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Attached and planktonic *Listeria monocytogenes* global proteomic responses and associated influence of strain genetics and temperature

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ABSTRACT:

Contamination of industrial and domestic food usage environments by the attachement of bacterial food-borne pathogen *Listeria monocytogenes* has public health and economic implications. Comprehensive proteomics experiments using label-free liquid chromatography/tandem mass spectrometry (LC-MS/MS) were used to compare the proteomes of two different *L. monocytogenes* strains (Siliken_1/2c and F2365_4b) showing very different capacities to attach to surfaces. Growth temperature and strain type were highly influential on the proteomes in both attached and planktonic cells. Based on the proteomic data it is highly unlikely specific surface proteins play a direct role in adherence to inanimate surfaces. Instead strain-dependent responses related to cell envelope polymer biosynthesis and stress response regulation likely contributes to a different ability to attach and also to survive external stressors. Collectively, the divergent proteome-level responses observed define strain- and growth-temperature-dependent differences relevant to attachment efficacy, highlight relevant proteins involved in stress protection in attached cells and suggests that strain differences and growth conditions are important in relation to environmental persistence.

Keywords: *Listeria monocytogenes*, attachment, biofilms, persistence, temperature, proteomics

INTRODUCTION

Listeria monocytogenes is a Gram-positive pathogenic bacterium responsible for human and animal listeriosis. Invasive listeriosis predominantly affects pregnant

women, immunocompromised individuals, and the elderly.[1,2] *L. monocytogenes* is notorious for its ability to survive within a diverse array of deleterious environments, such as acidic food, gastric secretions, freezing temperature, high salinity, and following phagocytosis. *L. monocytogenes* is particularly adept at initiating an acid-tolerance response (ATR) that cross-protects it against a range of stressors.[3-6] In addition, *L. monocytogenes* is able to adhere to a variety of surfaces, such as polystyrene, polypropylene, glass, stainless steel, quartz, marble, granite and bunan rubber, materials commonly used in food processing equipment. On these surfaces it forms simple monolayer biofilms.[7,8] Attachment contributes to the persistence of the species within the food supply chain. Listerial contamination presents an economic burden within the food processing industry due to the perceived public health risk.[9,10]

Biofilm structures can confer protection to bacterial cells and decrease the efficiency of cleaning and disinfection procedures.[11] *L. monocytogenes* is unable to form thick multilayer biofilms, which have as many as 10^9–10^{12} CFU/cm^2 . Instead surface monoculture populations of *L monocytogenes* reach only 10^4–10^7 CFU/cm^2 [12], thus in this study instead of using the term “biofilm” we will refer to *L. monocytogenes* adhered cells simply as “attached cells”. *L. monocytogenes* strains do not form classic complex biofilms and the attachment process primarily relies on adherence by means of van der Waals forces, electrostatic forces, and hydrophobic interactions. After a certain time interval cell dispersion occurs, with subsequent colonization of other surfaces.[13] Several studies have shown that the main factors that influence the ability of *L. monocytogenes* to adhere to inanimate surfaces include the physicochemical properties of the contact surface (electrostatic charge and hydrophobicity), the strain serotype, which likely correlates to cell wall properties, the presence of flagella and the

environmental conditions (pH, temperature, and culture medium)[10,14-19] However, molecular mechanisms of how environmental and strain-derived intrinsic factors modulate *L. monocytogenes* surface attachment ability are still not well understood. Proteomic analysis provides a powerful tool to broadly assess the protein complement of attached and planktonic (swimming/free-floating cells) providing a means to connect known phenotypic aspects of *L. monocytogenes* adherent cells to information regarding specific changes in protein abundance.

The aim of the present study was to compare broadly proteomic responses of two *L. monocytogenes* strains with different capacities to attach to surfaces. Label-free proteomics by nano HPLC-MS/MS has been previously used to examine responses of *L. monocytogenes* in several different contexts, and is an effective means to interrogate changes in bacterial proteomes between different conditions.[20-22] Strains from two different *L. monocytogenes* genetic lineages with very different attachment abilities were assessed to determine what proteins can be associated with attachment and the relative influence of strain type. In addition, two temperature conditions (25°C and 37°C) were also tested since at 37°C attachment is usually enhanced due to greater cell wall hydrophobicity and suppression of motility[23]. Proteomic data provided a perspective of the importance of these variables and was able to demonstrate that growth conditions and strain type results in different protein responses as well as demonstrates the significance of specific proteins in attached cells that likely confer enhanced survival and thus persistence to cells.

MATERIAL AND METHODS

L. monocytogenes Strains and Experimental Growth Conditions

For these experiments two *L. monocytogenes* strains were chosen. Siliken 204231/1 (referred from here as "Siliken_1/2c"), a serotype 1/2c industry isolate of the same MLST sequence type (ST9)[10,24] as genome sequenced strain EGD-e, was selected because it proficiently attaches to inanimate surfaces including polystyrene and glass wool. The second strain, F2365_4b, was chosen for its tendency to only poorly attach to inanimate surfaces. This serotype 4b (MLST ST1) strain has a completely sequenced genome.[25] The experiment was conducted at two temperatures (25°C and 37°C) and focused on cultures in the stationary growth phase, as cell attachment plateaus at this point.[26-28]

To prepare physiologically homogeneous experimental inoculum of both strains, replicate cultures were grown in 10 mL of Brain-Heart Infusion broth (CM225, 'BHI'; OXOID, Australia), incubated at 25ºC and at 37ºC overnight. The cultures were sub-cultured for a period of five days by transferring each day a 100 µL aliquot of the cultures to 9.9 mL of fresh BHI broth.

Harvesting Biomass from Planktonic and Attached Cells

The substratum used in the attachment experiments was glass wool (GW), which provides enough surface area for harvesting sufficient biomass for protein extraction.[29,30] The density of the GW used in this study was experimentally estimated, assuming a surface area of 290 cm^2 per gram of GW. For the establishment of planktonic glass wool cells (PGW) and attached glass wool cells (AGW), 3 mL of 24 h cultures (OD_{600} of 1.3 to 1.4 - cell concentrations ~5×10^9 CFU/mL) was added to 300 mL of BHI containing 6 g of glass wool. All strains were grown without agitation and harvested after 24h at 25°C and 37°C. PGW cells were recovered from the culture medium (ten 15mL tubes with 12 mL PGW culture each) by centrifugation at 5000 x *g*

for 15 min and pellets were washed twice in PBS. The cells were concentrated into one final tube per biological replicate. Glass wool was aseptically removed from the BHI after incubation and gently washed twice in 30 mL PBS and blotted dry on filter paper to remove loosely attached and planktonic cells. The dried glass wool was placed in a sterile flask containing 60g of glass beads (6 mm in diameter) and 30 mL of PBS, and shaken vigorously by hand for 10 min to detach the cells attached to the glass wool. The liquid phase was collected in three 15 mL tubes with 10 mL AGW biomass harvesting and centrifuged at 5000 x g for 15 min to pellet the detached cells. The pellets were washed twice in sterile PBS and the cells were concentrated in one final tube. Both PGW and AGW cells were stored at -20°C until protein extraction. One of the replicate pellets was used for the quantification of biomass levels.

Microtitre plate biofilm production assay and Quantification of Biomass

The formation of attached cells was measured in triplicate (3 biological replicates and 3 technical replicates) for each of the two tested strains using the colorimetric 96-well microtiter plate method described by Djordjevic et al.[31] with the modifications recommended by Borucki et al.[32] (Fig. 1). Briefly, all isolates were recovered from frozen storage by culture on Brain–Heart Infusion (BHI) agar (Oxoid CM0225B, Oxoid Australia, Adelaide, with 1.5% agar) and incubated at 25 °C for 24 h. Following this, each strain was transferred into four 10mL BHI broths (pH 7.3 ± 0.1) and incubated for 24 h at 25°C and 37°C. After incubation, concentrations of the inoculum were adjusted to 10^8 CFU. ml $^{-1}$ and then, 100µL of each culture was added to fresh 9.9mL BHI broths, gently mixed, and 200µL aliquots were transferred to wells of four 96-well polystyrene (PS) microtiter plates (Greiner Scientific, Sigma-Aldrich, Australia). The plates were incubated statically at their corresponding treatment temperature to

allow attachment and growth to occur, Further, the culture media were discarded and the procedures of washing plates, fixation and distaining with crystal violet solution of 1% of the attached cells were carried out. Optical density at 595 nm (OD_{595}) was measured. The growth of PGW cells was characterized by determining colony forming units (CFU) per mL via the pour plate technique. The pour plate technique was also used to estimate attached cells (AGW) counts as $CFU.cm^2$ of GW. All determinations were performed from three separate samples.

Protein Extraction and Digestion

PGW (planktonic) and AGW (attached) cells were collected for protein extraction as described previously.[29-30] Two hundred microliter cell suspensions were lysed by beating with 0.5 g of glass beads (0.1 mm) using a mini-bead beater (MBB-8; Biospec Products) for 3-5 min with cooling on ice for 5 min. Cellular debris was removed by centrifugation and the collected supernatant was centrifuged at 15000 × *g* for 30 min at 4°C.[33] Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Victoria, Australia) according to the manufacturer's instructions. Volumes of protein extract containing 50 µg of protein were transferred to clean Protein LoBind microcentrifuge tubes (Eppendorf South Pacific, North Ryde, NSW, Australia), frozen with liquid nitrogen, and freeze-dried for 2 h using a Dynavac mini ultra-cold vacuum freeze drier (Technolab, Kingston, Tasmania, Australia). The concentrated protein samples were alkylated with iodoacetamide, reduced, and then digested overnight with trypsin, then transferred to HPLC vials (Waters, USA).[21]

Peptide identification

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis was performed using an LTQ-Orbitrap XL tandem mass spectrometer using

chromatographic and data-dependent MS parameters as previously described[36]. Briefly, trypsin digest products were loaded onto a C18 trapping column at 50 µl/min (Peptide CapTrap, Michrom Bioresources) then separated on an analytical nanoHPLC column packed with 5 µm C18 media (PicoFrit Column, 15 µm i.d. pulled tip, 10 cm, New Objective) controlled using a Surveyor MS Pump Plus (ThermoFisher Scientific). A flow splitter was used to achieve a final flow rate of 200 nL/min. Proteins in the sample were identified by matching the MS2 spectra against trypsin and the complete *L. monocytogenes* non-redundant databases of strains EGD-e for Siliken_1/2c (NCBI accession code NC_003210, 2857 proteins) and F2365_4b (NC_002973, 2849 proteins) using the Labkey server Version 8.1 (www.labkey.org). A precursor ion tolerance of 20 ppm and a fragment-ion mass tolerance of 0.5 Da were employed. In addition we used a decoy database based on reverse peptides derived from the EGD-e and F2365 protein sequences to assess the level of false positive identifications.

Statistical analysis

Relative protein abundances between growth conditions were determined using the spectral counting method[37]. Statistical significance of the differences in spectra abundances for protein identifications between samples were assessed using a likelihood ratio test for independence (G-test) adjusted using the William's correction to reduce the false positive rate.[38-39] A scatter plot of fold-change of 2.5 was determined to be significant at $p<0.05$ by adapting the LOESS method[40] and only proteins greater than or equal to both criteria were considered.[10] In order to gain a broad understanding of the sources of differences in dataset the individual biological replicates were initially analyzed using non-parametric multi-dimensional scaling (nMDS) plots to establish that biological factors such as temperature, strain type and

attachment state were testable hypotheses. Canonical analysis of principal coordinates (CAP)[41] was then used to visualize these predefined factors in a scatter plot and determine whether the centroids of the groups (multidimensional vector of means) did or did not differ, with the latter indicting a null hypothesis. The *a priori* hypotheses were then assessed by a permuted analysis of variance (PERMANOVA), which can then be used to parametrically define a significance value. These statistical tests were performed in the Primer-6 v. 6.1.12/PERMANOVA+ v. 1.02 software package (Primer-E, Plymouth, UK). For PERMANOVA, pairwise tests and 9999 permutations were used for analysis of the design table.

The proteomics responses were also examined at the functional level with identified proteins classified into functionally allied sets on the basis of gene ontology.[6,21] The T-Profiler method was used to statistically test the significance of changes in overall protein abundance within the sets.[42] A t-value of >+3 and <-3 can be considered statistically significant for all sets, regardless of size ($p<0.01$). The t-values generated from T-Profiler were clustered by unsupervised hierarchical analysis to form a heat map as done previously.[6, 21] Venn diagrams were created using VENNY (J. C. Oliveros, 2007, http://bioinfogp.cnb.csic.es/tools/venny/index.html)

qRT-PCR validation. Seven genes considered significant in relation to attachment processes were analysed by quantitative real-time reverse transcription-PCR (qRT-PCR) for each treatment type. Forward and reverse PCR primers were designed using Primer3Plus software[4] (listed in supplementary datafile 4). The 16S rRNA gene was used for normalization since its expression has been described as largely stable in *L. monocytogenes* under a range of stress conditions[44]. Biomass was generated according to the above approach and RNA was obtained using the micro

RNeasy RNA extraction kit (Qiagen) and treated with RNase-free DNAse to remove contaminating DNA. RNA was quantified on a Fragment Analyzer (Advantec Analytical techniologies Inc., Ames, IA). RT-PCR was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) following the manufacturer's instructions. Real-time PCR was performed using dilutions (10^{-2}, 10^{-4}, 10^{-6}) of the template cDNA. RT-PCR reaction mixes contained a total volume of 25 µl and consisted of 0.5 µl mastermix, RNA (from 0.005 to 5 ng), 0.5 µM each forward and reverse primer, and the remainder nuclease-free water. All RT-PCR reactions were set-up using a CAS1200N liquid handling station (Corbett Robotics) and thermocycling and data recording performed on a Rotor-Gene Q (Qiagen) under the following conditions: 50°C for 30 min (reverse transcription step), 95°C for 15 min, 40 consecutive cycles consisting of 94°C for 15 s and 60°C for 30 s, and 72°C for 30 s. Melting curve analysis (55 to 95°C, increment of 0.11°C/sec) was performed to ensure PCR specificity. The PCR efficiencies obtained were >0.89. Linearity observed between diluted samples had a linear regression values (r^2) of >0.97. The method described by Pfaffl[45] was employed to determine the expression fold changes of the target gene in cultures in each treatment with comparison made agains RNA extracted from planktonic samples grown at 25°C.

Tolerance to acid and oxidative shock. Tolerance to acidic conditions and oxidative stress of planktonic and attached cells was deternined by survival of biomass gown above that was diluted to a concentration of approximately 10^9 CFU/ml. Cell suspensions were fully dispersed in 10 ml BHI broth in 15 ml sterile stubes that had been adjusted to either pH 2.0 (using HCl) or included 15% (vol/vol) absolute ethanol. All tubes, including controls (which were not exposed to low pH broth or peroxides), were incubated for 2 h at 37°C statically. Cell viability was assessed by standard plate

counting on BHI agar plates with plates incubated at 37°C. Data was obtained from two independent experiments, each performed in duplicate.

RESULTS AND DISCUSSION

Experiments were performed to determine if the proteomes of attached *L. monocytogenes* strains (supplementary information files 1 and 2) were markedly altered when compared to the corresponding planktonic cells, with variables including strain genetic type, attachment ability and growth temperature considered. The clear differences between the attachment capacity of the Siliken_1/2c and F2365_4b at 25°C and 37°C after 24 hours of incubation (Fig. 1) demonstrates their different capacities to adhere to glass wool as well as the detectable temperature dependency of the attachment. From cells harvested from this system the total number of proteins identified and quantified that passed filtration criteria are shown in Table 1. They represent approximately 20-30% of the proteome and were largely cytosolic. Alterations in protein abundance and gene expression do not necessarily lead to different phenotypes, instead changes can be compensatory in nature maintaining extant phenotypes. That said, based on existing literature *L. monocytogenes* behaves in particular ways that allows us to interpret the proteomic data to determine relevant phenotypes associated with the different growth conditions and strain differences comprising the dataset described here.

Comparisons of proteomes from attached and planktonic cells of different *L. monocytogenes* strains. Since temperature and strain are known to interact to affect attachment[28], PERMANOVA and CAP analysis, following *a priori* testing using nMDS, was used to determine the greatest sources of differences the datasets. The

PERMANOVA analysis results (Table 2), with data visualized within a CAP plot (Fig. 2), indicated that the overall protein profiles between attached and planktonic cells were largely similar when strains and temperatures are not considered as independent variables (p>0.14). However, when treated independently, temperature, attachment state, and strain genetic type were all distinct variables (p<0.0013). Incubation temperature contributed the single largest source of difference in the dataset (pseudo-F=21.4, p<0.001) and independent of strain-distinct differences between planktonic and attached cell derived datasets (p=0.007) can be observed. Strain genetic differences and attachment state when treated independently imparted smaller, similar degrees of difference (pseudo-F=6.2-6.8, Table 2). There was more dispersion and distinctiveness of protein profiles of the individual treatments from 25°C grown cultures, which may reflect the lower amount of retrievable biomass from glass wool with higher levels of loosely attached cells potentially creating a less balanced dataset.

A heat map based on hierarchically clustered *t*-values (Fig. 3) provided both an indication of functional trends but also unsupervised comparisons of the strains and treatments in relation to a designated control state, in which cells were grown at 25°C and harvested as planktonic cells. The clustering revealed similar treatment relationships to that of the CAP plot (Fig. 2), in which significant alterations in protein abundance were most readily apparent in cultures grown at 37°C. Based on the heat map *t*-value data the functionally-related changes associated to attachment (in comparison to planktonic cells) are observable. At 37°C attachment promotes, independent of strain, proteins associated with carbohydrate related metabolism and central glycolytic pathways, while DNA replication, tRNA/ribosome processing, ribosomal and tRNA aminoacyl synthesis protein groups are reduced in overall

abundance. Reduced abundance of motility/chemotaxis proteins suggests the development of a sessile state since at 25°C cells are normally peritrichously flagellated. At 25°C the differences between attached and planktonic states was much more divergent between strains. The large effect of temperature on the *L. monocytogenes* proteomes is consistent with previous studies that have used phenotypic and transcriptomic approaches[10,46,47] Strain genetics and attachment states have discernible influence on proteomes that are most visible at 37°C where attachment is most greatly promoted and large differences in attachment levels occur between the strains studied (Fig. 1).

Virulence proteins and the effect of attachment status. The ability of *L. monocytogenes* to cause disease involves proteins that allow attachment to host cells, cell invasion, evasion of the immune system, and cell-to-cell spread[46]. However, our data suggests that attachment is only associated with specific virulence-associated factors and provides no evidence for attachment via virulence factors associated with cell adhesion or internalisation. Neither is there evidence that "virulence capacity" per se is promoted at 37°C or when attached. Virulence-associated proteins increased by attachment of both strains at 37 (Fig. 4) included listeriolysin (LLO) and bile salt hydrolase (Bsh) in addition to proteins with a broader house-keeping role (protein turnover - ClpC, ClpE, ClpP; oligopeptide uptake – OppA, OppD, OppF; protein lipolylation – LplA). Comparisons between attached and planktonic cells otherwise showed no consistent patterns especially for cells grown at 37°C. Proteins with connection to GI tract and host-associated survival, such as Bsh, were found to be more abundant in both planktonic and attached Siliken_1/2c cells at 37°C when compared with cultures grown at 25°C (up to 20 fold more abundant) and the pore-

forming protein LLO was also more abundant, significantly in the case of Siliken_1/2c attached cells grown at 37°C (3-6 fold). These proteins showed strain dependent and attachment dependent responses, however, the other proteins relevant to virulence that have only general support roles were likely primarily promoted at 37°C due to the induction of stress responses at that temperature, such as increased rates of mis-folded and aggregated proteins. Other virulence proteins were not detected due to extraction bias and due to culture conditions suppressing their synthesis.

The differentially affected protein complement in attached versus planktonic cells. The numbers of proteins that increased and decreased significantly in abundance in attached Siliken_1/2c and F2365_4b cells compared to planktonic counterparts are shown in Venn diagrams (Fig. 5) and in supplementary information file 3. Only 8 proteins demonstrated substantial changes in common between both strains and both temperatures tested. These included the following proteins: GroEL (Hsp60 chaperonin large subunit), DnaK (molecular chaperone), PtsH (phosphotransferase system phosphocarrier Hpr), PdxS (pyridoxine biosynthesis lyase subunit), and Pgi (glucose 6-phosphate isomerase). RpsB, RpsD, and RpsP (small subunit ribosomal proteins) all showed decreases in abundance. These results suggest in general attached cells potentially have increased protein folding, and increased carbohydrate uptake and metabolism but lower rates of protein translation. This response is indicative of lower growth rates since increased protein folding via GroEL and DnaK has very high energy expenditure in the cell[48], which would likely forces more carbohydrate metabolism to be directed away from anabolic processes. RT-PCR analysis of *groEL* and *rpsP* showed that these genes also had increased and decreased expression in attached cells (supplementary data file S3).

a) Effect of attachment on motility/chemotaxis proteins. Surface attachment is clearly associated with changes in cellular flagellation and thus related to changes in the electrostatic properties of the cell.[27] Our experiments showed that attached cells grown at 25°C had 3 to 9-fold less abundance of flagellin Fla than planktonic cells as well as the flagella hook protein FlgE (8-10-fold less) suggesting attached cells have overall reduced flagellation. At 37°C, in both planktonic and attached samples, Fla was either undetected or present at very low levels (Fig. 6) suggesting cells were generally denuded of flagella. Interestingly, the results suggest that at 25°C the attached cells undergo flagella synthesis repression where normally flagellin synthesis is maximal. At either 25°C or 37°C the abundance of flagella motor switch protein FliM remained relatively constant in both strains suggesting the flagella motor protein complex itself does not seem substantially affected in attached cells. Chemotaxis sensor proteins CheA and CheY were also reduced in abundance in attached cells grown at 25°C indicating most attached cells enter a sessile state with presumably reduced active tactic behavior (Fig. 6). RT-PCR analysis of *fla* expression showed good agreement with the proteomic data (supplementary data file S3).

Cell wall biogenesis related changes. At a growth temperature of 25°C there were little consistent changes in cell wall biogenesis-related protein abundances, either between attachment states or between strains. The results do suggest that, in general, peptidoglycan biosynthesis seems to be repressed in attached cells, which may reflect reduction of cellular growth rates. D-alanylation of teichoic acid (TA), which determines the distribution of TA throughout the cell wall, seems also to be affected. This process is carried out by 4 proteins (DltABCD) coded within a single operon. DltB, being a transmembrane protein, was underrepresented in the dataset.

DltA was also at low abundance, but was most abundant in Siliken_1/2c planktonic cells grown at 37°C but not detected in attached cells, while in F2365_4b the highest abundance of DltA occurred in attached cells grown at 25°C. In both strains, especially Siliken_1/2c, increased DltD and DltC abundance at 37°C (Fig. 7). RT-PCR data suggests that both temperature and attachment promotes the expression of the *dltD* gene (supplementary data file 4) confirming the proteomic data in principal. The DltD protein could be linked to promotion of adherence due to potentially increased D-alanylation of TA as observed in other related bacteria.[49] Elimination of the gene *dltD* has been shown to nearly abolish adherence of *L. monocytogenes* cells[50] The difference in the abundance of DltD, which promotes the transfer of D-alanine to the ligation site[51] could be a factor in the greater attachment of Siliken_1/2c compared to F2365_4b to polystyrene and glass wool surfaces. Assuming D-alanine residues are surface exposed, increased D-alanylation of TA could reduce cell surface electrostatic interactions that would act to impede initial attachment of cells due to less interaction with eDNA surface deposits (see below). The chemical distribution of D-alanine esterification and the effect of glycosylation of teichoic acid needs further study since it is unclear to what degree they influence adherence and whether this is reflected in physiologically or genetically imposed differences in cellular adhesion.

Cell surface proteins. Besides proteins involved in cell wall biogenesis, *Listeria* secretes a range of proteins that are covalently anchored to peptidoglycan via sortases, some of which have membrane insertions. Most are exposed on the cell surface to some degree. Various surface exposed proteins have possible adhesin-like roles.[52] Besides well studied internalins (such as InlA and InlB) that have roles in host cell adhesion and invasion, little information is available about individual contributions

of surface proteins to adherence to inanimate surfaces. Only one peptidoglycan-bound protein, termed BapL (biofilm-associated protein, lmo0435) has been linked directly to surface adherence in strains belonging to lineage II (such as Siliken_1/2c) but absent in lineage I strains (F2365_4b)[53] For Siliken_1/2c it was observed that BapL abundance, though low, was not enriched in attached cells and was only slightly more abundant at 25°C (not statistically significant), suggesting a direct role in adherence by BapL seems doubtful. RT-PCR data also indicates gene expression of *bapL* in Siliken_1/2c (supplementary data file 4) was repressed at 37°C and when attached further suggesting this protein does not seem to have a direct role in attachment. No other cell surface protein were found to be enriched in the attached cell samples of either strain suggesting no one surface protein seems to be directly linkable to surface attachment.

Extracellular DNA. Extracellular DNA (eDNA) of high molecular weight interacting with cell wall-derived N-acetylglucosamine has been shown to promote early attachment of *Listeria*.[19] In another recent study eDNA deposition seems to be carried out by the DNA translocase FstK[54], which is normally involved in cell division. Due to the membrane-bound nature of FtsK (lmo1386) and extraction bias it was uncertain that FtsK was more abundant in attached cells since few spectra were detected (Fig. 8). RT-PCR analysis; however indicated the *ftsK* gene had greater expression in attached cells of both (9-50 fold) (supplementary data file 4). Autolysins normally associated with peptidoglycan turnover and with a potential to allow DNA to leak from cells were not promoted in either planktonic or attached cells of either strain. DNA synthesis associated enzymes were also at the same or lower abundance between attachment states. Thus our data does not provide a compelling case that

eDNA is a determinant aspect of *L. monocytogenes* attachment efficacy. We suspect that eDNA deposition is more of a passive process though our results support a connection with FtsK.

Quorum sensing related proteins. Adherence to glass has also been linked to the *agr* quorum sensing gene cluster that encodes a two component sensory system (AgrAC) and a quorum sensory peptide and its exporter (AgrDB). The function of the agr system is still poorly understood but is known that *agr* gene expression is enhanced in attached cells in a growth phase dependent manner while this is not the case in planktonic cells. Deletion of genes *agrA* or *agrD* results in *L. monocytogenes* strain EGD-e having poorer adherence[55]. We observed that, at both temperatures, Siliken_1/2c attached cells showed substantially more abundance of the quorum sensing peptide processing endopeptidase AgrB while in F2365 this was significant only at 25°C (Fig. 8). AgrB is required for production of the mature quorum sensory peptide AgrD as determined in an analogous version of the agr system in *Staphylococcus aureus*[56] thus it is assumed AgrB abundance is indicative of greater synthesis of AgrD However, AgrD could not be detected in our experiments and further work is needed to unravel the role of this system.

The enzyme S-adenosylhomocysteine/5'-methylthioadenosine nucleosidase (Mtn) converts S-adenosylhomocysteine (SAH), a toxic metabolite derived from the important cofactor S-adenosylmethionine, to S-ribosylhomocysteine (SRH). S-ribosylhomocysteine lyase (LuxS) converts SRH back to L-homocysteine. SRH has been considered to be the precursor of the chemical messenger autoinducer-2, however in *L. monocytogenes* (and many other bacteria) SRH is only part of the SAH detoxification and methionine salvage cycle and has yet to be shown to have a quorum sensory

role[57]. Despite this a mutant forming a truncated *luxS* product was found to be substantially more adherent[58]. Mtn levels were higher at 37°C then 25°C in both strains while LuxS abundance was not substantially altered (Fig. 8). RT-PCR of the *luxS* gene suggests gene expression was repressed (supplementary data file 4), which would agree with the concept that low levels of the *luxS* transcript seems to lead to a hyper-attaching state. This difference with the proteomic data may suggest LuxS protein is relatively stable in the cell. The persistent level of LuxS likely maintains a capacity for the cell to deal with any accumulation of SAH.

The proteomics-based results do circumstantially suggest a possible role of the Agr system quorum sensing and more indirectly the Mtn/LuxS systems in attachment in *Listeria*, however the downstream gene cascade of the regulatory responses and associated physiological and metabolic links remain rather unclear. Furthermore, the data suggests Mtn/LuxS abundance is temperature dependent and potentially is linked with temperature-dependent influences on rates of attachment observed for various strains of *L. monocytogenes*[28], however further work is needed to understand whether Mtn/LuxS-derived metabolites such as SAH and SRH directly or indirectly influence attachment or are the consequence of other adherence-influencing metabolic adjustments.

Growth rate deceleration and stress induction in attached cells. We observed that attached cells have a temperature-dependent altered metabolism based on changes in proteins which has overall similar to the state achieved during the acid tolerance response[6]. Firstly, a large number of proteins involved with lipid-related metabolism, nucleic acid and nucleotide metabolism, DNA replication, transcription, mRNA turnover, tRNA amino-acyl synthesis, ribosomal proteins, protein synthesis,

protein turnover and cell division demonstrate reduced relative abundance in attached cells of both strains at both temperatures, especially at 37°C (summarized in supplementary data file 3). This general response is strongly indicative of slowed growth rates since most cell building block processes have decreased in relative terms. However, proteins associated with carbohydrate uptake and fermentation, the TCA cycle, and nascent protein folding (GroES, DnaK) are overall more abundant in attached cells (summarized in supplementary data file 3). This is a typical observation in cells that have undergone an acid tolerance response since in order to develop and maintain pH homeostasis, for example H^+ extrusion by the F_1F_0-ATPase complex, more cellular energy is funneled away from anabolic processes resulting in slowed growth[6]. The SigB regulon, which mediates acid resistance in *L. monocytogenes*[59], was also enhanced in attached Siliken_1/2c cells when grown at 25°C compared to planktonic cells while the proteins making up the regulon were more abundant in Siliken_1/2c compared to strain F2365_4b (Fig. 9). Promotion of SigB regulated proteins in Siliken_1/2c was equal between planktonic and attached cells cultured at 37°C. The data suggests a SigB-related attachment response seems to occur at least in Siliken_1/2c. SigB-dependent glutamate decarboxylase GadB2 (lmo2434), which aids in acid shock protection via H^+ removal was more abundant at 37°C than at 25°C. However, the abundance of this protein was not enhanced in attached cells independent of temperature. The SigB-*independent* glutamate decarboxylase GadB1 that provides constitutive pH homeostasis[6] was promoted in attached cells at 25°C (7-fold and 2-fold greater abundance in Siliken_1/2c and F2365_4b, respectively) but not altered at 37°C. This suggests the SigB-associated responses could be related to the consequences of the attachment.

At 37°C a range of stress protective proteins are also elevated in abundance compared to that at 25°C, while a smaller difference was observed between attached and planktonic cells. At 37°C the peroxide stress regulatory protein PerR and its associated regulon[60] were more abundant in general while the Fur regulon[61], which controls iron acquisition and storage is more abundant in attached cells (Fig. 10). Both Fur and PerR act together to tightly control iron uptake since excessive levels causes oxidative stress via the Fenton reaction.[61] The results suggest that attached cells (at 37°C) exhibit oxidative stress related responses. The increased level of Dps likely acts to protect DNA from oxidative damage.[62] These changes are consistent with a study that demonstrated superoxide dismutase (Sod) has an important role in *Listeria* biofilms.[6e] Attached cells were found to have increased abundance of a number of other stress protective proteins though the responses were either strain- and temperature dependent. At 25°C both strains showed increases in the non-heme iron-binding protein Fri/Dps (4-5 fold), conjugated bile salt hydrolase (3-19 fold) and a DJ-1/PfpI protein family YhbO-like protein (lmo2256) that in *E. coli* and *Bacillus subtilis* is required for either protection or repair of oxidation, pH, and UV stress associated stress damage.[64] Acid (HCl, pH 2.0) and ethanol (15% vol/vol) shock experiments (Fig. 11) confirmed that attached cells of both strains were more resistant than planktonic cells to acid and oxidative stress at 25°C. At 37°C only Siliken_1/2C showed a higher level of resilience when attached. Strain Siliken_1/2c also had overall more stress tolerance then F2365_4b. Since SigB regulon interacts with both the Fur and Per regulons[60,61] the results suggest possible indirect involvement of SigB-regulated genes and proteins in the attachment process that is strain dependent in terms of the intensity of the response. It has been clearly shown that *L. monocytogenes* strains have

different stress tolerance capacities and also differ in terms of environmental persistence[28] The SigB regulon incorporates several genes that are strain dependent[65] and include several of unknown function that potentially have influential roles in attachment processes. Taking these features into account the results here may suggest that differences in regulatory architecture must be important in dictating attachment and persistence and thus further studies would need to focus on determining if consistent genetic programs can be shown to occur between different genetic groups with *L. monocytogenes* coupled to attachment and stress tolerance phenotypes.

CONCLUSIONS

The proteomics approach that was used here provides the advantage of providing an overview of the cell population under particular circumstances including strain type, culture conditions, and physical interactions. All impact the proteomes of *L. monocytogenes*, and strain differences can be quite large despite the strains having mostly the same gene complement. Also it must be assumed within the population the physiology of cells is not identical rather they form a distribution of responses thus the information captured here effectively is an “average” of the physiological state of a bacterial population. Though this inevitably leads to a lot of biological noise we can still estimate relative abundances of specific proteins with known (or suspected) properties that are potentially relevant to attachment processes and surmise their role at the protein-level. In this respect based on the data here listerial attachment to inanimate surfaces is not dictated at least as far as we can determine by any one specific surface protein but rather a more systematic modification of the cell surface. A good example of such a modification is D-alanylation of teichoic acid, which alters surface properties

of the cell causing them to become more hydrophobic[66]. Increases in D-alanylation have also been associated with cell wall thickening that leads to resistance to antibiotics, including antimicrobial peptides[67] and conceivably other chemicals, such as ethanol and those used in disinfectants. Other differences observed in attached cell proteomes also suggests changes to stress defense protein abundances occurs consistent with the observation that attached cells are more chemically resistant. The strain-dependent aspects of these changes also correlate to the fact that chemical resistance also varies considerably between strains. Thus the ability to attach is only part of a more complex adaptation regime that likely is both dynamic but also constrained by the inherent genetic and phenotypic capacity of a given strain. In the end strain-specific context must be acknowledged when exploring persistence and chemical-dependent stress resistance in bacteria. Overall, several variables play roles in determining a strain's inherent capacity for stress tolerance once attached and subsequent chance for persistence. Persistence cannot therefore be defined on the basis of specific gene-encoded properties. Only by comparing different strains can we capture the genetic predispositions relevant to complex phenomena such as environmental persistence. Given this could include a large array of observed responses, ideally multi-strain analyses will be a powerful means in understanding how bacteria adapt and interact within complex systems.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Data Files 1 and 2 includes the proteome data for strains Siliken_1/2c and F2365_4b, respectively. This includes Uniprot protein assignation via X!Tandem; ProteinProphet scores and associated error and number of unique peptides identified against L. monocytogenes proteomes ;raw, pooled and normalised spectral counts mapped to EGD-e or F2365 loci; fold-change ratios between treatments, G-Test base and William's corrected significance values; protein name and functional assignation; assigned functional class; amino acid length; and the total percentage protein coverage obtained for each identified protein.

Data File 3 lists proteins that have abundances significantly affected under the experimental conditions organized on the basis of strain and temperature of incubation. Fold-change and significance values included.

Data File 4 lists primers used in RT-PCR experiments and the fold changes compared between attachment state at 37°C relative to planktonic cells grown at 25°C.

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Supporting Information Available Statement

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org

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Table 1- Treatments and Protein numbers quantified in *L. monocytogenes* strains Siliken_1/2c and F2365_4b.

Strain	Treatment	Non-singleton proteins identified
Siliken_1/2c		
	37°C planktonic	724
	37°C attached	620
	25°C planktonic	475
	25°C attached	419
F2365_4b		
	37°C planktonic	580
	37°C attached	612
	25°C planktonic	438
	25°C attached	555

Table 2- PERMANOVA analysis comparisons of *L. monocytogenes* examining interactions between the proteome profiles of two different strains (Siliken_1/2c, F2365_4b), incubation temperatures (25°C, 37°C) and attachment states.

Variable	df	Sum of Squares	Pseudo-F	P(permuted)
Strain (St)	1	2.10E+05	6.2481	**0.0013**
Temperature (Te)	1	7.22E+05	21.451	**0.0001**
Attachment state (At)	1	2.29E+05	6.8223	**0.0006**
St × Te	1	57405	1.7066	0.1412
St × At	1	43375	1.2895	0.2391
Te × At	1	1.55E+05	4.6064	**0.0067**
St × Te × At	1	47325	1.407	0.212
Residuals	26	8.75E+05		
Total	33	2.33E+06		

Figure Legends:

Figure 1. The attachment capacity of *L. monocytogenes* strains F2365_4b and Siliken_1/2c grown at 25°C and 37°C measured using the crystal violet assay indicated the superior attachment ability of strain Siliken_1/2C is independent of temperature..

Figure 2. Canonical analysis of principal coordinates plot in which protein spectral count abundance profiles obtained from *L. monocytogenes* strains F2365_4b (black symbols) and Siliken _1/2c (open symbols) are compared against primary biological factors investigated in this study comprising growth temperature (25°C and 37°C) and attachment state (planktonic cells attached to glass wool). These factors were determined *a priori* to be significantly influential on protein profiles after initial testing by non-parametric multidimensional scaling and analysis of similarity.

Figure 3. Heat map showing abundance change trends in functional groups of proteins in *L. monocytogenes* strains Siliken_1/2c and F2365_4b grown in a planktonic (plank) or attached state (att; attached to glass wool), at two different temperatures (25°C and 37°C). Cells grown at 25°C planktonically was used as the baseline control condition. The color scale is based on t-values a statistical scoring method[42] for assessing the degree of change of a given functionally-allied protein set. The dendrograms on the top and left of the heat map, determined using the complete linkage method indicate the pattern of clustering of the samples analysed and also infer broader functional associations. The overall consensus indicates that both temperature and attachment states are very influential on cell functionality.

Figure 4. The relative change in abundance of virulence associated proteins obtained from glass wool attached and planktonic cells of *L. monocytogenes* strains Siliken_1/2a and F2365_4b grown at either 25°C or 37°C. Asterisks indicate a significant change in relative abundance ($p<0.05$) of the indicated protein. Overall, proteiuns most affected are support proteins for virulence and virulence proteins themselves show little significant change.

Figure 5. Venn diagrams showing the number of proteins that exhibited significant increased (top diagram) and decreased (bottom diagram) abundance (>2-fold, $p<0.05$) between *L. monocytogenes* strains Siliken_1/2c and F2365_4b grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum. Essentially the data indicates that fundamentally the variables lead to divergent proteomes and few proteins show collectively similar responses. Only 5 proteins (grey section) were significantly more abundant for both strains when attached at both incubation temperatures, including GroEL (Hsp60 chaperonin large subunit), DnaK (molecular chaperone), PtsH (phosphotransferase system phosphocarrier Hpr), PdxS (pyridoxine biosynthesis lyase subunit), and Pgi (glucose6-phosphate isomerase). Similarly, RpsB, RpsD, and RpsP (small subunit ribosomal proteins) were the only proteins found to be significantly less abundant when all variables were considered.

Figure 6. Relative abundance (normalized spectral counts) of proteins associated with either motility or chemotaxis processes. Data is derived from two *L. monocytogenes*

strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum. Values on the bars for FlaA are here out of the scale (>60) SpC values.

Figure 7. Relative abundance (normalised spectral counts) of proteins associated with teichoic acid D-alanylation (DltA, DltB, DltC, DltD). Data is derived from two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum.

Figure 8. Relative abundance (normalised spectral counts) of proteins associated with quorum sensing (AgrB of the accessory *agrACBD* gene system) as well as proteins associated with putative chemical messenger-like or detoxification activities including Mtn (5-methylthioribose kinase) and LuxS (S-ribsyl-homocysteine lyase). Data is derived from two *L. monocytogenes* strains grown at either 37°C or 25°C with biomass investigated either taken from planktonic or attached fractions of cultures containing glass wool as an adherent substratum.

Figure 9. Changes in the relative abundance of proteins coded by genes belonging to the SigB core regulon[59]. The abundance changes are analysed using the T-profiler method[42]. Asterisks indicate significant differences between treatments (** $p<0.01$) and the control condition which includes cells grown at 25°C that are fully planktonic.

Figure 10. Changes in the relative abundance of proteins coded by genes belonging to the PerR[60] and Fur[61] regulons. The abundance changes are analysed using the T-profiler method[42]. Asterisks indicate significant differences between treatments (* P<0.05) and the control condition which includes cells grown at 25°C that are fully planktonic.

Figure 11. The reduction of viable planktonic or attached *L. monocytogenes* biomass following sudden exposureof cells to acid stress (pH 2.0, 2 hours) shown in the top graph and to ethanol stress (15% vol/vol ethanol, 1 hour) in the lower graph. The reduction is expressed as viable cell count obtained by the agar plate dilution method. Cell numbers are expressed as the logarithm of the estimated number of colony forming units (CFU) per ml. l. The letters above the bars indicate significant differences between values; bars that do not share the same letters have significantly different ($p<0.05$) values as determined by ANOVA analysis.

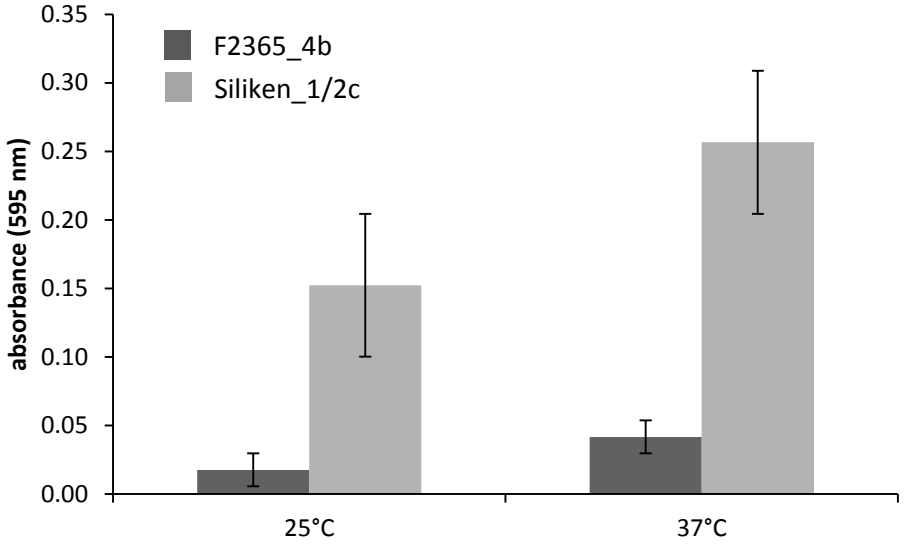

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
absorbance (595 nm)
F2365_4b
Siliken_1/2c
25°C
37°C

Figure 1

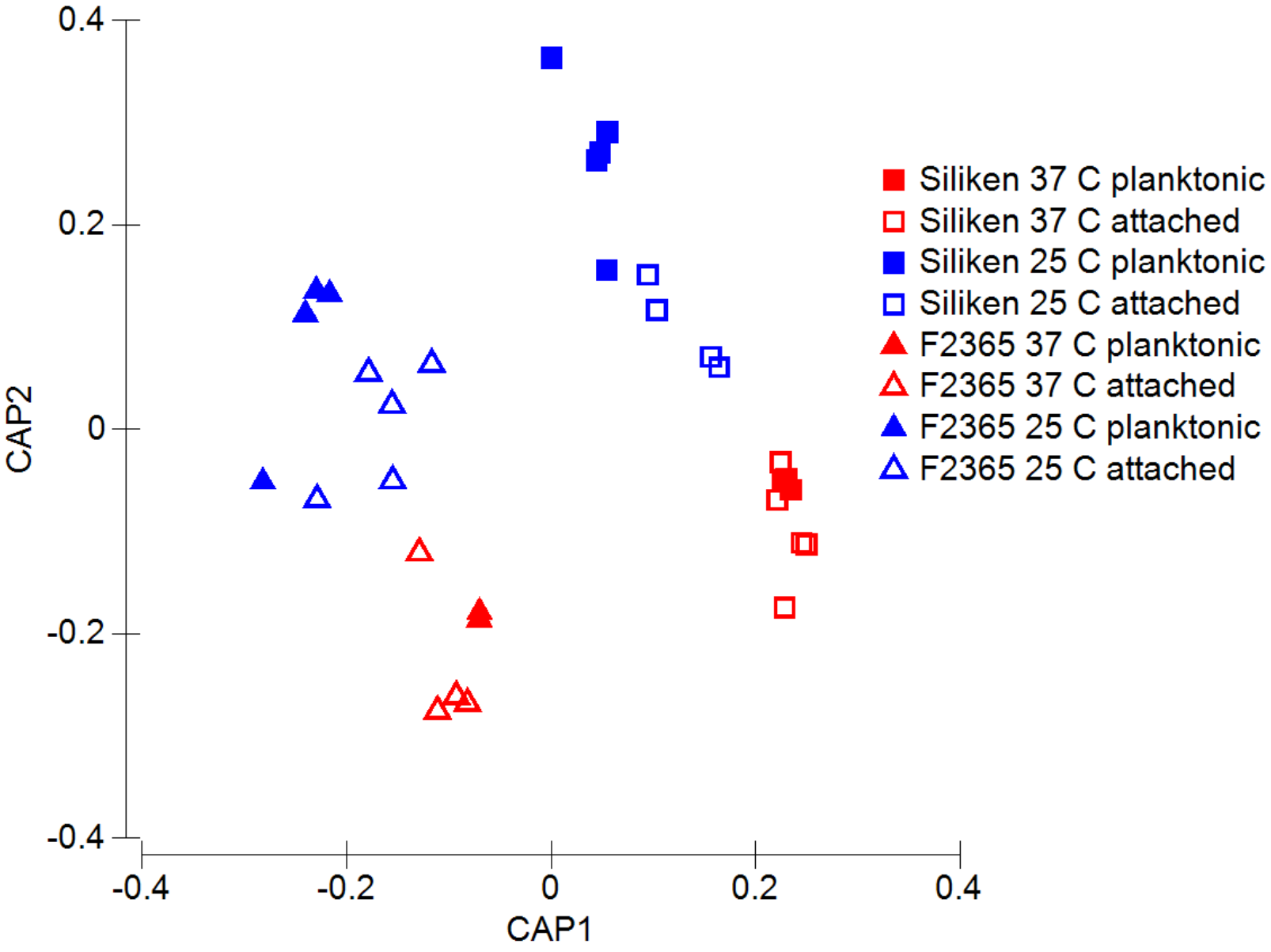

0.4
0.2
0
-0.2
-0.4
CAP2
-0.4
-0.2
0
0.2
0.4
CAP1
Siliken 37 C planktonic
Siliken 37 C attached
Siliken 25 C planktonic
Siliken 25 C attached
F2365 37 C planktonic
F2365 37 C attached
F2365 25 C planktonic
F2365 25 C attached

Figure 2

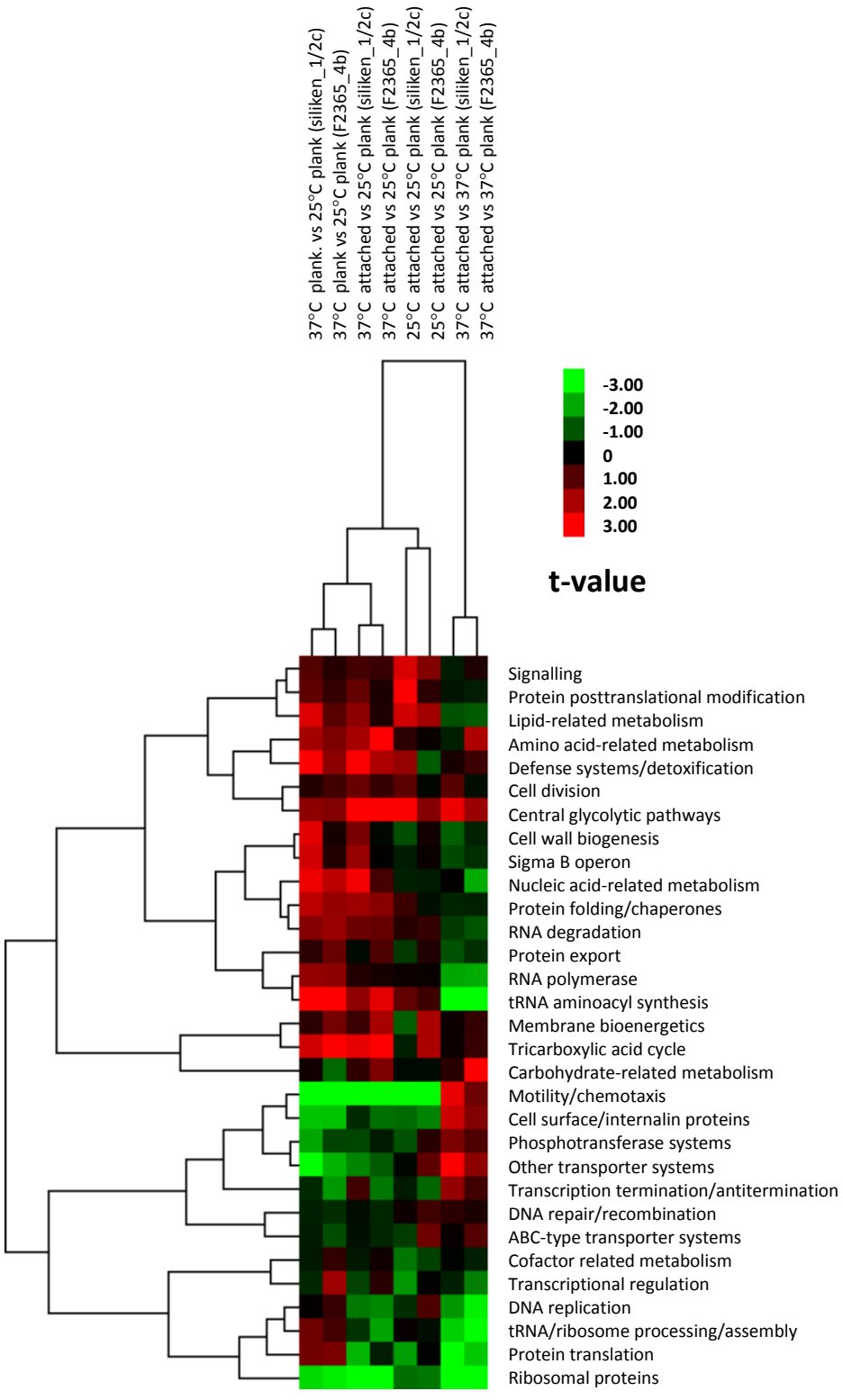

37°C plank. vs 25°C plank (siliken_1/2c)
37°C plank vs 25°C plank (F2365_4b)
37°C attached vs 25°C plank (siliken_1/2c)
37°C attached vs 25°C plank (F2365_4b)
25°C attached vs 25°C plank (siliken_1/2c)
25°C attached vs 25°C plank (F2365_4b)
37°C attached vs 37°C plank (siliken_1/2c)
37°C attached vs 37°C plank (F2365_4b)
-3.00
-2.00
-1.00
0
1.00
2.00
3.00
t-value
Signalling
Protein posttranslational modification
Lipid-related metabolism
Amino acid-related metabolism
Defense systems/detoxification
Cell division
Central glycolytic pathways
Cell wall biogenesis
Sigma B operon
Nucleic acid-related metabolism
Protein folding/chaperones
RNA degradation
Protein export
RNA polymerase
tRNA aminoacyl synthesis
Membrane bioenergetics
Tricarboxylic acid cycle
Carbohydrate-related metabolism
Motility/chemotaxis
Cell surface/internalin proteins
Phosphotransferase systems
Other transporter systems
Transcription termination/antitermination
DNA repair/recombination
ABC-type transporter systems
Cofactor related metabolism
Transcriptional regulation
DNA replication
tRNA/ribosome processing/assembly
Protein translation
Ribosomal proteins

Figure 3

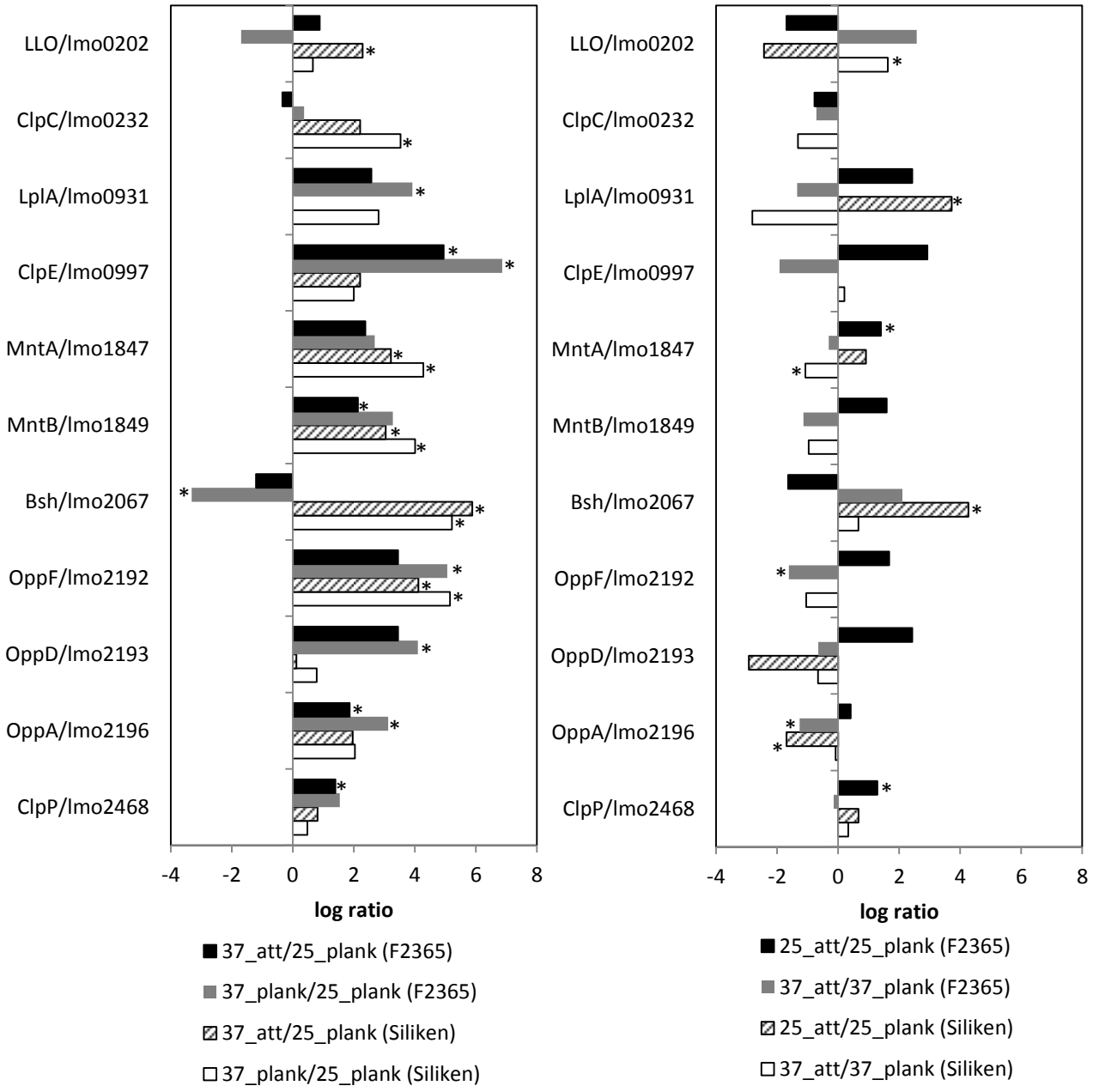
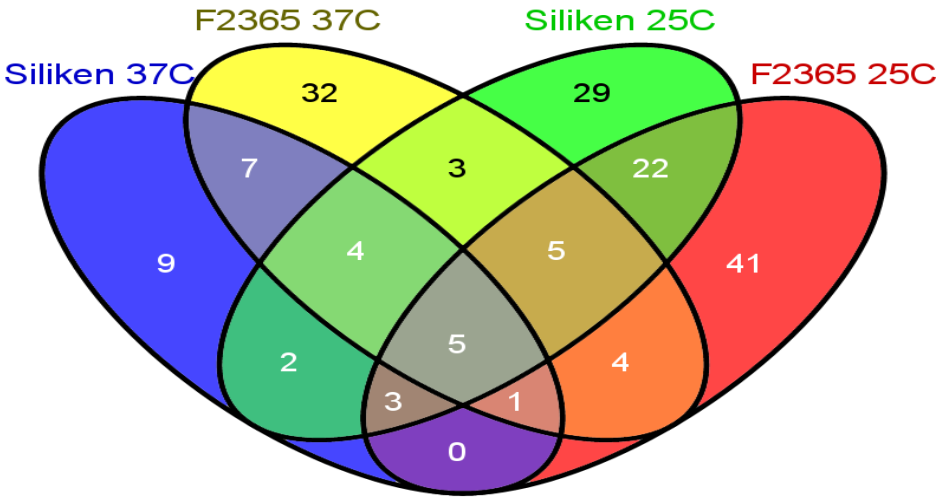

LLO/lmo0202
ClpC/lmo0232
LplA/lmo0931
ClpE/lmo0997
MntA/lmo1847
MntB/lmo1849
Bsh/lmo2067
OppF/lmo2192
OppD/lmo2193
OppA/lmo2196
ClpP/lmo2468
-4 -2 0 2 4 6 8
log ratio
37_att/25_plank (F2365)
37_plank/25_plank (F2365)
37_att/25_plank (Siliken)
37_plank/25_plank (Siliken)
LLO/lmo0202
ClpC/lmo0232
LplA/lmo0931
ClpE/lmo0997
MntA/lmo1847
MntB/lmo1849
Bsh/lmo2067
OppF/lmo2192
OppD/lmo2193
OppA/lmo2196
ClpP/lmo2468
-4 -2 0 2 4 6 8
log ratio
25_att/25_plank (F2365)
37_att/37_plank (F2365)
25_att/25_plank (Siliken)
37_att/37_plank (Siliken)

Figure 4

Proteins with increased abundance:



Proteins with reduced abundance:

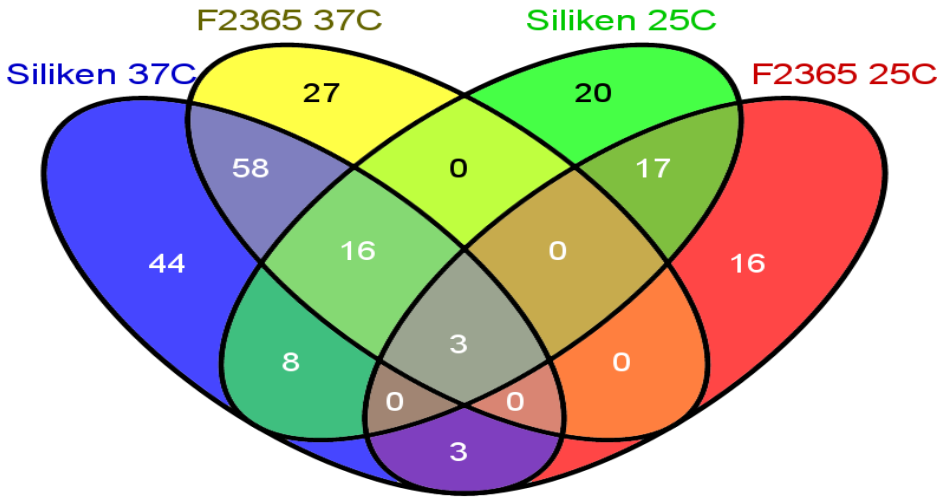


Figure 5

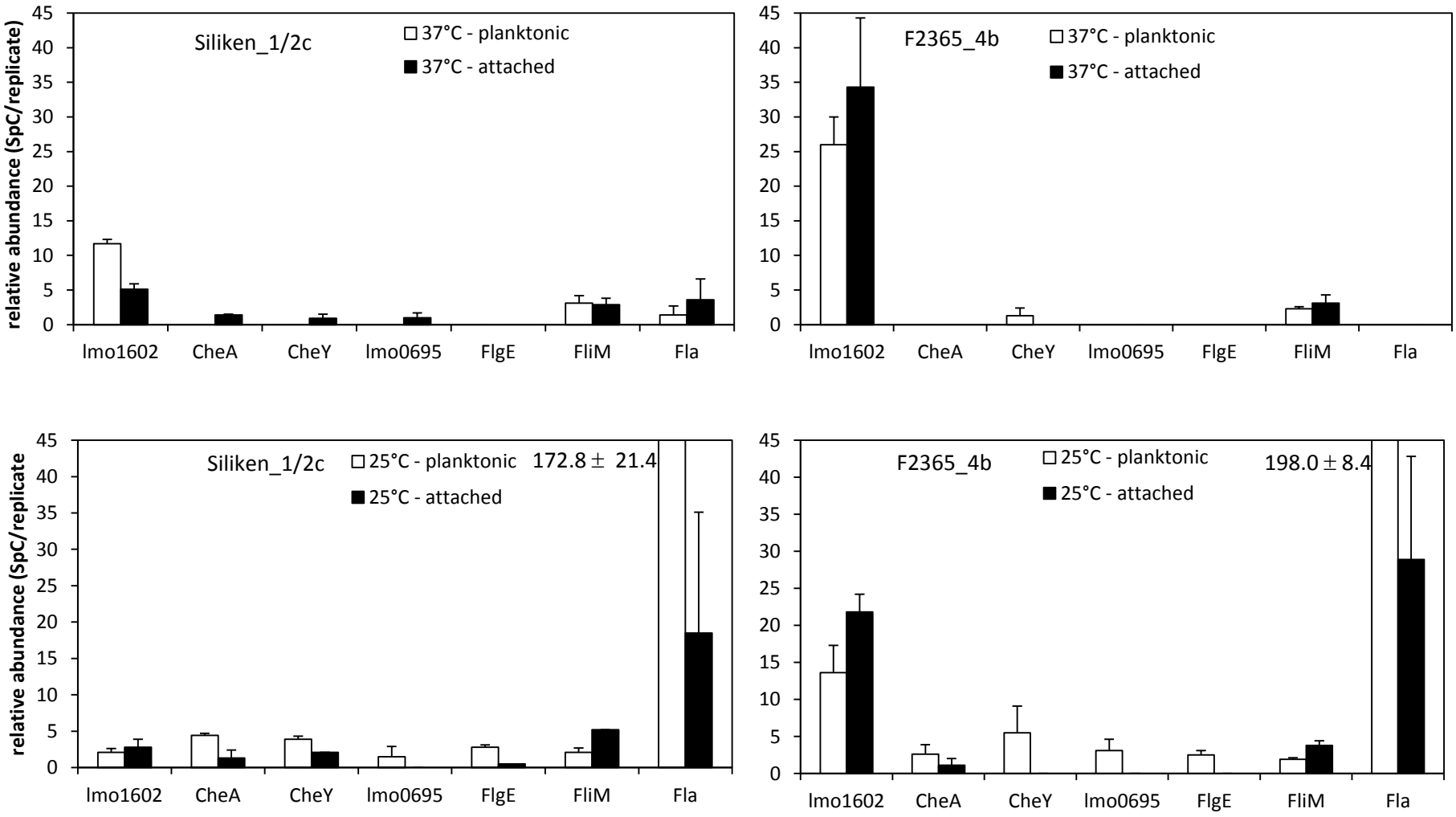

Siliken_1/2c
37°C - planktonic
37°C - attached
relative abundance (SpC/replicate)
lmo1602
CheA
CheY
lmo0695
FlgE
FliM
Fla
F2365_4b
37°C - planktonic
37°C - attached
lmo1602
CheA
CheY
lmo0695
FlgE
FliM
Fla
Siliken_1/2c
25°C - planktonic
172.8 ± 21.4
25°C - attached
relative abundance (SpC/replicate
lmo1602
CheA
CheY
lmo0695
FlgE
FliM
Fla
F2365_4b
25°C - planktonic
198.0 ± 8.4
25°C - attached
lmo1602
CheA
CheY
lmo0695
FlgE
FliM
Fla

Figure 6

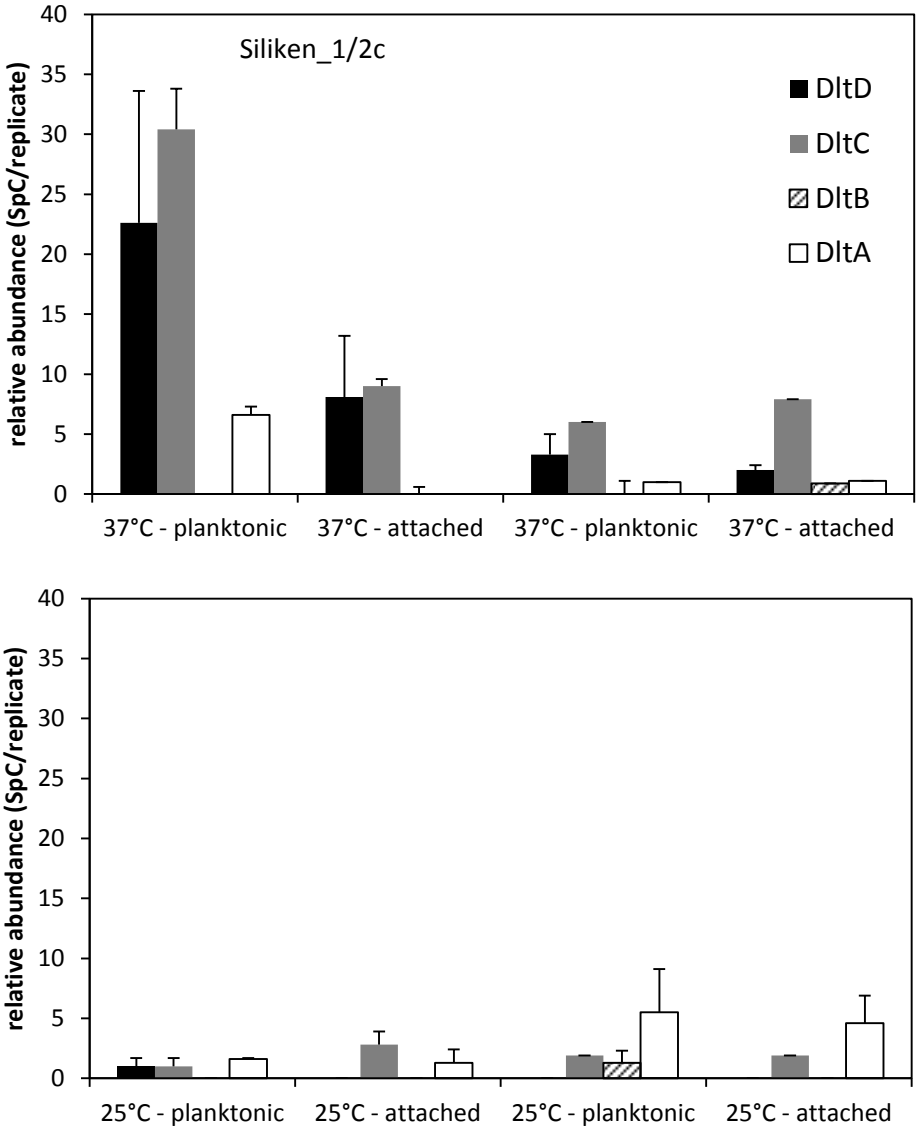

Siliken_1/2c
DltD
DltC
DltB
DltA
relative abundance (SpC/replicate)
0
5
10
15
20
25
30
35
40
37°C - planktonic
37°C - attached
37°C - planktonic
37°C - attached
relative abundance (SpC/replicate)
0
5
10
15
20
25
30
35
40
25°C - planktonic
25°C - attached
25°C - planktonic
25°C - attached

Figure 7

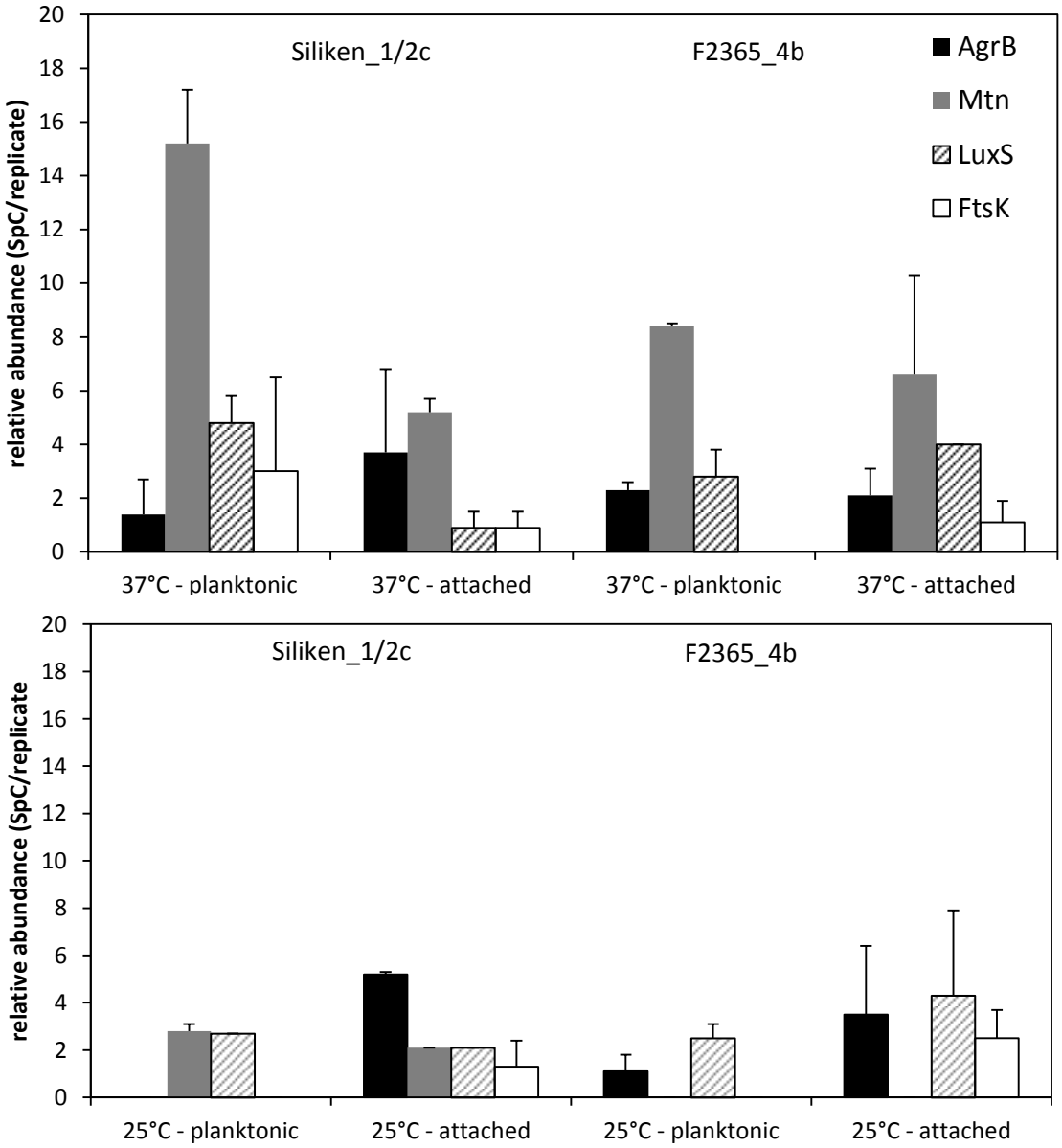

Siliken_1/2c
F2365_4b
AgrB
Mtn
LuxS
FtsK
relative abundance (SpC/replicate)
0
2
4
6
8
10
12
14
16
18
20
37°C - planktonic
37°C - attached
37°C - planktonic
37°C - attached
Siliken_1/2c
F2365_4b
relative abundance (SpC/replicate)
25°C - planktonic
25°C - attached
25°C - planktonic
25°C - attached

Figure 8

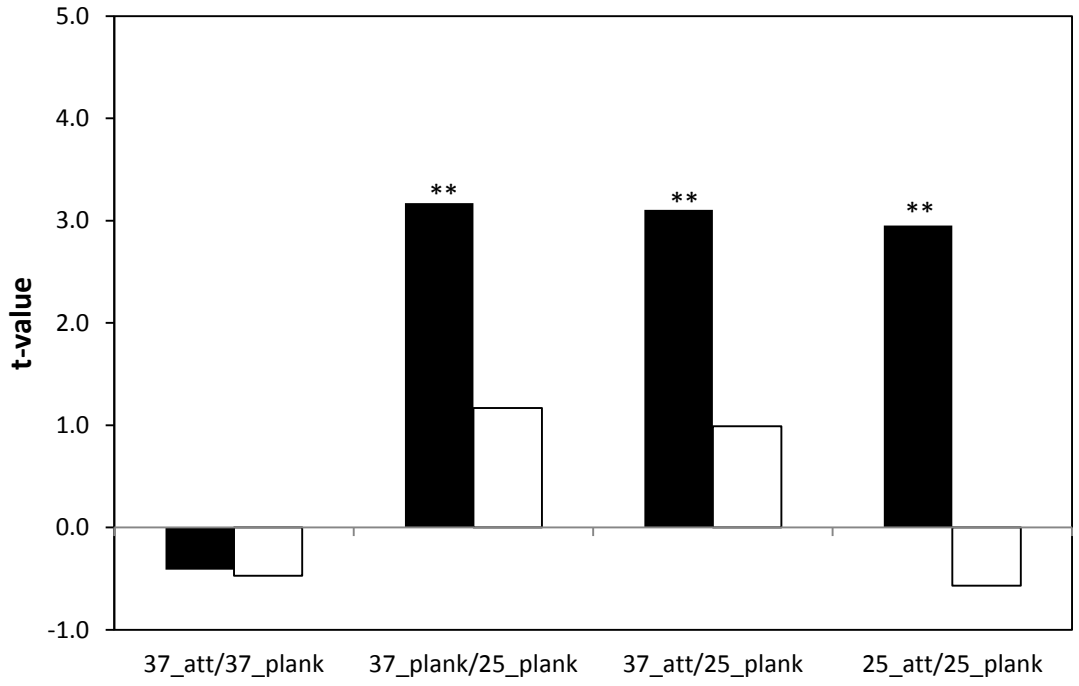

5.0
4.0
3.0
2.0
1.0
0.0
-1.0
t-value
**
**
**
37_att/37_plank
37_plank/25_plank
37_att/25_plank
25_att/25_plank

Figure 9

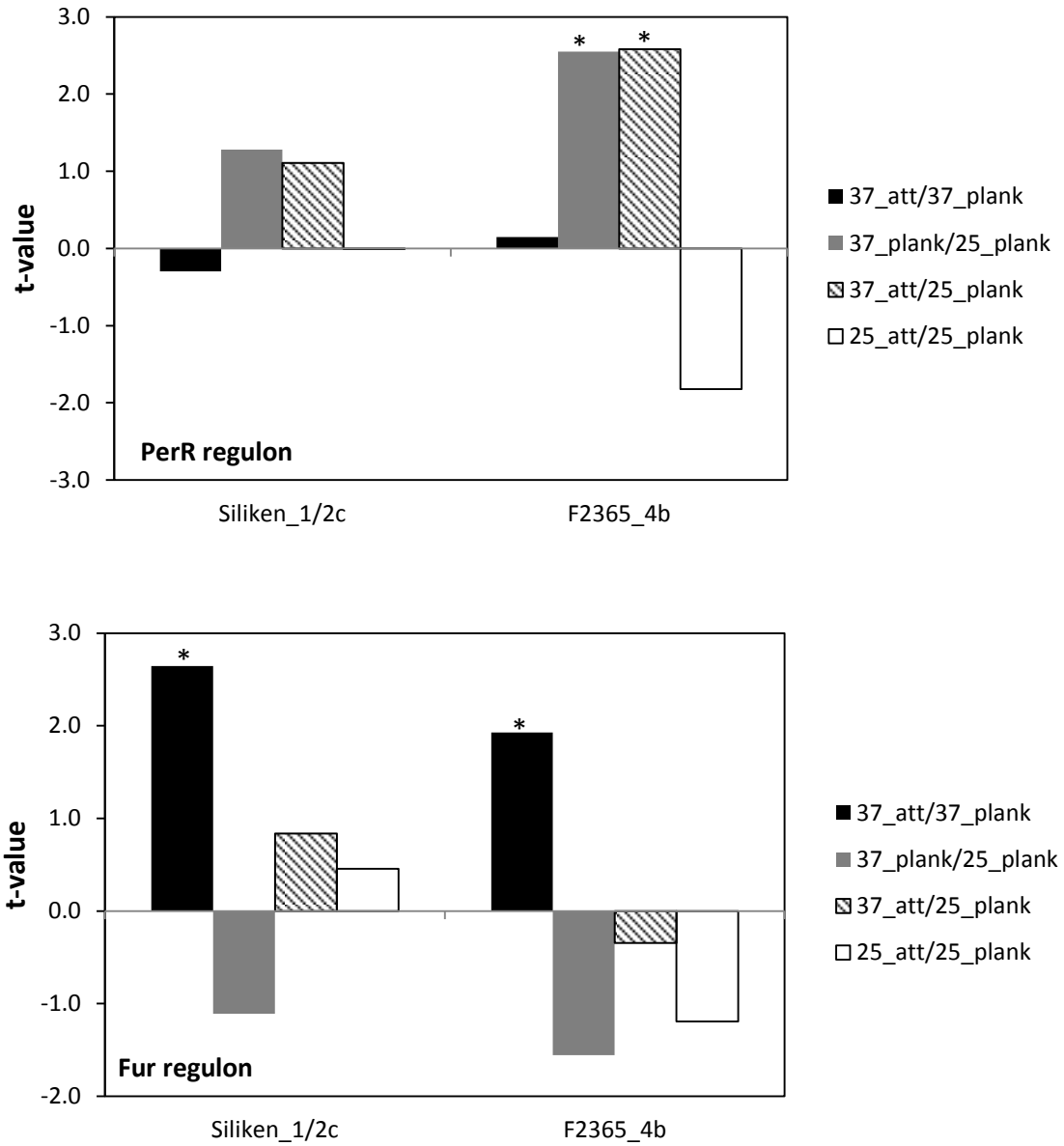

3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
t-value
*
*
PerR regulon
Siliken_1/2c
F2365_4b
37_att/37_plank
37_plank/25_plank
37_att/25_plank
25_att/25_plank
3.0
2.0
1.0
0.0
-1.0
-2.0
t-value
*
*
Fur regulon
Siliken_1/2c
F2365_4b
37_att/37_plank
37_plank/25_plank
37_att/25_plank
25_att/25_plank

Figure 10

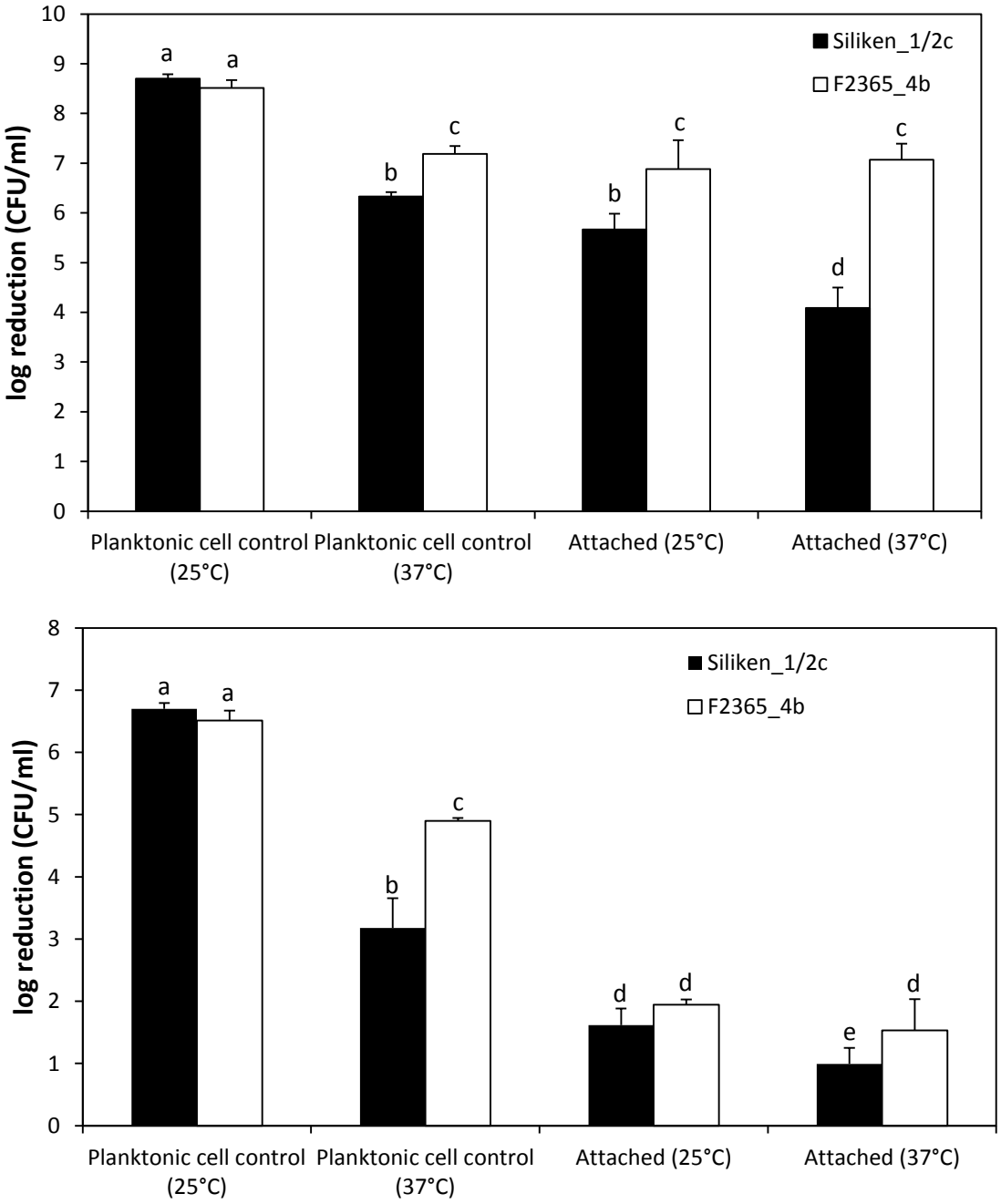
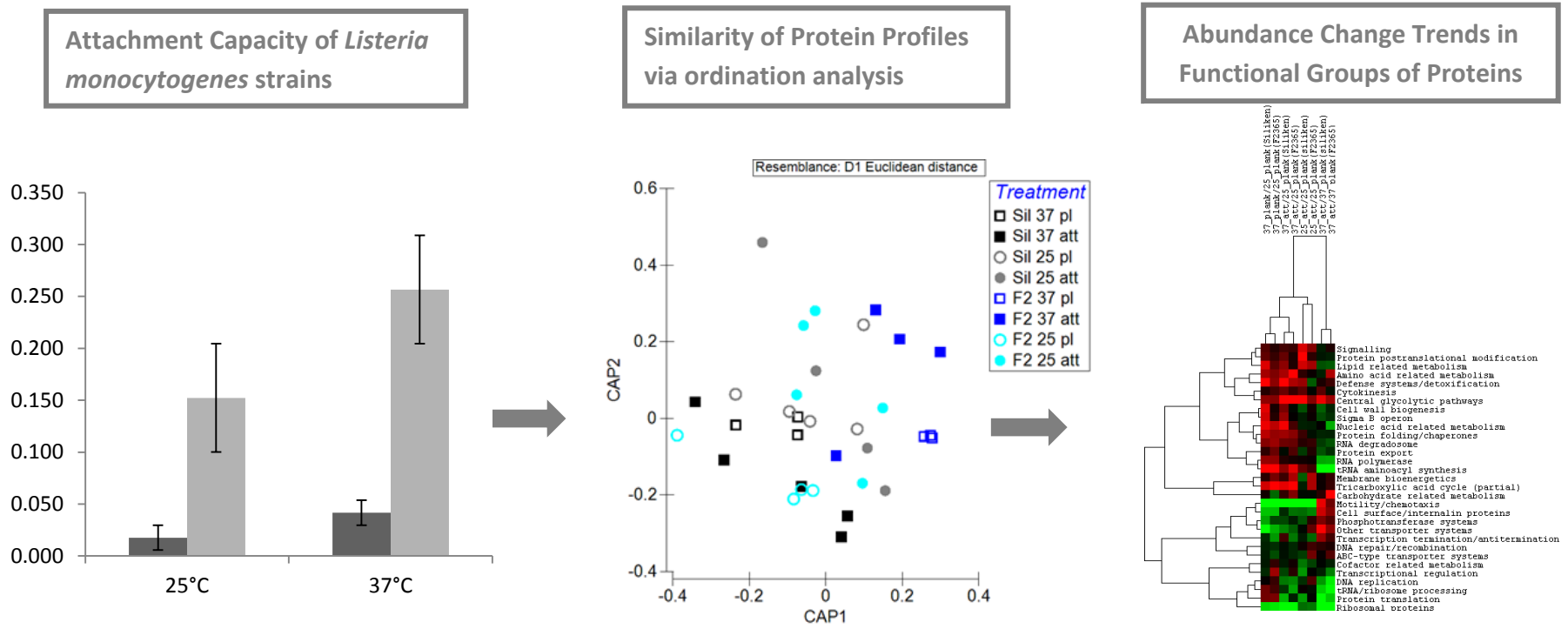

log reduction (CFU/ml)
Siliken_1/2c
F2365_4b
a
a
b
c
b
c
d
c
Planktonic cell control (25°C)
Planktonic cell control (37°C)
Attached (25°C)
Attached (37°C)
log reduction (CFU/ml)
Siliken_1/2c
F2365_4b
a
a
b
c
d
d
e
d
Planktonic cell control (25°C)
Planktonic cell control (37°C)
Attached (25°C)
Attached (37°C)

Figure 11



Graphical Abstract