lipoprotein carrier protein precursor | on |
| PROKKA_03402 | Enhancing lycopene biosynthesis protein 2 | on |
| PROKKA_04332 | General stress protein 18 | on |
| PROKKA_05350 | Lipoprotein-releasing system ATP-binding protein LolD | on |
| PROKKA_05353 | putative L,D-transpeptidase YbiS precursor | on |
| PROKKA_02545 | Lon protease | 8.10 |
| PROKKA_00281 | Beta-Ala-Xaa dipeptidase | 6.64 |
| PROKKA_01089 | Metalloprotease LoiP precursor | 3.91 |
| PROKKA_04925 | FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase | 3.73 |
| PROKKA_01048 | peptidase PmbA | 3.49 |
| PROKKA_04593 | Tail-specific protease precursor | 3.18 |
| PROKKA_00118 | putative lipoprotein YiaD precursor | 3.01 |
| PROKKA_01952 | Peptidyl-prolyl cis-trans isomerase A precursor | 2.64 |
| PROKKA_02544 | ATP-dependent Clp protease ATP-binding subunit ClpX | 2.21 |
| PROKKA_03302 | hypothetical protein | 2.15 |
| | | |
| ***Protein synthesis*** | | |
| PROKKA_00031 | 30S ribosomal protein S17 | on |
| PROKKA_00040 | 50S ribosomal protein L30 | on |
| PROKKA_00425 | Ribosomal RNA small subunit methyltransferase D | on |
| PROKKA_00477 | Ribosomal RNA small subunit methyltransferase E | on |
| PROKKA_00620 | tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG | on |
| PROKKA_01468 | GTPase Der | on |
| PROKKA_01493 | S-adenosylmethionine:tRNA ribosyltransferase-isomerase | on |
| PROKKA_02042 | Alanine--tRNA ligase | on |
| PROKKA_02645 | tRNA-specific 2-thiouridylase MnmA | on |
| PROKKA_02772 | Phenylalanine--tRNA ligase alpha subunit | on |
| PROKKA_03142 | tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein MnmC | on |
| PROKKA_03188 | 23S rRNA (guanosine-2'-O-)-methyltransferase RlmB | on |
| PROKKA_03266 | Ribosomal protein L11 methyltransferase | on |
| PROKKA_03741 | tRNA pseudouridine synthase B | on |
| PROKKA_03845 | Peptide chain release factor 1 | on |

| | | |
|---|---|---|
| PROKKA_04684 | 30S ribosomal protein S21 | on |
| PROKKA_05082 | 50S ribosomal protein L32 | on |
| PROKKA_04931 | GTPase Obg | 23.36 |
| PROKKA_00012 | 50S ribosomal protein L1 | 15.56 |
| PROKKA_00022 | 50S ribosomal protein L3 | 7.04 |
| PROKKA_00036 | 30S ribosomal protein S8 | 5.94 |
| PROKKA_00013 | 50S ribosomal protein L10 | 5.16 |
| PROKKA_05366 | Elongation factor P | 4.58 |
| PROKKA_00045 | 30S ribosomal protein S4 | 4.31 |
| PROKKA_03838 | 50S ribosomal protein L25 | 4.21 |
| PROKKA_03189 | 30S ribosomal protein S6 | 4.07 |
| PROKKA_00037 | 50S ribosomal protein L6 | 4.01 |
| PROKKA_00039 | 30S ribosomal protein S5 | 3.70 |
| PROKKA_02661 | Serine--tRNA ligase | 3.37 |
| PROKKA_00025 | 50S ribosomal protein L2 | 3.33 |
| PROKKA_01019 | 50S ribosomal protein L13 | 3.12 |
| PROKKA_03739 | Translation initiation factor IF-2 | 3.02 |
| PROKKA_02775 | Translation initiation factor IF-3 | 2.99 |
| PROKKA_00044 | 30S ribosomal protein S11 | 2.96 |
| PROKKA_00033 | 50S ribosomal protein L24 | 2.82 |
| PROKKA_01111 | Ribosomal RNA small subunit methyltransferase H | 2.68 |
| PROKKA_04982 | 30S ribosomal protein S16 | 2.64 |
| PROKKA_00011 | 50S ribosomal protein L11 | 2.35 |
| PROKKA_04769 | 30S ribosomal protein S2 | 2.35 |
| PROKKA_00026 | 30S ribosomal protein S19 | 2.32 |
| PROKKA_03192 | 50S ribosomal protein L9 | 2.30 |
| PROKKA_00041 | 50S ribosomal protein L15 | 2.28 |
| PROKKA_01626 | Valine--tRNA ligase | 2.19 |
| PROKKA_00023 | 50S ribosomal protein L4 | 2.08 |
| PROKKA_01492 | Queuine tRNA-ribosyltransferase | 2.07 |
| | | |
| ***Purines, pyrimidines, nucleosides, and nucleotides*** | | |
| PROKKA_01833 | Adenine deaminase | on |
| PROKKA_01914 | Phosphoribosylformylglycinamidine cyclo-ligase | on |
| PROKKA_03338 | Deoxyuridine 5'-triphosphate nucleotidohydrolase | on |
| PROKKA_03359 | Xanthine phosphoribosyltransferase | on |
| PROKKA_03727 | Carbamoyl-phosphate synthase small chain | 14.90 |
| PROKKA_03728 | Carbamoyl-phosphate synthase large chain | 9.94 |
| PROKKA_04963 | Phosphoribosylformylglycinamidine synthase | 6.91 |
| PROKKA_04494 | Cytidylate kinase | 6.20 |
| PROKKA_04751 | CTP synthase | 4.92 |
| PROKKA_01075 | Uracil phosphoribosyltransferase | 4.06 |
| PROKKA_04767 | Uridylate kinase | 3.27 |
| PROKKA_00702 | N5-carboxyaminoimidazole ribonucleotide synthase | 3.11 |

| | | |
|---|---|---|
| PROKKA_03839 | Ribose-phosphate pyrophosphokinase | 3.10 |
| PROKKA_02340 | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 2.69 |
| PROKKA_05100 | Ribonucleoside-diphosphate reductase 1 subunit alpha | 2.40 |
| PROKKA_04977 | Phosphoribosylglycinamide formyltransferase 2 | 2.27 |
| PROKKA_02643 | Adenylosuccinate lyase | 2.26 |
| PROKKA_03181 | Adenylosuccinate synthetase | 2.17 |
| | | |
| ***Regulatory functions*** | | |
| PROKKA_00530 | Response regulator PleD | on |
| PROKKA_01026 | HTH-type transcriptional regulator LutR | on |
| PROKKA_03320 | Hydrogen peroxide-inducible genes activator | on |
| PROKKA_03008 | GTP-binding protein TypA/BipA | 4.27 |
| PROKKA_00527 | Alginate biosynthesis transcriptional regulatory protein AlgB | 2.85 |
| PROKKA_01641 | Sigma 54 modulation protein / S30EA ribosomal protein | 2.60 |
| PROKKA_02008 | putative HTH-type transcriptional regulator YdfH | 2.33 |
| PROKKA_02562 | putative transcriptional regulatory protein | 2.31 |
| | | |
| ***Signal transduction*** | | |
| PROKKA_01056 | Nitrogen regulatory protein | on |
| ***Transcription*** | | |
| PROKKA_02227 | NADPH-dependent 7-cyano-7-deazaguanine reductase | on |
| PROKKA_03548 | hypothetical protein | on |
| PROKKA_03419 | hypothetical protein | 6.72 |
| PROKKA_03738 | hypothetical protein | 2.39 |
| PROKKA_00010 | hypothetical protein | 2.25 |
| | | |
| ***Transport and binding proteins*** | | |
| PROKKA_00683 | Cystine-binding periplasmic protein precursor | on |
| PROKKA_01304 | Fatty acyl-CoA reductase | on |
| PROKKA_01319 | Hemin-binding periplasmic protein HmuT precursor | on |
| PROKKA_02724 | Macrolide export protein MacA | on |
| PROKKA_03184 | Iron-utilization periplasmic protein precursor | on |
| PROKKA_03225 | Bacterioferritin | on |
| PROKKA_04601 | Periplasmic solute binding protein family protein | on |
| PROKKA_05015 | putative ABC transporter ATP-binding protein | on |
| PROKKA_04499 | LPS O-antigen length regulator | 4.76 |
| PROKKA_05330 | hypothetical protein | 3.00 |
| PROKKA_03683 | putative ABC transporter ATP-binding protein | 2.89 |
| PROKKA_04287 | Glycine betaine-binding periplasmic protein precursor | 2.37 |

842 [1] Function predicted by Prokka annotation[2] “on ” exclusively identified under 15 °C

**Table 2.** Proteins repressed at 15 °C in comparison to 30 °C.

| Identifier | Function[1] | Fold change[2] UT15/UT30 |
|---|---|---|
| ***Amino acid biosynthesis*** | | |
| PROKKA_01520 | Histidinol-phosphate aminotransferase 2 | off |
| PROKKA_05541 | O-succinylhomoserine sulfhydrylase | 0.45 |
| PROKKA_05608 | Ornithine carbamoyltransferase, catabolic | 0.44 |
| PROKKA_01071 | ATP phosphoribosyltransferase | 0.44 |
| PROKKA_00904 | Tryptophan synthase beta chain | 0.42 |
| PROKKA_02986 | Imidazoleglycerol-phosphate dehydratase | 0.32 |
| ***Biosynthesis of cofactors, prosthetic groups, and carriers*** | | |
| PROKKA_03863 | Ferrochelatase | off |
| PROKKA_03405 | Delta-aminolevulinic acid dehydratase | 0.48 |
| PROKKA_04486 | Ubiquinone biosynthesis O-methyltransferase | 0.47 |
| PROKKA_02094 | Electron transfer flavoprotein-ubiquinone oxidoreductase | 0.46 |
| PROKKA_02662 | Siroheme synthase | 0.46 |
| PROKKA_03811 | Gamma-glutamyltranspeptidase precursor | 0.44 |
| ***Cell envelope*** | | |
| PROKKA_01669 | D-alanyl-D-alanine carboxypeptidase DacC precursor | off |
| PROKKA_04822 | Glucans biosynthesis protein D precursor | 0.43 |
| PROKKA_03534 | Outer membrane protein W precursor | 0.32 |
| PROKKA_02028 | Outer membrane porin F precursor | 0.29 |
| PROKKA_03767 | Penicillin-binding protein 1B | 0.29 |
| PROKKA_01622 | Lipopolysaccharide export system permease protein LptF | 0.28 |
| PROKKA_00116 | Outer membrane lipoprotein SlyB precursor | 0.27 |
| ***Cellular processes*** | | |
| PROKKA_00520 | mce related protein | off |
| PROKKA_00991 | Glycine betaine/carnitine/choline-binding protein OpuCC precursor | off |
| PROKKA_01099 | Paraquat-inducible protein B | off |
| PROKKA_01211 | Copper-transporting P-type ATPase | off |
| PROKKA_01766 | Flagellar M-ring protein | off |
| PROKKA_02002 | Chromosome partition protein Smc | off |
| PROKKA_03354 | 2-aminomuconate deaminase | off |
| PROKKA_04562 | Filamentous hemagglutinin | off |
| PROKKA_00072 | putative efflux pump membrane transporter TtgB | 0.46 |
| PROKKA_00073 | putative efflux pump outer membrane protein TtgC precursor | 0.45 |
| PROKKA_01105 | Osmotically-inducible protein Y precursor | 0.44 |
| PROKKA_01773 | Flagellar hook-associated protein 2 | 0.43 |
| PROKKA_03782 | Paraquat-inducible protein B | 0.38 |
| PROKKA_05563 | DNA protection during starvation protein 2 | 0.33 |

| | | |
|---|---|---|
| PROKKA_01775 | B-type flagellin | 0.32 |
| PROKKA_00253 | heat-inducible protein | 0.31 |
| PROKKA_05581 | Filamentous hemagglutinin | 0.25 |
| PROKKA_04405 | Methyl-accepting chemotaxis protein PctC | 0.24 |
| PROKKA_05548 | Methyl-accepting chemotaxis protein PctA | 0.20 |
| PROKKA_03040 | Methyl-accepting chemotaxis protein McpS | 0.20 |
| | | |
| ***Central intermediary metabolism*** | | |
| PROKKA_00421 | Coniferyl aldehyde dehydrogenase | off |
| PROKKA_01466 | (R)-stereoselective amidase | off |
| PROKKA_01967 | Aerotaxis receptor | 0.35 |
| | | |
| ***DNA metabolism*** | | |
| PROKKA_00722 | DNA-binding protein HU-beta | off |
| PROKKA_02651 | Cold shock-like protein CspD | off |
| PROKKA_03709 | Type-1 restriction enzyme R protein | off |
| PROKKA_05569 | Holliday junction ATP-dependent DNA helicase RuvA | off |
| PROKKA_03154 | DNA topoisomerase 4 subunit B | 0.46 |
| PROKKA_04489 | DNA gyrase subunit A | 0.42 |
| PROKKA_03156 | DNA topoisomerase 4 subunit A | 0.30 |
| | | |
| ***Energy metabolism*** | | |
| PROKKA_00350 | 2-hydroxy-3-oxopropionate reductase | off |
| PROKKA_01785 | Phenylalanine-4-hydroxylase | off |
| PROKKA_01980 | Ubp3 associated protein Bre5 | off |
| PROKKA_02062 | Phosphorylated carbohydrates phosphatase | off |
| PROKKA_02464 | Anthranilate 1,2-dioxygenase large subunit | off |
| PROKKA_02627 | NADH-quinone oxidoreductase subunit M | off |
| PROKKA_02727 | Methionine gamma-lyase | off |
| PROKKA_03974 | Trehalose synthase/amylase TreS | off |
| PROKKA_04974 | 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase | off |
| PROKKA_05460 | Aminomethyltransferase | off |
| PROKKA_01971 | Cytochrome C oxidase, mono-heme subunit/FixO | 0.50 |
| PROKKA_05189 | L-glyceraldehyde 3-phosphate reductase | 0.50 |
| PROKKA_01176 | Methylmalonate-semialdehyde dehydrogenase [acylating] | 0.45 |
| PROKKA_01021 | Ubiquinol-cytochrome c reductase iron-sulfur subunit | 0.44 |
| PROKKA_01023 | Cytochrome b/c1 | 0.43 |
| PROKKA_00630 | ATP synthase gamma chain | 0.42 |
| PROKKA_02507 | Dihydrolipoyl dehydrogenase | 0.42 |
| PROKKA_00337 | Citrate synthase | 0.39 |
| PROKKA_00360 | Cytochrome c-type biogenesis protein CcmH precursor | 0.38 |
| PROKKA_01973 | Cbb3-type cytochrome c oxidase subunit CcoP1 | 0.37 |
| PROKKA_05220 | putative enoyl-CoA hydratase echA8 | 0.36 |

| | | |
|---|---|---|
| PROKKA_03975 | 1,4-alpha-glucan branching enzyme GlgB | 0.35 |
| PROKKA_02639 | Isocitrate lyase | 0.35 |
| PROKKA_01666 | Ureidoglycolate lyase | 0.33 |
| PROKKA_05652 | Glucose-6-phosphate 1-dehydrogenase | 0.29 |
| PROKKA_01667 | Homogentisate 1,2-dioxygenase | 0.25 |
| PROKKA_01972 | Cbb3-type cytochrome oxidase component FixQ | 0.22 |
| PROKKA_01022 | Cytochrome b | 0.17 |
| | | |
| ***Fatty acid and phospholipid metabolism*** | | |
| PROKKA_01388 | Glucose 1-dehydrogenase 1 | off |
| PROKKA_01846 | 3-ketoacyl-CoA thiolase | off |
| PROKKA_02583 | Long-chain-fatty-acid--CoA ligase | off |
| PROKKA_03054 | Poly(hydroxyalcanoate) granule associated protein (phasin) | off |
| PROKKA_03347 | putative cardiolipin synthase YwiE | off |
| PROKKA_01444 | Acetyl-CoA acetyltransferase | 0.49 |
| PROKKA_01847 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | 0.49 |
| PROKKA_01803 | Putative outer membrane protein precursor | 0.35 |
| PROKKA_02046 | 2-methylcitrate synthase | 0.32 |
| PROKKA_05504 | Acyl-CoA dehydrogenase | 0.32 |
| PROKKA_00270 | Acyl-coenzyme A dehydrogenase | 0.26 |
| | | |
| ***No classification*** | | |
| PROKKA_00089 | Low-affinity inorganic phosphate transporter 1 | off |
| PROKKA_00582 | D-galactonate dehydratase | off |
| PROKKA_01488 | hypothetical protein | off |
| PROKKA_01798 | DNA recombination protein RmuC | off |
| PROKKA_02422 | lysozyme inhibitor | off |
| PROKKA_02678 | META domain protein | off |
| PROKKA_02708 | hypothetical protein | off |
| PROKKA_02806 | Gamma-glutamylputrescine oxidoreductase | off |
| PROKKA_03014 | hypothetical protein | off |
| PROKKA_03106 | Mechanosensitive channel MscK precursor | off |
| PROKKA_03158 | hypothetical protein | off |
| PROKKA_03231 | E3 ubiquitin-protein ligase ipaH3 | off |
| PROKKA_03294 | putative deoxyribonuclease RhsA | off |
| PROKKA_03473 | Acyl-homoserine lactone acylase QuiP precursor | off |
| PROKKA_03583 | Peptidase C13 family protein | off |
| PROKKA_03650 | hypothetical protein | off |
| PROKKA_03686 | hypothetical protein | off |
| PROKKA_03994 | hypothetical protein | off |
| PROKKA_04403 | Methyl-accepting chemotaxis protein PctB | off |
| PROKKA_04561 | Hemolysin transporter protein ShlB precursor | off |
| PROKKA_04806 | hypothetical protein | off |
| PROKKA_04808 | hypothetical protein | off |

| | | |
|---|---|---|
| PROKKA_04959 | Maltoporin precursor | off |
| PROKKA_05161 | hypothetical protein | off |
| PROKKA_05230 | putative 4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase ArnD | off |
| PROKKA_05325 | General stress protein 69 | off |
| PROKKA_05560 | HIT domain protein | off |
| PROKKA_03698 | hypothetical protein | 0.49 |
| PROKKA_00381 | hypothetical protein | 0.49 |
| PROKKA_05649 | Porin B precursor | 0.48 |
| PROKKA_03317 | hypothetical protein | 0.48 |
| PROKKA_00323 | hypothetical protein | 0.48 |
| PROKKA_03224 | putative assembly protein | 0.48 |
| PROKKA_04269 | hypothetical protein | 0.47 |
| PROKKA_00112 | hypothetical protein | 0.47 |
| PROKKA_04341 | hypothetical protein | 0.47 |
| PROKKA_03542 | hypothetical protein | 0.47 |
| PROKKA_00138 | NAD-specific glutamate dehydrogenase | 0.46 |
| PROKKA_04556 | hypothetical protein | 0.45 |
| PROKKA_04669 | hypothetical protein | 0.44 |
| PROKKA_03678 | Inner membrane protein YjiY | 0.43 |
| PROKKA_00373 | putative lipoprotein YgdR precursor | 0.41 |
| PROKKA_01012 | hypothetical protein | 0.41 |
| PROKKA_03272 | hypothetical protein | 0.41 |
| PROKKA_03325 | hypothetical protein | 0.40 |
| PROKKA_00542 | hypothetical protein | 0.39 |
| PROKKA_02030 | hypothetical protein | 0.38 |
| PROKKA_04259 | hypothetical protein | 0.38 |
| PROKKA_01066 | putative phospholipid ABC transporter-binding protein MlaD | 0.36 |
| PROKKA_05259 | hypothetical protein | 0.36 |
| PROKKA_02996 | molybdopterin biosynthesis protein MoeB | 0.32 |
| PROKKA_03278 | hypothetical protein | 0.30 |
| PROKKA_00547 | hypothetical protein | 0.28 |
| PROKKA_01962 | ATP-dependent RNA helicase HrpB | 0.27 |
| PROKKA_03134 | Putative glycosyltransferase EpsF | 0.27 |
| PROKKA_03149 | hypothetical protein | 0.27 |
| PROKKA_03572 | Inner membrane protein YghB | 0.25 |
| PROKKA_04656 | Imelysin | 0.24 |
| PROKKA_03843 | bacteriophage N4 receptor, outer membrane subunit | 0.21 |
| PROKKA_01609 | Chagasin family peptidase inhibitor I42 | 0.17 |
| | | |
| ***Protein fate*** | | |
| PROKKA_00361 | Thiol:disulfide interchange protein DsbE | off |
| PROKKA_00681 | Extracellular serine protease precursor | off |
| PROKKA_01742 | Chemotaxis protein CheA | off |

| | | |
|---|---|---|
| PROKKA_03062 | ATP-dependent protease subunit HslV | off |
| PROKKA_03165 | Motility protein B | off |
| PROKKA_03297 | Chaperone protein ClpB | off |
| PROKKA_02089 | putative lipoprotein YiaD precursor | 0.50 |
| PROKKA_01619 | Signal peptidase I | 0.50 |
| PROKKA_04665 | 60 kDa chaperonin | 0.50 |
| PROKKA_01469 | Outer membrane protein assembly factor BamB precursor | 0.49 |
| PROKKA_01103 | putative lipoprotein YiaD precursor | 0.47 |
| PROKKA_01205 | Peptidoglycan-binding protein ArfA | 0.47 |
| PROKKA_01608 | Lon protease | 0.46 |
| PROKKA_05085 | Putative signal peptide peptidase SppA | 0.45 |
| PROKKA_05384 | Carboxypeptidase G2 precursor | 0.44 |
| PROKKA_03307 | hypothetical protein | 0.43 |
| PROKKA_04761 | Outer membrane protein assembly factor BamA precursor | 0.40 |
| PROKKA_04617 | Aminopeptidase | 0.39 |
| PROKKA_02677 | Thiol-disulfide oxidoreductase ResA | 0.37 |
| PROKKA_03179 | Modulator of FtsH protease HflC | 0.37 |
| PROKKA_01491 | preprotein translocase subunit YajC | 0.34 |
| PROKKA_04708 | Esterase EstA precursor | 0.34 |
| PROKKA_03842 | Outer-membrane lipoprotein LolB precursor | 0.31 |
| PROKKA_00840 | Extracellular serine protease precursor | 0.30 |
| PROKKA_02401 | Leukotoxin | 0.30 |
| PROKKA_03486 | Aminopeptidase YwaD precursor | 0.28 |
| PROKKA_03900 | Protease HtpX | 0.27 |
| PROKKA_01321 | Protease PrtS precursor | 0.22 |
| PROKKA_04762 | Regulator of sigma-E protease RseP | 0.19 |
| PROKKA_00839 | Extracellular serine protease precursor | 0.15 |
| PROKKA_02779 | Peptidase inhibitor I78 family protein | 0.12 |
| | | |
| ***Protein synthesis*** | | 0.45 |
| PROKKA_04929 | 50S ribosomal protein L21 | 0.37 |
| PROKKA_05231 | Bifunctional polymyxin resistance protein ArnA | 0.30 |
| PROKKA_00604 | Glycine--tRNA ligase alpha subunit | 0.30 |
| | | |
| ***Purines, pyrimidines, nucleosides, and nucleotides*** | | |
| PROKKA_05372 | phosphoribosylglycinamide formyltransferase | off |
| PROKKA_04118 | allantoicase | 0.20 |
| | | |
| ***Regulatory functions*** | | |
| PROKKA_02036 | Phosphoenolpyruvate synthase regulatory protein | off |
| PROKKA_01793 | Transcriptional regulator SlyA | 0.50 |
| | | |
| ***Signal transduction*** | | |
| PROKKA_00715 | Phosphate regulon sensor protein PhoR | off |

| | | |
|---|---|---|
| PROKKA_02681 | Signal transduction histidine-protein kinase BarA | off |
| PROKKA_03108 | Methyl-accepting chemotaxis protein McpS | off |
| | | |
| ***Transcription*** | | |
| PROKKA_00015 | DNA-directed RNA polymerase subunit beta | 0.47 |
| | | |
| ***Transport and binding proteins*** | | |
| PROKKA_00170 | C4-dicarboxylate-binding periplasmic protein precursor | off |
| PROKKA_00278 | Ferrichrome-iron receptor precursor | off |
| PROKKA_00710 | Phosphate transport system permease protein PstA | off |
| PROKKA_00999 | Dipeptide transport system permease protein DppC | off |
| PROKKA_01003 | Periplasmic dipeptide transport protein precursor | off |
| PROKKA_01670 | Ferrichrome-iron receptor precursor | off |
| PROKKA_02762 | Ferrichrome-iron receptor precursor | off |
| PROKKA_02892 | Arginine transport ATP-binding protein ArtM | off |
| PROKKA_02907 | ABC transporter glutamine-binding protein GlnH precursor | off |
| PROKKA_03323 | Biopolymer transport protein ExbB | off |
| PROKKA_04345 | Oligopeptide-binding protein AppA precursor | off |
| PROKKA_04650 | Hemin receptor precursor | off |
| PROKKA_04962 | Membrane-bound lytic murein transglycosylase F precursor | off |
| PROKKA_05091 | Aerobic C4-dicarboxylate transport protein | off |
| PROKKA_05128 | Phosphate import ATP-binding protein PstB 3 | off |
| PROKKA_05751 | Glutathione-regulated potassium-efflux system protein KefC | off |
| PROKKA_01004 | Periplasmic dipeptide transport protein precursor | 0.49 |
| PROKKA_00997 | putative D,D-dipeptide transport ATP-binding protein DdpF | 0.49 |
| PROKKA_00544 | Methionine import ATP-binding protein MetN | 0.48 |
| PROKKA_00071 | putative efflux pump periplasmic linker TtgA precursor | 0.48 |
| PROKKA_01001 | Periplasmic dipeptide transport protein precursor | 0.48 |
| PROKKA_03761 | Hemin-binding periplasmic protein HmuT precursor | 0.47 |
| PROKKA_05370 | Ferripyoverdine receptor precursor | 0.42 |
| PROKKA_01982 | putative copper-importing P-type ATPase A | 0.40 |
| PROKKA_01000 | Dipeptide transport system permease protein DppB | 0.37 |
| PROKKA_04423 | Fe(3+) dicitrate transport protein FecA precursor | 0.37 |
| PROKKA_02687 | biopolymer transport protein ExbD | 0.33 |
| PROKKA_03247 | Ferric enterobactin receptor precursor | 0.29 |
| PROKKA_02904 | Macrolide export protein MacA | 0.28 |
| PROKKA_00711 | Phosphate import ATP-binding protein PstB | 0.27 |
| PROKKA_03129 | Ferrichrome-iron receptor precursor | 0.26 |
| PROKKA_02686 | Biopolymer transport protein ExbB | 0.24 |
| PROKKA_03961 | Multidrug resistance protein MdtA precursor | 0.23 |
| PROKKA_00709 | Phosphate transport system permease protein PstC | 0.13 |

ACCEPTED MANUSCRIPT

843 [1] Function predicted by Prokka annotation[2] “off ” exclusively identified under 30 °C

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845 **Table 3.** Proteins induced by HLF-treatment at 15°C and 30°C.

| Identifier | Function[1] | Fold change[2] 15°C | 30°C |
|---|---|---|---|
| ***Aminoacid biosynthesis*** | | | |
| PROKKA_02989 | 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase | 2.29 | on T |
| PROKKA_05528 | 3-isopropylmalate dehydratase small subunit 1 | 3.79 | on T |
| ***Biosynthesis of cofactors, prosthetic groups, and carriers*** | | | |
| PROKKA_02355 | tRNA-modifying protein YgfZ | 2.06 | on T |
| PROKKA_04334 | DNA nickase | 2.63 | on T |
| ***Cell envelope*** | | | |
| PROKKA_05232 | Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase | 6.42 | 6.02 |
| PROKKA_05226 | UDP-glucose 6-dehydrogenase TuaD | on T | on T |
| PROKKA_02028 | Outer membrane porin F precursor | 3.50 | 2.10 |
| PROKKA_00116 | Outermembrane lipoprotein SlyB precursor | 5.22 | 5.40 |
| PROKKA_05229 | Undecaprenyl phosphate-alpha-4-amino-4-deoxy-L-arabinose arabinosyl transferase | 5.56 | 6.49 |
| ***Cellular processes*** | | | |
| PROKKA_03442 | UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase | 5.49 | 5.13 |
| PROKKA_01782 | Flagellar basal-body rod protein FlgG | on T | on T |
| PROKKA_00073 | putative efflux pump outer membrane protein TtgC precursor | 4.02 | 4.74 |
| PROKKA_04999 | Bacterial virulence protein (VirJ) | on T | on T |
| PROKKA_03935 | Multidrug resistance protein MexB | 3.46 | 3.74 |
| ***Energy metabolism*** | | | |
| PROKKA_00423 | 3-mercaptopyruvate sulfurtransferase | on T | on T |
| PROKKA_04385 | Disulfide-bond oxidoreductase YfcG | 2.19 | on T |
| PROKKA_00628 | ATP synthase subunit delta | 2.22 | 6.22 |
| PROKKA_04874 | Ferredoxin--NADP reductase | 2.80 | 2.38 |
| PROKKA_04485 | Phosphoglycolate phosphatase | on T | on T |
| PROKKA_00568 | 5'-nucleotidase | on T | on T |
| ***Fatty acid and phospholipid metabolism*** | | | |
| PROKKA_02061 | Cyclic-di-GMP-binding biofilm dispersal mediator protein | on T | on T |

| | | | |
|---|---|---|---|
| ***No classification*** | | | |
| PROKKA_00069 | N-ethylmaleimide reductase | 2.90 | 2.27 |
| PROKKA_05230 | putative 4-deoxy-4-formamido-L-arabinose phosphoundecaprenol deformylase ArnD | on T | 3.13 |
| PROKKA_05000 | Phosphatidyl glycerol lysyl transferase | on T | on T |
| PROKKA_00573 | Limonene 1,2-monooxygenase | 2.98 | on T |
| PROKKA_00705 | Putative glucose-6-phosphate 1-epimerase | 2.40 | on T |
| PROKKA_05594 | Polyketide cyclase / dehydrase and lipid transport | on T | on T |
| PROKKA_00161 | MltA-interacting protein MipA | on T | on T |
| PROKKA_01044 | Hypotheticalprotein | 2.21 | on T |
| PROKKA_01517 | Hypothetical protein | 4.58 | on T |
| PROKKA_04800 | Hypothetica lprotein | on T | on T |
| ***Protein fate*** | | | |
| PROKKA_05085 | Putative signal peptide peptidase SppA | 2.91 | 2.06 |
| PROKKA_00645 | FtsH protease regulator HflC | on T | on T |
| PROKKA_02543 | ATP-dependent Clp protease proteolytic subunit | 2.69 | on T |
| PROKKA_04332 | General stress protein 18 | 2.98 | on T |
| PROKKA_01103 | putative lipoprotein YiaD precursor | 2.73 | 2.28 |
| PROKKA_02658 | Outer-membrane lipoprotein carrier protein precursor | 2.35 | on T |
| ***Protein synthesis*** | | | |
| PROKKA_03192 | 50S ribosomal protein L9 | 2.81 | 6.34 |
| PROKKA_04796 | tRNA threonyl carbamoyl adenosine biosynthesis protein TsaB | on T | on T |
| ***Purines, pyrimidines, nucleosides, and nucleotides*** | | | |
| PROKKA_05544 | Formyl tetrahydrofolate deformylase | on T | on T |
| PROKKA_01914 | Phosphoribosyl formylglycin amidinecyclo-ligase | 2.11 | on T |
| ***Regulatory functions*** | | | |
| PROKKA_02350 | Sigma factor AlgU negative regulatory protein | on T | on T |
| ***Transport and binding proteins*** | | | |
| PROKKA_03184 | Iron-utilization periplasmic protein precursor | 3.05 | on T |
| PROKKA_01064 | putative ABC transporter ATP-binding protein | on T | on T |
| PROKKA_03936 | Multidrug resistance protein MexA precursor | 2.35 | 2.99 |

846 [1] Function predicted by Prokka annotation[2] "on T" exclusively identified after HLF treatment

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848 **Table 4.** Proteins repressed by HLF-treatment at 15°C and 30°C.

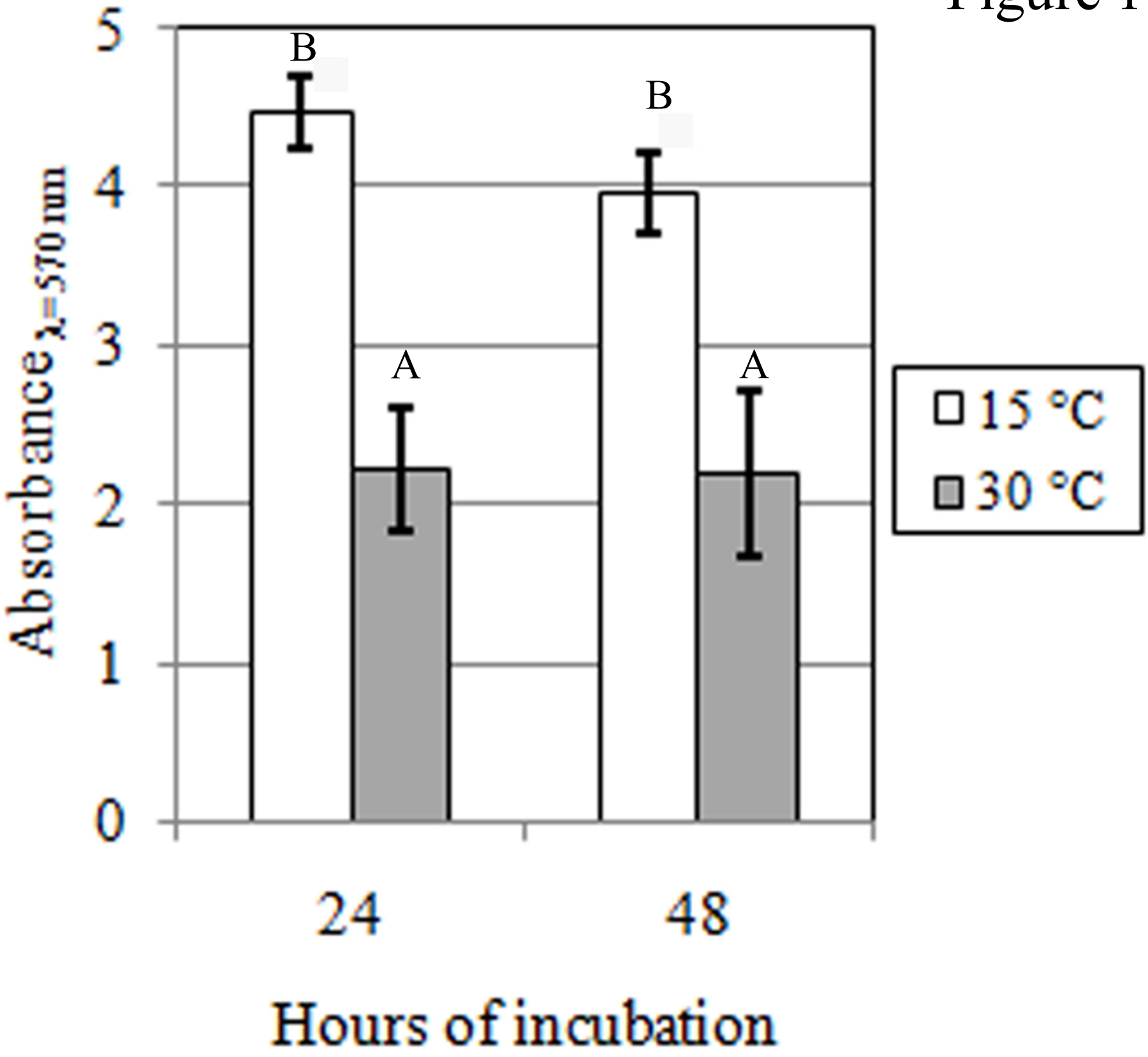
| Identifier | Function[1] | Fold change[2] | |
|---|---|---|---|
| | | **15°C** | **30°C** |
| ***Aminoacid biosynthesis*** | | | |
| PROKKA_00203 | Cystathionine beta-lyase | 0.23 | off T |
| PROKKA_01787 | Aspartateaminotransferase | 0.45 | 0.48 |
| ***Biosynthesis of cofactors, prosthetic groups, and carriers*** | | | |
| PROKKA_03463 | Omega-amino acid--pyruvate aminotransferase | 0.28 | 0.18 |
| PROKKA_03147 | Phosphomethylpyrimidinesynthase | off T | off T |
| PROKKA_02039 | putative adenylyltransferase/sulfurtransferase MoeZ | off T | off T |
| ***Cell envelope*** | | | |
| PROKKA_04557 | Poly-beta-1,6-N-acetyl-D-glucosamine export protein precursor | off T | off T |
| ***Cellular processes*** | | | |
| PROKKA_00897 | UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase | off T | off T |
| PROKKA_00898 | UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase | off T | off T |
| PROKKA_05548 | Methyl-accepting chemotaxis protein PctA | off T | 0.34 |
| PROKKA_04511 | ComE operon protein 1 | off T | off T |
| PROKKA_00251 | Acyl-homoserine lactone acylasePvdQ precursor | off T | off T |
| PROKKA_01272 | Chitinase D precursor | off T | off T |
| ***Central intermediary metabolism*** | | | |
| PROKKA_02553 | Carbon-nitrogen hydrolase | off T | off T |
| ***DNA metabolism*** | | | |
| PROKKA_04783 | Major cold shock protein CspA | off T | off T |
| ***Energy metabolism*** | | | |
| PROKKA_03027 | Imidazolone propionase | off T | off T |
| PROKKA_01666 | Ureidoglycolate lyase | off T | off T |
| PROKKA_03025 | Histidine ammonia-lyase | 0.23 | 0.31 |
| PROKKA_00268 | Glutathione S-transferase GstB | 0.46 | 0.44 |
| PROKKA_05600 | Aminomethyl transferase | 0.19 | 0.47 |
| PROKKA_00695 | Aspartateammonia-lyase | 0.35 | 0.50 |
| PROKKA_04586 | Cytochrome c | off T | 0.10 |
| PROKKA_05220 | putative enoyl-CoA hydratase echA8 | 0.29 | 0.10 |
| PROKKA_03584 | putative enoyl-CoA hydratase 1 | 0.09 | 0.18 |
| PROKKA_02048 | Methylisocitrate lyase | 0.14 | 0.19 |
| PROKKA_02580 | Hydroxycinnamoyl-CoAhydratase-lyase | off T | 0.21 |

| | | | |
|---|---|---|---|
| PROKKA_00641 | Phospholipase YtpA | off T | 0.23 |
| PROKKA_05262 | Methionyl-tRNA formyltransferase | 0.21 | 0.32 |
| PROKKA_02508 | Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex | off T | 0.12 |
| PROKKA_02639 | Isocitrate lyase | 0.25 | 0.30 |
| PROKKA_01237 | Fumarate hydratase class II | 0.43 | 0.42 |
| | | | |
| ***Fatty acid and phospholipid metabolism*** | | | |
| PROKKA_05217 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | 0.13 | 0.17 |
| PROKKA_05216 | Acetyl-coenzyme A synthetase | off T | off T |
| PROKKA_00502 | Acyl-CoA dehydrogenase | off T | off T |
| PROKKA_02582 | Acyl-CoA dehydrogenase | off T | 0.17 |
| PROKKA_01442 | putative succinyl-CoA:3-ketoacid coenzyme A transferase subunit A | off T | 0.32 |
| PROKKA_01803 | Putative outer membrane protein precursor | 0.48 | 0.41 |
| PROKKA_05661 | Long-chain-fatty-acid--CoA ligase | 0.13 | 0.43 |
| | | | |
| ***No classification*** | | | |
| PROKKA_05738 | Nucleoside-specific channel-forming protein tsx precursor | off T | off T |
| PROKKA_00717 | Secreted repeat of unknown function | off T | off T |
| PROKKA_03447 | CYTH domain protein | off T | off T |
| PROKKA_00834 | Serralysin precursor | off T | off T |
| PROKKA_04692 | PrkA AAA domain protein | 0.48 | 0.19 |
| PROKKA_00895 | Indole-3-glycerol phosphate synthase | off T | 0.21 |
| PROKKA_04821 | Inner membrane protein YebE | 0.46 | 0.41 |
| PROKKA_05559 | Porin D precursor | 0.30 | 0.45 |
| PROKKA_01146 | Serine 3-dehydrogenase | off T | 0.48 |
| PROKKA_05645 | putative sugar-binding periplasmic protein precursor | 0.32 | 0.48 |
| PROKKA_03131 | Decarbamoyl novobiocin carbamoyltransferase | 0.37 | 0.49 |
| PROKKA_02387 | Hypothetical protein | off T | off T |
| PROKKA_04269 | Hypotheticalprotein | off T | off T |
| PROKKA_05138 | Hypothetical protein | off T | off T |
| PROKKA_05429 | Hypothetical protein | off T | off T |
| PROKKA_01958 | Hypothetical protein | off T | 0.06 |
| PROKKA_01096 | Hypothetical protein | 0.04 | 0.17 |
| PROKKA_04669 | Hypothetical protein | off T | 0.23 |
| PROKKA_01095 | Hypothetical protein | off T | 0.24 |
| PROKKA_04341 | Hypothetical protein | 0.35 | 0.30 |
| PROKKA_04546 | Hypothetical protein | 0.14 | 0.39 |
| PROKKA_04556 | Hypothetical protein | 0.47 | 0.44 |
| | | | |
| ***Protein fate*** | | | |
| PROKKA_01321 | Protease PrtSprecursor | off T | off T |
| PROKKA_00900 | putative succinyl-diaminopimelate desuccinylase | off T | off T |
| PROKKA_05384 | Carboxypeptidase G2 precursor | off T | 0.46 |

| | | | |
|---|---|---|---|
| PROKKA_01697 | Extracellular serine protease precursor | off T | off T |
| PROKKA_00839 | Extracellular serine protease precursor | off T | off T |
| PROKKA_00840 | Extracellular serine protease precursor | off T | 0.09 |
| PROKKA_03486 | Aminopeptidase YwaD precursor | off T | 0.11 |
| PROKKA_04761 | Outer membrane protein assembly factor BamA precursor | 0.40 | 0.23 |
| PROKKA_04708 | Esterase EstA precursor | off T | 0.24 |
| | | | |
| ***Protein synthesis*** | | | |
| PROKKA_00026 | 30S ribosomal protein S19 | off T | off T |
| | | | |
| ***Purines, pyrimidines, nucleosides, and nucleotides*** | | | |
| PROKKA_04118 | allantoicase | 0.42 | 0.06 |
| PROKKA_03623 | AMP nucleosidase | 0.48 | 0.34 |
| | | | |
| ***Regulatory functions*** | | | |
| PROKKA_00716 | Phosphate regulon transcriptional regulatory protein PhoB | off T | off T |
| | | | |
| ***Transport and binding proteins*** | | | |
| PROKKA_00340 | Glycine betaine-binding protein OpuAC precursor | off T | off T |
| PROKKA_02443 | Glycine betaine-binding protein OpuAC precursor | 0.48 | off T |
| PROKKA_00101 | Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein precursor | 0.19 | 0.25 |
| PROKKA_00709 | Phosphate transport system permease protein PstC | off T | off T |
| PROKKA_05125 | Phosphate-binding protein PstS precursor | 0.21 | 0.10 |
| PROKKA_00708 | Phosphate-binding protein PstS precursor | 0.08 | 0.12 |
| PROKKA_00711 | Phosphate import ATP-binding protein PstB | off T | 0.12 |
| PROKKA_05370 | Ferripyoverdine receptor precursor | off T | off T |
| PROKKA_04201 | Oligopeptide-binding protein AppA precursor | off T | off T |
| PROKKA_04286 | putative TonB-dependent receptor BfrD precursor | off T | off T |
| PROKKA_05140 | Magnesium-transporting ATPase, P-type 1 | 0.07 | 0.10 |
| PROKKA_04423 | Fe(3+) dicitrate transport protein FecA precursor | off T | 0.16 |
| PROKKA_01001 | Periplasmic dipeptide transport protein precursor | 0.10 | 0.22 |
| PROKKA_00997 | putative D,D-dipeptide transport ATP-binding protein DdpF | off T | 0.46 |
| PROKKA_04604 | putative periplasmic iron-binding protein precursor | off T | off T |

849 [1] Function predicted by Prokka annotation[2] "off T" exclusively identified under control condition

Figure 1


5
4
3
2
1
0
Absorbance λ=570 nm
B
B
A
A
15 °C
30 °C
24
48
Hours of incubation

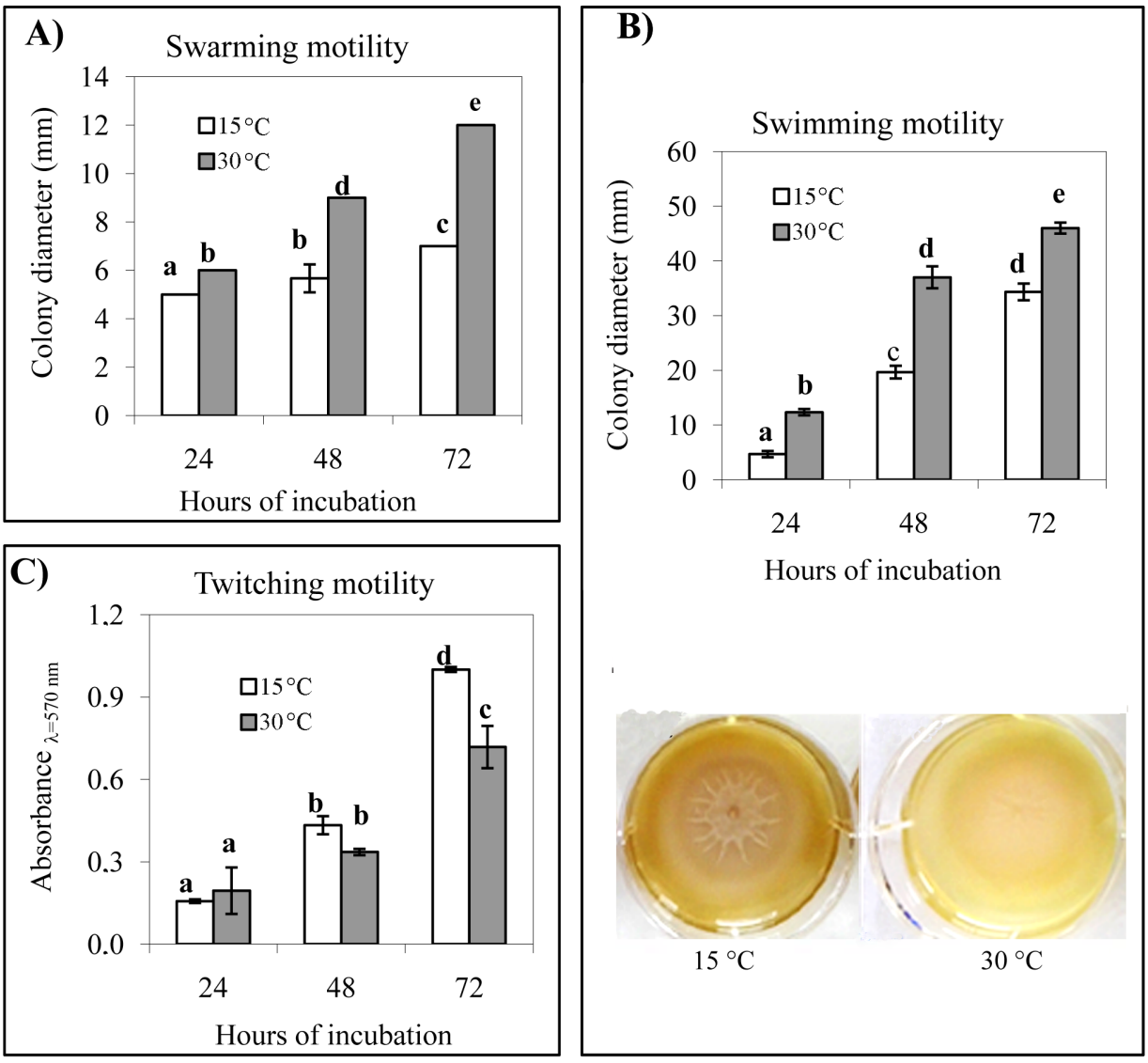

A)
Swarming motility
Colony diameter (mm)
15 °C
30 °C
Hours of incubation
B)
Swimming motility
Colony diameter (mm)
15 °C
30 °C
Hours of incubation
15 °C
30 °C
C)
Twitching motility
Absorbance λ=570 nm
15 °C
30 °C
Hours of incubation

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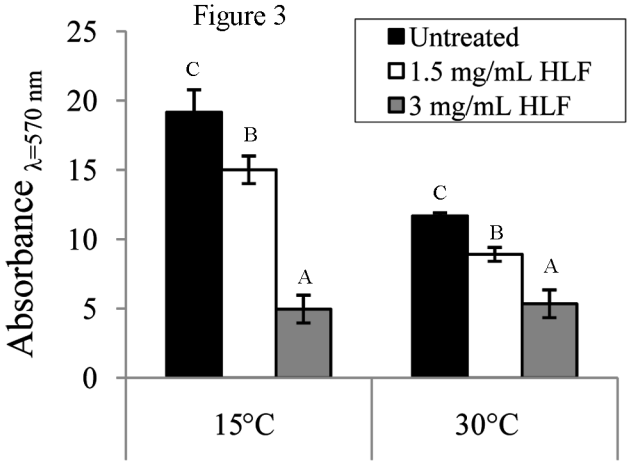
Figure 3

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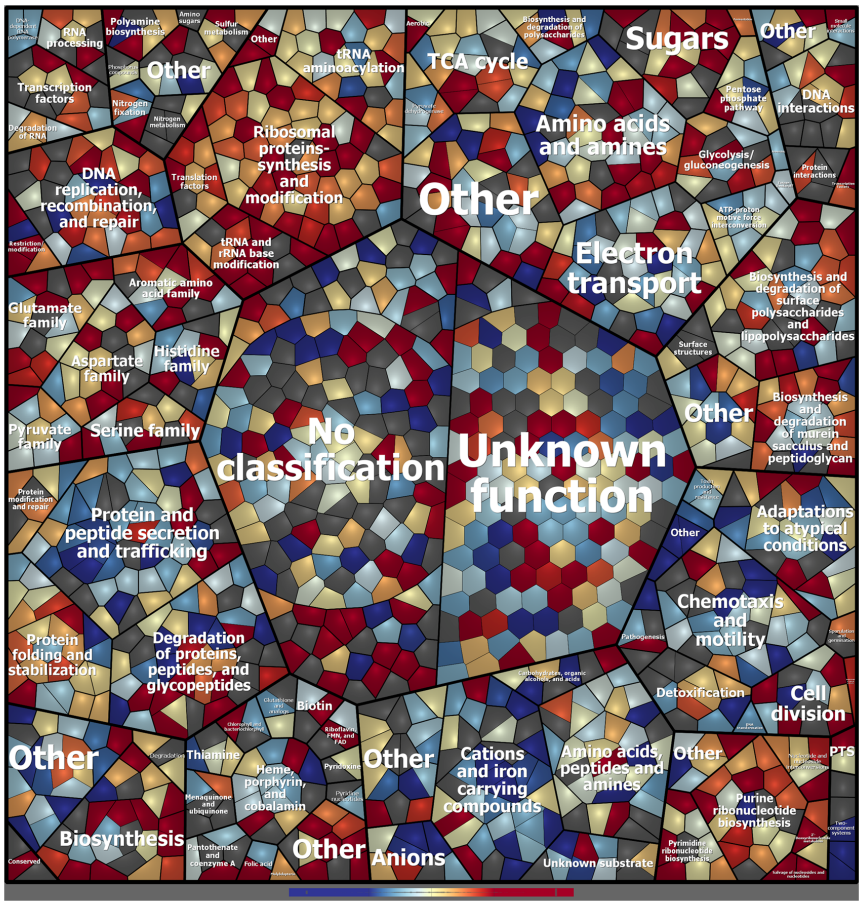


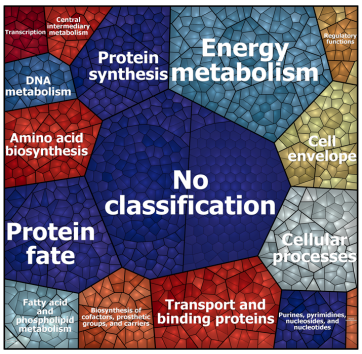


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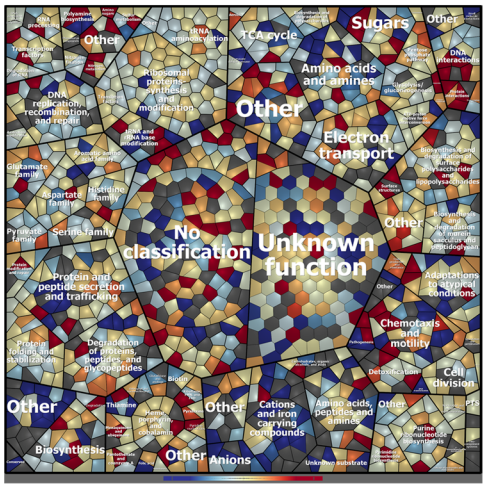


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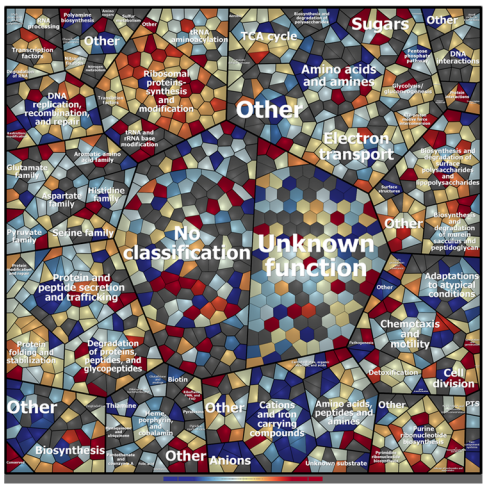


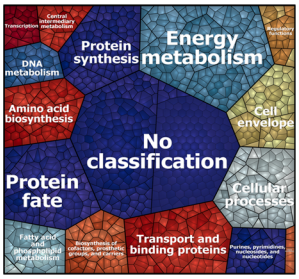


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## HIGHLIGHTS

- Biofilm biomass by *P. fluorescens* increased at 15 °C compared to 30 °C
- Bovine lactoferrin hydrolysate reduced biofilm regardeless growth temperature
- Bovine lactoferrin hydrolysate affected swarming, twiching, swimming motility
- Comparative proteomic analysis revelead the affected pathways

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