Fever-like temperature impacts on *Staphylococcus aureus* and *Pseudomonas aeruginosa* interaction, physiology, and virulence both *in vitro* and *in vivo*

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Abstract

Staphylococcus aureus and *Pseudomonas aeruginosa* are two bacterial species that cause a wide variety of infections and coinfections. The interaction between these species is complex and involves the production of different metabolites and metabolic changes. The impact of elevated body temperature, such as fever, on the physiology and interaction of these pathogens remains poorly understood. Therefore, the aim of this work was to analyze the effect of moderate fever-like temperatures (39°C) on *S. aureus* USA300 and *P. aeruginosa* PAO1 mono- and co-cultures in comparison with 37°C, by using RNAseq and physiological assays in microaerobiosis. Both bacterial species showed metabolic alterations in response to temperature but also in response to the

competitor. Organic acid production and nitrite content in the supernatant were altered both by the presence of the competitor and the incubation temperature. Interaction ANOVA showed that, in *S. aureus*, gene expression presented interaction between the temperature and the presence of the competitor. Among these genes, the most relevant were the *agr* operon and three of its direct target genes *psmβ*, *psmβ2* and *hld*. In the A549 epithelial lung cell line, fever-like temperatures impacted *in vitro* virulence, antibiotic resistance, cell invasion, and cytokine production. In agreement with the *in vitro* assays, the survival of mice that had been intranasally inoculated with *S. aureus* monocultures pre-incubated at 39°C showed reduced survival after 10 days. An even higher mortality of around 30% was observed in mice that had been inoculated with co-cultures pre-incubated at 39°C. The bacterial burden in the lungs, kidney, and liver was higher for both species when the mice were infected with co-cultures previously incubated at 39°C. Our results highlight a relevant change in the virulence of bacterial opportunistic pathogens exposed to fever-like temperatures, opening new questions related to bacteria-bacteria and host-pathogen interactions and coevolution.

Author summary

In mammals, fever plays a role in defense against infection. The ability to withstand fever-like temperatures is thus important for bacterial survival and host colonization. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two opportunistic human bacterial pathogen species that can cause infections and even coinfections. In this study, we demonstrated that culturing these bacterial species as mono- or co-cultures at 39°C for 2 h differentially impacted on their metabolism, virulence, antibiotic resistance, and cellular invasion. More importantly, mouse survival was affected by the bacterial culture conditions, including temperature. Our findings show the importance of fever-

like temperatures in the interaction and *in vivo* virulence of these bacterial species and open new questions about the host-pathogen interaction.

Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are two opportunistic bacterial species globally distributed(1,2), which cause a variety of infections ranging from mild to severe, including skin and respiratory infections (3,4). Additionally, these bacteria are a major cause of both morbidity and mortality in cystic fibrosis patients, causing acute and chronic infections that lead to severe respiratory damage and (or perhaps due to) a continuous inflammatory response.

The interaction between *S. aureus* and *P. aeruginosa* has been recently reviewed (5) and it is traditionally described as antagonistic due to the secretion of different anti-staphylococcal factors by *P. aeruginosa*. Among these factors, the pigment pyocyanin, quinoline N-oxides, and cyanhydric acid inhibit *S. aureus* respiratory chain, rhamnolipids cause biofilm dispersion, while the extracellular protease LasA affects the cell wall of *S. aureus*, leading to cell lysis (6). Unlike many nonpathogenic staphylococcal species, *S. aureus* is sensitive to respiratory inhibitors secreted by *P. aeruginosa*. That is because the cytochrome bd quinol oxidase, which oxidizes ubiquinol and reduces oxygen as a part of the electron transport chain, is pyocyanin- and cyanide-sensitive in *S. aureus* (7).

Despite this disadvantage, in co-culture with *P. aeruginosa*, *S. aureus* changes its metabolism to a fermentative state leading to the formation of small colony variants (SCVs). In this state *S. aureus* isis resistant to pyocyanin and cyanide and able to survive in co-culture with *P. aeruginosa*. *S. aureus* cells can also shift to L-forms to escape the cell wall lytic activity of LasA (8,9). However, during the last years, the

coexistence of both bacteria has also been detected in cystic fibrosis patients and *in vivo* experiments, revealing even a cooperative interaction (10–12). A key metabolite in this coexistence, which has been found in cystic fibrosis sputum, is acetoin, a compound produced by clinical *S. aureus* isolates, and catabolized by *P. aeruginosa* (9–11). Although the interaction between both bacteria has been less explored under low oxygen conditions, microerobiosis is relevant in cystic fibrosis and other pulmonary diseases due to the presence of mucus and oxygen gradients (13). In this context, Pallet et al. (2019) (14)reported that anoxia leads to a phenotype of *P. aeruginosa* that is less dominant over *S. aureus*.

Besides oxygen and nutrients, another key factor for bacterial survival and physiology is temperature (15,16). Particularly, pathogenic species display virulence factors depending on several factors, including the host temperature(17,18). Previous studies have analyzed the effects of temperature transitions on *S. aureus* and *P. aeruginosa* physiology by using transcriptomic and physiological approaches(19,20). In bacteria, the main temperature sensing mechanisms are RNA thermometers, which regulate the expression of genes at the post-transcriptional level through structured RNAs (18). Several researchers have reported that RNA thermometers regulate different physiological features like the virulence factor *tviA* in *Salmonella typhi* or the type three injectisome in *Yersinia pseudotuberculosis* (18,21,22)Temperature-dependent traits, mainly associated with virulence, host colonization and survival, have been recently reviewed in *Pseudomonas* spp., including *P. aeruginosa* (23).

In mammals, fever or pyrexia is defined as the increase in body temperature above homeostatic values, and, in the context of infections, is considered a beneficial process for the host (24,25). For example, in three different *Salmonella enterica* serovars, fever-like temperatures impair infective characteristics, suggesting that fever

is a signal for a persistent state (26). In *S. aureus* USA300 strain AH1263, Bastok et al. (20) analyzed three different temperatures, including extreme pyrexia, and found alterations in some relevant phenotypes related to virulence. However, these authors focused mainly on the differences between 34°C and 37°C under aerobic conditions.

Based on the above, the aim of the present study was to analyze the effect of fever-like temperatures (39°C) on the interaction between *S. aureus* and *P. aeruginosa* by using a sequential culture scheme to analyze the RNA expression profile *in vitro* as well as virulence traits both *in vitro* and in a mouse model. Our data support that fever-like temperatures increase virulence *in vitro* and *in vivo*, particularly when *S. aureus* nd *P. aeruginosa* are co-cultured.

Results

S. aureus and *P. aeruginosa* survival in co-cultures under microaerobic conditions and fever-like temperatures

We first investigated bacterial survival in microaerobic monocultures and co-cultures by using KNO_3 as an alternative electron acceptor to oxygen. The cultures were incubated at 37°C either for 4 h (herein called 37) or for 2 h, followed by incubation at 39°C for 2 h (herein called 39) (**Fig. S1**). The survival of *S. aureus* USA300 (herein called SA) in monoculture at 39°C ($1.0x10^{10} \pm 4.1x10^{9}$ CFU ml^{-1}) was similar to that of the control cultures at 37°C ($6.2x10^{9} \pm 2.7x10^{9}$ CFU ml^{-1}) (Fig. 1A), indicating that bacterial viability was not compromised by temperature in these conditions. Similar results were observed for *P. aeruginosa* PAO1 (herein called PA), for which $9.6x10^{8} \pm 2.8x10^{8}$ CFU ml^{-1} were observed in monoculture at 37°C and $8.0x10^{8} \pm 3.8x10^{8}$ CFU ml^{-1} at 39°C (Fig. 1B).

Regarding co-culture experiments, after 4 h at 37°C $1.1x10^{8} \pm 1.83x10^{7}$ CFU ml^{-1}were observed for SA, i.e., a value lower than that observed for monocultures at the same temperature (Fig. 1A). PA survival in co-cultures was also lower than in monocultures at 37°C, with a value of $4.2x10^{8} \pm 1.6x10^{8}$ CFU ml^{-1} (Fig. 1B). When comparing co-cultures at different temperatures, SA survival was similar at both temperatures ($6.6x10^{7} \pm 1.3x10^{7}$ at 39°C and $1.0\ 10^{8} \pm 1.8x10^{7}$ at 37°C; Fig. 1A). The same results were obtained for PA grown in co-cultures, since $1.9x10^{8} \pm 7.6x10^{7}$ CFU ml^{-1}were observed at 39°C, a value similar to that obtained for co-cultures at 37°C (Fig. 1B). Our results showed that under the conditions evaluated, the differences in survival depend on the presence of the competitor rather than on the temperature. Additionally, we performed plate competence assays, which confirmed that competence was not affected by the temperature (Fig. 1C).

Metabolic reshaping of *S. aureus* and *P. aeruginosa* after exposure to fever-like temperatures

To investigate how these bacterial species adapt their physiology to fever-like temperatures, we next carried out total RNAseq experiments and physiological assays for SA and PA monocultures and SA-PA co-cultures, following the same culture scheme as that described above (Fig. S1).

When comparing the gene expression profiles of SA monocultures at 39°C and 37°C (herein called SA mono 39°C vs 37°C), we identified 121 upregulated genes and 608 downregulated genes. These genes represent approximately 38 % of the total genes. Differently, when comparing the gene expression profiles of *P. aeruginosa* monocultures at 39°C and 37°C (herein called PA mono 39°C vs 37°C), we found that only seven genes were upregulated and 24 downregulated (representing 0.5 % of the

total genes). These results suggest that in monocultures, SA was more sensitive to fever-like temperatures than PA (Tables S1 and S2, and Fig. S2A).

We also performed Gene Ontology Enrichment (GOE) analysis, which allows the detection of metabolic pathways represented within the differentially expressed genes, even if not all the genes involved present a P and $Q < 0.05$ in the RNAseq analysis. Rather, it allows detecting a global trend of the metabolic pathway or branch. The GOE analysis in the SA mono 39°C vs 37°C expression dataset showed an enrichment in the arginine and tricarboxylic acid (TCA) cycle pathways (Tables S3 and S4). Particularly, in tryptic soy broth (TSB) cultures, we found that some genes related to arginine metabolism, such as *argG*, *argH*, *rocD*, and *gubD*, were upregulated at 39°C, and that others, such as *argJ*, *rocF*, and *putA*, followed the same trend, although the differences were not significant (Table S3, Fig. 2A). Reslane et al. (2022) (27) reported that when glucose and arginine are absent, SA is a functional prototroph. These authors also showed that in the presence of glucose, and even in the absence of arginine, *S. aureus* behaves like an arginine auxotroph showing that absence of arginine alone is not enough to trigger arginine biosynthesis. The authors suggested that, during abscess infection, where active macrophages are found, arginine and glucose are depleted, thus activating arginine biosynthesis from proline (27). Our hypothesis is that incubation of SA at a fever-like temperature for 2 h triggers some physiological responses similar to those displayed after immune system activation even though glucose is present.

To test our hypothesis, SA monocultures were cultured in complete defined medium (CDM) supplemented with glucose and KNO_3 but without arginine or proline, following the same temperature scheme as that described above. Results showed that bacterial count in glucose-supplemented medium without arginine or proline was higher at 39°C

than at 37°C (Fig. 2B), supporting the RNAseq observations and suggesting that arginine biosynthesis is triggered by fever-like temperatures.

The GOE analysis also revealed a differential expression of genes related to the TCA cycle and fermentation pathways in SA monocultures at 39°C (Tables S1 and S3, Fig. 3A). Regarding the TCA cycle pathway, we found that some genes such as *mqo*, *gltA*, *icd*, *sucA*, and *sucB* were upregulated and that others such as *acnA*, *sucC*, *sdhA* and *sdhB* also followed the same trend but the differences were not significant. The *gudB* and *hutG* genes, which are related to peripheral feeding pathways, were also upregulated. In concordance with the GOE analysis (Table S3, Fig. 3A), which considers a global change in a certain pathway, the *hutH*, *hutI*, *hutU*, *rocA* and *putA* genes showed a non-significant increase in their expression (Fig. 3A). Some genes encoding proteins related to fermentation branches, including *ldh*, *pflAB* and *ackA*, were downregulated (Table S1 and Fig. 3A). Expression of the formate dehydrogenase subunit *fdhD* was upregulated at 39°C. Also, the expression of the *fdh* and *fdhA* genes was upregulated, albeit non-significantly.

Although the cultures were incubated under microaerobic conditions in the presence of nitrate, some genes encoding nitrate and nitrite reductase, including *narK*, the operon *narHIJ*, *nirBD* and *nirR*, as well as the responsive nitrogen regulation system NreABC, which activates nitrate and nitrite dissimilation, were downregulated in mono 39°C vs 37°C (28). In concordance with the gene expression profile, nitrite accumulation in the supernatant of SA monocultures was significantly decreased at 39□C as compared to 37□C (Fig. 3C) (28). The expression of cytochrome D ubiquinol oxidase subunits in SA monocultures at 39°C was repressed, while the rest of the genes encoding cytochrome subunits were similarly expressed at 39°C and 37°C (Table S1, Fig. S2B). Additionally, when we investigated organic acid production in SA monocultures in CDM

supplemented with glucose at 37°C and 39°C under microaerobic conditions, we detected a decrease in lactate, pyruvate, and acetate content in the culture supernatant, but an increase in succinate and formate at 39°C vs 37°C (Fig. 3D). The integration of results suggests that SA monocultures feed the TCA cycle at 39□C through α-ketoglutarate to obtain energy, probably through cytochrome quinol and the succinate oxidase complex (Fig. 3A).

When PA was incubated at 39°C, only 31 genes presented differential expression in TSB (Fig. S2A). Among them, an operon that included *fumC1* and *sodM* was upregulated at 39°C. It is important to point out that *fumC1*, which encodes a fumarate reductase, has been reported to be upregulated *in vivo* in cystic fibrosis patients. Another key *Pseudomonas* metabolic pathway, the periplasmic glucose oxidation, was repressed at 39°C, with almost all the genes repressed (*kgut*, *kguk*, *kgD*, and *kgnt*) (Table S2, Fig. 3B). In contrast, the GOE analysis showed that the gluconeogenesis pathway and glyoxylate shunt were represented at 39°C, with several related genes presenting an increase in their expression, although the differences were non-significant (Tables S2 and S4).

Nitrite production was similar for PA monocultures incubated at both temperatures (Fig. 3C). Since *P. aeruginosa* strains are in general able to perform denitrification, nitrite accumulation is dynamic, and this species produces gaseous nitrogen molecules. In the Durham tube assay, gas production after 48 h in monocultures was lower at 39°C, although growth was similar to that observed at 37°C (Fig. 3E). The organic acid profile in CDM supplemented with KNO_3 and glucose showed a very low concentration of organic acids in the supernatants of PA monocultures (Fig. 3D). Cytochrome expression showed no significant differences between temperatures (Table S2, Supp. Fig. 2C).

The results obtained when comparing the expression profiles between co-cultures and monocultures at 37°C (further called co vs mono 37) were different. SA presented 172 upregulated and 59 downregulated genes (Fig. S1B; Table S1). For co vs mono at 37°C, the GOE analysis showed five over-represented categories, including purine metabolism, amino acid biosynthetic process, siderophore biosynthesis, and heme transport (Table S3). Particularly, enrichment in the catabolic and biosynthetic amino acid pathways was observed among the upregulated genes (Table S3). In the catabolic amino acid pathways, L-glutamine, L-serine, L-threonine, and L-alanine degradation were over-represented (Table S3). Interestingly, these catabolic pathways produce pyruvate, which can subsequently be fermented to produce ATP and acetate, lactate, or ethanol. As expected, in co-cultures where PA was present, SA displayed fermentative metabolism, with the *ldhA*, *pflA*, and *pflB* genes being upregulated (Table S1).

The heme degradation and staphyloferrin B biosynthesis pathways, which are related to iron acquisition from heme, the most abundant iron source in the host and a key feature in the competition with PA(29), were also represented among the upregulated genes in co-cultures, when compared with monocultures at 37°C (Tables S1 and S3).

In the same condition (co vs mono 37), the PA transcriptomic profile and GOE analysis showed enrichment in the gluconeogenesis pathway, with the *pckA* gene being significantly upregulated and other genes showing a non-significant increase but a clear trend in the entire pathway (Tables S2 and S4). Genes involved in the peripheral fructose catabolic pathway, including *fruA*, *fruK* and *fruI*, were repressed. Additionally, the GOE analysis showed enrichment in cysteine metabolism, with the *cysD*, *cysN* and *cysH* genes, which belong to the assimilatory sulfonate reduction superpathway, and alkane-monooxygenase-coding genes being upregulated. In addition, the GOE analysis showed increased expression of genes of the guanosine nucleotide metabolism in co vs

mono 37. This pathway, which allows *de novo* biosynthesis of guanine nucleotides in *Pseudomonas*, included the overexpression of *gmk* and a non-significant 1.5-2-fold increase in the expression of several genes such as *guaA*, *guaB*, *nrdA*, *nrdB*, *nrdD* and *nrdJAB* operons (Tables S2 and S4).

RNAseq analysis of co vs mono 39 for SA showed upregulation in glycolysis-related genes, including *glk*, *pfka*, *tpiA*, *gap*, *pgk*, *pgm*, *eno* and *pyk*, and higher expression of *pgi* and *fbaA* in co-cultures, albeit non-significant (Table S1). These results were different from those found in co vs mono 37 datasets, which were in concordance with previous studies (5). Interestingly, TCA-enzyme-coding genes and the cytochrome coding genes *sdhC*, *sdhA* and *sdhB* were repressed (Table S1) and concomitantly, *pflA*, *pflB* and *ldh* expression showed a strong increase of 121, 113 and 43-fold, respectively, in co vs mono at 39. In concordance, the lactate levels as determined by high performance liquid chromatography (HPLC) were increased at 39°C in co-cultures (Fig. 3D). Our results for SA showed a difference in gene expression of some upper metabolic branches since glycolysis-related genes were upregulated in co vs mono 39, while amino acid catabolism showed a general increase in co vs mono 37. Additionally, at 39°C, SA co-cultured with PA showed a strong increase in genes encoding fermentative enzymes and a repression of TCA (Table S1). Although a fermentative metabolism was expected in the presence of PA, the effect at 39°C was even sharper than at 37°C (Table S1).

Analysis of the PA expression profile in the same conditions (co vs mono 39) revealed 140 upregulated and 267 downregulated genes (Fig. S1). Among the repressed genes, there were *gdc*, *kgut*, *kguK*, *kguD* and *glk*, which are involved in the periplasmic glucose oxidation pathway (Table S2). In contrast, we found overexpression of genes belonging to the glyoxylate shunt, including *icl*, which encodes the isocitrate lyase, and of the

genes encoding the succinate dehydrogenase subunit C and D (Table S2), suggesting that in PA, glyoxylate could feed the TCA cycle through succinate in co vs mono 39.

Finally, we compared RNAseq results of SA-PA co-cultures at 39°C vs SA-PA at 37°C (co 39 vs 37) and found only 4 upregulated and 122 downregulated genes at 39°C for SA, and 108 upregulated and 201 downregulated genes at 39°C for PA (Tables S1 and S2; Fig. S2A).

In SA co-cultured with PA, fever-like temperatures (co 39 vs 37) led to the repression of genes belonging to transcription and translation processes as well as envelope modification, and of the enterotoxin-coding genes *seq* and *sek* (Tables S1 and S3). In contrast, only genes comprising the PTS type II ascorbate-specific operon, which has been reported to alter the properties of SA cell wall and thereby modulate virulence (30) were upregulated at 39°C (Table S1). In general, when PA was present, SA central metabolic genes were not affected by temperature. Some exceptions were *ldh*, *budB*, and the cytochrome D ubiquinol oxidase subunits, which were downregulated at 39□C (Table S1, Fig. 3A).

For PA, co 39 vs 37 expression profile analysis showed changes in key metabolic features. Genes belonging to the ethanol oxidation pathway, which is a secondary metabolic branch present in *Pseudomonas* species for energy production through oxidation of ethanol (31), were upregulated at 39°C. These included genes belonging to the PQQ biosynthesis operon (*pqqC*, *pqqD*), the *exaC* gene, which encodes an iron-containing aldehyde dehydrogenase, and the response regulator *erbR*. Additionally, we observed a non-significant increase in the expression of *pqqA*, *pqqB* and *pqqE* and the sensor *erbS* (Table S2; Fig. 3B). Accordingly, cytochrome C expression, related to the ethanol oxidation pathway, was higher in co-cultures incubated at 39°C while other cytochrome-coding genes were repressed (Table S2, and Fig. S2C). Ethanol oxidation

has been recently described as important in low oxygen environments (32). Traditional anaerobic metabolic genes were repressed in co-cultures at 39°C in comparison with 37°C, even when both were incubated under low oxygen conditions in the presence of nitrate. These genes included *arcD*, *nrdD*, *narK1*, *narK2*, *dnr*, *aer*2 and *hcnB*, all of which are target genes of the globar regulator Anr (Table S2). The expression of this global regulator was not affected by temperature; thus, the repression effect could be due to other regulators or to an alteration of Anr activity.

Nitrite accumulation in co-cultures was similar at both temperatures and lower than in monocultures after 4 h of incubation (Fig. 3C). Gas accumulation in Durham tubes after 48 h showed a similar bubble size and similar growth when comparing co 39 vs 37, but smaller when compared with monocultures (Fig. 3E). Lactate and acetate contents in the whole supernatant were increased in co-cultures at 39°C (Fig. 3D).

In summary, regarding the metabolic reshaping of SA and PA after exposure to fever-like temperatures, SA monocultures showed a decrease in nitrate reduction, acetate and lactate metabolism and the overexpression of arginine biosynthesis and TCA-related genes, whereas PA monocultures showed a decrease in the expression of genes belonging to the periplasmic glucose oxidation pathway and a decrease in denitrification. The expression profiles of SA and PA co-cultures compared with monocultures at 37ºC showed mainly metabolic changes towards a fermentative metabolism in the case of SA in co-culture. In PA, the GOE analysis showed genes involved in *de novo* nucleotide biosynthesis and cysteine and sulfonate metabolism, which are related to sulfur starvation and metabolism, and important during stress conditions (33). Co-cultures incubated at different temperatures showed a more intense fermentative state than at 37°C.

Temperature has a different impact on *S. aureus* but not *P. aeruginosa* depending on the culture conditions

RNAseq analysis showed a differential response to temperature depending on the presence of the competitor. To confirm our observations, we performed a principal component analysis (PCA) (Fig. S5A and B). For SA, the PCA showed that the first PC discriminated culture condition (mono or co), while the grouping by temperature was less evident in any of the first three components (Fig. S5A), and the first and second components explained 89.8 % of the data variation (Fig. S5A). For PA, the first and second components explained 88.6 % of the data variation (Fig. S5B). Additionally, we performed a multifactorial ANOVA for each gene present in the RNAseq data with false discovery rate (FDR) correction method and found no statistical interaction between the culture condition (mono or co) and temperature for PA (Fig. S5D). In contrast, for SA, this analysis showed an interaction between these factors and 35 genes, with a $P<0.01$ (Fig. S5C; Table S7). However, as we detected differential expression of *agr* genes in the different data sets, we reanalyzed the data and found that the *agr* operon and three of its target genes, *psmβ*, *psmβ2* and *hld*, crucial for SA virulence, had a $P<0.05$ for the interaction between temperature and culture condition. Therefore, these genes were further analyzed (Table 1).

In *S. aureus* strains, Agr is the quorum system that controls virulence functions. In SA monoculture, the *agrABCD* operon was overexpressed at 39°C in comparison with 37°C (four-fold on average, Table S1). As expected, *agr* overexpression caused an increase in the expression of its target genes, including *psmβ1*, *psmβ2* and *hld* (Table 1; Fig. 4A). Additionally, other genes also described to be under direct or indirect control of *agr*, such as *spa*, *isaA*, *mnhA*, *mnhG*, and *mnhH*, and other genes affected by *agr*, including *eta*, were found to be repressed (Table S1, Fig. 4A).

When we compared the SA expression profile of co 39 vs 37 or co vs mono 39, we found that *agr* genes presented a lower expression at 39°C. The *psmβ1*, *psmβ2* and *hld* genes also showed lower expression, although the differences were not significant. However, in co vs mono 37, the same genes presented a non-significant increase (Table S1).

The SA transcriptional data related to virulence are summarized in Figure 4A. These data show that the expression profile of the master regulator system *agr* was affected by the fever-like temperature depending on the presence of the competitor and that the most virulent expression profile was found in SA monocultured at 39°C.

Fever-like temperatures trigger virulence factors and alter antibiotic resistance in *S. aureus* and *P. aeruginosa in vitro*

Considering the impact of fever-like temperature (39°C) on gene expression, we next tested its impact on some virulence factors of SA monocultures *in vitro,* including DNAse activity, pigment production, haemolysis, biofilm formation and colony phenotype in Red-Congo agar. The biosynthetic operon staphyloxanthin includes the *crtN*, *crtM*, *crtP*, *crtQ* and *crtO* genes, all of which were overexpressed at 39°C (Table S1) (34,35).

In vitro staphyloxanthin production was higher at 39°C, similar to what observed in the competence plates shown in Fig. 1A, in which the SA lawns presented a strong yellow color (Fig. 4B). Extracellular DNAse activity in DNA agar plates was similar at both temperatures after 24 h of incubation (Fig. 4C). We also tested the colony phenotype in Congo red plates, where SA presented similar, black-pigmented colonies both at 37°C and 39°C after 24 h of incubation. Haemolysis as tested in blood agar plates was higher at 39°C (Fig. 4D). Biofilm formation, as assayed in polystyrene multiwell plates using

TSB medium supplemented with KNO_3, was lower at 39°C, in line with our RNAseq data (Fig. 4E) and with the upregulation of *agr* under the same conditions (36).

Antibiotic resistance was screened using the Sensititre plate multi-test for *S. aureus* monocultures incubated at 37°C or 39°C during the entire test. We found that at 39ºC, the Minimal Inhibitory Concentration (MIC) for moxifloxacin was higher than at 37°C (Table S5). The expression of topoisomerase IV, the main target of moxifloxacin (37), was repressed at 39°C, which could explain the increase in the MIC for this antibiotic. Daptomycin, an important antibiotic against methicillin-resistant *S. aureus* strains, whose resistance has been related to the interruption of two genes in *S. aureus* (HG003, SAOUHSC_02441 and SAOUHSC_0036 (38), showed at least one-fold increase in its MIC in SA monoculture at 39°C (Table S5). In *S. aureus* USA300, SAUSA300_0377, which is the gene orthologous to SAOUHSC_0036, was repressed at 39□C. This result may explain the phenotype observed (Table S1). We also tested oxidative stress resistance in SA monocultures in agar plates and found similar results at both temperatures (Fig. 4F).

For PA mono 39 vs 37, RNAseq analysis related to the virulence factors summarized in Fig. 4G showed that genes related to the quorum sensing *pqs* system were repressed at 39°C. In PA monocultures, extracellular DNAse activity, lipase and rhamnolipid production were similar at both temperatures (Fig. 4B, I and H). Extracellular protease activity of PA monocultures at 39°C was significantly higher while motility was lower (Fig. 4J and K). Antibiotic resistance at different temperatures was also tested using the Sensititre panel for Gram negative bacteria. We showed a decrease in cefotaxime MIC and an increase in resistance to minocycline at 39□C (Table S6). Oxidative stress resistance was also tested, and results showed that it was similar at both temperatures (Fig. 4F).

In co-cultures, the PA type III secretion system (TSS3) regulators *exsA* and *exsC*, as well as the TSS3-related *pcrV* were upregulated at 39°C (Table S2, Fig. 4G). The phenazine production genes and the extracellular lipase coding gene *lipA* followed the same trend, being upregulated at 39°C (Table S2, Fig. 4G).

The extracellular lipase activity in co-cultures showed higher activity at 39°C while extracellular protease activity showed similar results at both temperatures (Fig. 4I and J). Motility, which is mostly driven by PA, decreased at 39°C in co-cultures, similar to what found in monocultures, thus showing a temperature effect despite the presence of SA.

To further investigate these *in vitro* observations, we analyzed cytokine production and cellular invasion on the epithelial lung cell line A549. Cells were infected with inocula grown following the same culture scheme as before (PA at 39°C and 37°C, SA at 39°C and 37°C and SA-PA co-cultures at 39°C and 37°C). When A549 cells were infected with PA monocultures at 37°C or 39°C, production of the proinflammatory cytokine IL-8 was similar to that of the anti-inflammatory cytokine IL-6 despite the temperature (Fig. 5A and B). In contrast, when A549 cells were infected with SA monocultures at 39ºC, proinflammatory IL-8 production was higher than that observed when cells were infected with SA monocultures at 37°C (Fig. 5A). These results are in line with the overexpression of *psmβ1* and *psmβ2* observed in SA mono 39 vs 37. When cells were infected with SA monocultures, IL-6 production was similar at both 37ºC and 39ºC (Fig. 5B). When cells were infected with SA-PA co-cultures, a similar production of IL-6 and IL-8 was observed regardless of the temperature, although a slight increment was observed for IL-8 production at 39°C (Fig. 5 A and B). IL-6 production was higher when cells were infected with PA monocultures at both temperatures compared with co-cultures (Fig. 5B).

When the cellular invasion assay was performed with SA monocultures incubated at 39□C, a decrease in cellular invasion was observed, in agreement with a more virulent profile (Fig. 5C). In contrast, cellular invasion of co-cultures was higher than that of monocultures, regardless of the culture temperatures for both species (Fig. 5C). Overall, our results show that fever-like temperature affects SA virulence, leading to an acute infective state when PA is absent.

In vivo virulence is affected by temperature and culturing in a mouse model

We next performed intranasal infections in mice, using SA and PA monocultures or SA-PA co-cultures grown as previously described. We tested both mouse survival and bacterial burden in the mouse lungs, kidneys, and liver. At the end of the experiment, survival was lower when mice were infected with SA monocultures preincubated at 39°C vs 37°C (Fig. 5D). In contrast, when mice were infected with PA monocultures, mouse survival was lower when the inoculum was grown at 37°C (Fig. 5D). Moreover, SA-PA co-cultures incubated at 39°C caused greater mortality than those incubated at 37°C. This was also the treatment that caused the highest mortality in mice (Fig. 5D).

Bacterial burden in the lungs, kidneys, and liver showed that PA co-cultures led to higher counts in the three organs at 39□C than at 37ºC, and in comparison, with monocultures (Fig. 5 E, F and G). SA count in these organs was similar when comparing between temperatures but was slightly higher when SA was in co-culture (Fig. 5 E, F and G). We re-considered these results in light of the *in vitro*-RNAseq data and other *in-vitro* experiments. Decreased survival of mice that had been inoculated with SA monoculture pre-incubated at 39°C in comparison with SA cultured at 37°C (Fig. 5D) is in line with the *in vitro* results, since under those conditions SA displayed an increase in expression of *agr* and in its target genes, an increase in IL-8 production

and a decrease in cellular invasion. As the bacterial burden in organs at the end point of the experiment was similar, we hypothesized that the difference in mortality was due to an increase in virulence factors leading to an inflammatory state when SA was cultured at 39°C rather than to an increase in the number of SA cells. In contrast, when mice were inoculated with PA monocultures, the effect was opposite to that of SA. Indeed, when the inoculum was incubated at 37°C, the mouse survival was lower than at 39°C. Again, the bacterial burden was similar between both temperatures and even higher at 39°C. The PA *in vitro* expression profile only showed repression of some genes related to quorum sensing at 39°C in comparison with 37°. Inflammatory interleukin production was higher at 37°C for PA monocultures, an effect that could lead to higher tissue damage *in vivo* and might explain the high mortality of mice and decreased bacterial burden when the inoculum was incubated at 37° C.

In our mouse model, inoculation with co-cultures incubated at 39° led to the lowest survival rate. When we compared the expression of SA virulence factors at co 39 vs 37, we found, at least *in vitro*, repression of the Sae operon *nuc*, *sek*, *seq*, *ecb*, *efb*, *psmβ1*, *psmβ2*, *pknB*, *scn*, *sak*, *hld* and *agrD*. The only exception was *chs*, which is important for immune system evasion. In mouse organs, SA counting was higher than in monocultures independently of the temperature, similar to what observed in the human cell invasion assay. The other actor in co-cultures was PA, which possesses different adhesin proteins and virulence factors underlying the high cytotoxicity observed in cell culture and animal models. As we prepared the inoculum for mouse infection by using the same culture scheme as for the RNAseq, we reanalyzed the expression data of virulence genes for PA in co-cultures and monocultures. The virulence effectors *exoS*, *exoT*, and *exoY* are regulated by *exsA*, *exsB*, *exsC* and *exsD* (39). When comparing co 39°C vs 37°C, we found that *exsA* and *exsC* were upregulated at 39°, while *exsB*, *exsD*,

exoS, *exoT* and *exoY* presented higher expression, albeit non-statistically significant (Fig. 4G). We hypothesize that the higher expression of these virulence effectors at 39°C in co-cultures could lead to the observed decrease in the survival of mice. However, we cannot rule out other factors impacting mouse survival since *in vivo* infection is a complex process. Human serum albumin is a major protein in human plasma that affects gene expression, virulence and survival in *Acinetobacter baumanii* (40,41). To understand whether human serum albumin (HSA) could alter the bacterial number in co-cultures at 39°C, we grew the bacteria following the same culture scheme (Fig. S1) but in the presence or absence of this protein. HSA did not affect SA or PA, which was expected since survival was not affected by 39°. In addition, we found no difference in the CFU count of SA or PA co-cultures at 37°C or 39°C in the presence or absence of HSA. However, as these experiments were carried out *in vitro* we cannot rule out an effect of HSA *in vivo.*

Finally, we wondered whether longer co-culture times could cause a different physiological response at 39°C in comparison with that observed at 37°C. Thus, we cultured SA, PA and PA-SA at 37°C and 39°C in the same microaerobic conditions but for 24 h. Surprisingly, co-cultures showed an evident, blue/green-colored medium typical of pyocyanin production but only at 39°C (Fig. S6 A). It has been reported at 37°C under low oxygen condition a decrease in pyocyanin production similar to that found in our model (12) leading to a major coexistence between SA and PA. However, at 39°C pigment production was observed under microaerobic conditions. The bluish pigment pyocyanin is a typical virulent factor that could increase virulence of PA at 39°C under low oxygen availability. More intriguing is the similar CFU count in the co-cultures after 24 h at both 39°C and 37°C, despite the different pyocyanin content (Fig. S6B).

Overall, we found that culturing PA and SA at fever-like temperatures for 2 h leads to a differential expression pattern of virulence factors, which could explain at least in part the decrease in the survival of mice inoculated with SA monocultures. The effect was even more evident in co-cultures at 39°C, opening new questions regarding host-bacteria and bacteria-bacteria interactions.

Discussion

The infection process and outcome depend on several factors, including the availability of nutrients, the microenvironment, the organ structure and the immune host response (42–44). Temperature is a physical factor that affects cellular physiology in the environment and during infection(45). Pyrexia or fever is the increase in the body temperature above a specific set-point controlled by the hypothalamus (46). This increase is often due to infection or other non-infectious causes such as inflammation or malignancy (46) In humans, fever-like temperatures have been classified as low-grade (37.3^{o}C to 38.0^{o}C), moderate-grade (38.1^{o}C -39.0^{o}C; used in this work), high-grade (39.1^{o}C to 41°C), and hyperthermia or hyperpyrexia when the temperature increases above 41°C (47). Fever is considered a beneficial process to resolve infections and increase host survival, whereas antipyretic treatments have been shown to increase mortality in influenza patients (48,49). Evans et al. (2015) reviewed the ways in which fever contributes to control infections, including direct effects like the increase in Gram-negative bacterial lysis caused by serum components and stimulation of the adaptive and innate immune system (49).

However, host-pathogen coevolution implies the selection of bacterial features that counteract fever effects. In *P. aeruginosa* PAO1, for example, genes encoding virulence factors have been found to increase when passing from the environment to the host,

with 6.4% of them, particularly genes related to quorum sensing, exoproteins and siderophores, being upregulated at 37□C as compared with 22°C (19). Particularly, genes related to quorum sensing, exoproteins and siderophores are upregulated at 37°C (17). *P. aeruginosa* PA14 has been found to show the same trend (50). For SA, Bastock et al. (2021) (20) reported an expression profile rearrangement in cultures incubated at 40°C during different times along with higher hemolysis and staphyloxanthin production. However, the authors performed human nasal epithelial infection experiments comparing only SA incubated at 34°C and 37°C. In our model, we chose a sequential 2-h incubation at 37°C followed by 2 h at 39°C to understand the early response of bacteria to host temperature followed by a fever-like situation. In monocultures, our results showed a different response of *P. aeruginosa* PAO1 and *S. aureus* USA300 to temperature. SA showed a differential expression of several central metabolic pathways, while PA only presented a decrease in the periplasmic glucose oxidation pathway. Recently, Hamamoto et al. (2022) (51) performed an *in vivo* RNAseq of *S. aureus* colonizing the mouse liver and reported that genes related to low oxygen levels are upregulated at 24 and 48 h post-infection. This shows the importance of microaerobiosis during infection, as that used in this work.

The most important SA response to 39°C under low oxygen conditions was the increase in the expression of the *agr* operon and the differential expression of its direct and indirect targets. In concordance with the work by Bastock et al. (2021) (20), our *in vitro* virulence assays for SA showed staphyloxanthin production, but, in contrast to these authors, we did observe a significant increase in genes related to staphyloxanthin biosynthesis. An important feature for virulence is hemolysis. In this work, we showed upregulation of hemolysin-coding genes at 39°C and an increase in hemolysis after overnight incubation at 39°C. Contradictory results regarding this issue can be found in

the literature. Bastock et al. (20) showed that when plates are incubated at 40°C for 30 minutes, hemolysis increases with temperature, in line with our results, while Palela et al. (52) reported that at 40.5^{o}C, hemolysis decreases because temperature affects hemolytic pore kinetics. Thus, results of this and other works show the complex scenario when virulence factors are evaluated. We found an effect of SA incubated at 39°C on IL-8 production, which was increased at 39°C along with a decrease in cellular invasion. Chekabab et al. (53) described an inhibitory effect of SA filtrated supernatants on IL-8 production in the airway epithelial cell line Beas-2B stimulated with *P. aeruginosa*. In this work, we showed a slight increase in IL-8 production in co-cultures at 39°C in A549 epithelial lung cells along with an increase in cellular invasion independent of culture temperature. Alves et al. (54) reported an intermediate interleukin production by cells infected with mixed biofilms of *S. aureus* and *P. aeruginosa* compared to mono-species biofilms. Additionally, in a wound model these authors described an impairment in the wound closure along with a proinflammatory response when mixed biofilms were used (54).

Our results revealed that in our mouse model bacteria pre-incubated at 39° showed a different behavior depending on the species and the culture condition (summary in Fig. 6). SA monocultures intranasally inoculated in mice caused higher lethality but with similar bacterial burden in the different organs. These results, along with our *in vitro* experiments, suggest an increased virulence and survival of bacteria, even in the absence of arginine or proline. In contrast, PA monocultures showed a different pattern with a more robust metabolism, with less differentially expressed genes in response to fever-like temperatures and some virulence features increased at 39°C in agar plates. However, they triggered less IL-8 production and caused lower mouse mortality.

However, the most evident *in vivo* effect was observed for co-cultures incubated at 39°C. These severely impacted on mouse survival rate and led to an increase in PA burden in all the organs tested. In contrast, for SA the results were similar and independent of the temperature but higher than those obtained with monocultures, in agreement with the cell line invasion assays. In a keratinocyte cell line, Alves et al. (54) demonstrated that *S. aureus* allows attachment of *P. aeruginosa* to cells, indicating a complex *in vivo* interaction between these bacterial species.

The mechanism by which these bacterial species sense the increase in 2°C and cause the phenotypes observed remains unknown. Thermoregulation through RNA thermometers has been described for SA but mostly for the environment-host transition and includes the *cspB*, *cspC*, and *cidA* genes (55,56). However, to our knowledge, the response to fever-like temperatures is still unknown. In PA, the sensing of temperature changes by RNA thermometers has also been reported for the virulence factors that are under the control of the quorum sensing regulator RhlR, including pyocyanin, elastase and rhamnolipid production but, again, the differences were observed following a temperature shift from 30°C to 37°C (57).

In summary, our results demonstrate that fever-like temperatures affect the virulence of *S. aureus (*SA) monocultures and *P. aeruginosa (*PA) and *S. aureus* co-cultures, mouse survival and bacterial burden in murine organs. Our results open new questions about the effect of fever-like temperatures on other pathogenic bacterial species and on the host-pathogen interaction.

Materials and Methods

Strains and culture conditions

The strains used throughout this work were *Staphylococcus aureus* USA300 (USA300_FPR3757) and *Pseudomonas aeruginosa* PAO1. Precultures were performed under aerobic conditions at 37°C in Tryptic Soy Broth (TSB; Oxoid). Microaerobic cultures were grown in TSB supplemented with 0.5% KNO_3, in sealed bottles, using a 1:4 medium-to-flask volume ratio without agitation. SA and PA monocultures were inoculated at an OD_{600nm} of 0.05 and incubated at 37°C for 2 h. After that, cultures were split in two and further incubated at 37°C (control) or 39°C (fever-like temperature) for 2 h. A similar protocol was used for co-culture experiments, where SA and PA were co-inoculated each at an OD_{600nm} of 0.05.

Plate competence assay

An overnight SA culture incubated at 37°C was adjusted to an OD_{600nm} value of 1 and then seeded on a Tryptic soy agar (TSA) plate. A 5-µl drop of a PA culture with an OD_{600nm} adjusted to 10 was placed in the center of the plate. After drying, the plates were incubated overnight at 37°C or 39°C.

RNA extraction and RNA library preparation

SA and PA monocultures or co-cultures were incubated at either 37°C or 39°C, following the experimental scheme shown in Fig. S1. Frozen bacterial pellets were homogenized with milliQ water by using mortar and pestle devices in Eppendorf tubes. After cell disruption, total RNA was extracted with the Trizol method followed by extraction using a commercial kit (Total RNA extraction kit - RBC bioscience). Samples were treated with DNAse I (Promega). To improve the quality of the readings, ribosomal RNA was depleted from the samples by Novogen services (CA, USA).

Libraries were constructed by Novogen Services (CA, USA). Mass sequencing was performed using the Illumina Novaseq 6000 platform with a paired-end protocol (Novogen Services; CA, USA). For each condition, triplicate independent RNA extraction and libraries were used.

RNA-seq data analysis

Reads were preprocessed using the Trimmomatic computer tool (58) by eliminating adapters and low-quality sequences. The reads quality was evaluated using the FastQC tool (www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Reads alignment and assembly, transcript identification and abundance quantification were carried out using the Rockhopper software for both monocultures and co-cultures (59). The reference genomes were *P. aeruginosa* PAO1 (AE004091.2) and *S. aureus* (USA300_FPR3757). Reads were normalized per kilobase per million mapped reads. To verify concordance between the independent replicates for each condition, a Spearman correlation analysis of normalized counts was performed. Differential gene expression was considered only with $P < 0.05$ and $Q < 0.05$. In the case of co-cultures, the RNA dataset was analyzed, and the reads aligned by separately using the PA or SA reference genomes.

Genes were sorted into functional classes using the KEGG (60), MetaCyc (61) and String (62) tools.

Cultures and determination of organic acid production

To determine organic acid production, bacteria were grown in CDM (63) supplemented with 7.5 mM glucose as a carbon source. Cultures were centrifuged at 13,000 rpm for 5 min and supernatants were diluted 1:5 in water and filtered through 0.22-µm syringe

filters (MSI, USA). Samples were analyzed by high performance liquid chromatography (HPLC) (LC-20AT Prominence; Shimadzu Corp., Japan) equipped with a UV detector (SPD-20AV; Shimadzu Corp.) using an Aminex HTX-87H column (Bio-Rad Laboratories, USA) at 50°C. The mobile phase consisted of 5 mM H_2SO_4 with a flow rate of 0.6 ml/min. Detection was performed at 210 nm and analytical standards (Sigma-Aldrich Co., USA) were used for quantification by external calibration curves.

SA growth in arginine and proline free medium

To assess arginine metabolism in SA, bacteria were grown in arginine- and proline-free modified CDM or in CDM supplemented with glucose 7.5 mM. SA was inoculated at an OD_{600nm} of 0.05 and cultures were incubated at 37°C or 39°C under microaerobic conditions, following the same scheme as described in Fig. S1. After 4 h of incubation, the CFU ml^{-1} in TSA was determined.

***In vitro* determination of virulence factors**

The carotenoid pigment staphyloxanthin was quantified as previously described (64). Briefly, cultures grown as described in Fig. S1, the OD_{600nm} was adjusted to 1, and then 10 ml was centrifuged at 8000 rpm for 10 min. Cell pellets were washed with sterile PBS and resuspended in 1 ml of methanol. The tubes were incubated overnight in the dark with agitation at 37°C. Then, the tubes were centrifuged to collect the supernatant containing the extracted pigments. Staphyloxanthin was quantified by measuring absorbance at 462 nm.

For the evaluation of virulence factors, precultures were grown at 37°C under aerobic conditions and the OD_{600nm} was adjusted to 1 for monocultures and for co-cultures in a 1:1 proportion for each bacterium, reaching an OD_{600nm} value of 1. With this bacterial

suspension, a 5-µl drop was placed in the corresponding plates and the plates were incubated at 37°C or 39°C for 24 h. DNAse agar (Britania) was used following product instructions to analyze extracellular DNAse activity. Extracellular protease was determined using TSA plates supplemented with 5% skimmed milk. Rhamnolipid production was analyzed in sheep-blood agar plates. Activity of extracellular lipases was analyzed using TSA plates supplemented with sterile egg-yolk suspension (5%). For all tests, the degradation halo or clear zone was measured in each case and the colony diameter was measured for normalization using the ImageJ software (65).

Antibiotic and H_2O_2 sensitivity assays

Antibiotic sensitivity was determined using the commercial panel Sensititre (ThermoFisher) Gram positive for *S. aureus* USA300 and Gram negative for *P. aeruginosa* PAO1. The test was carried out strictly following the manufacturer's instructions, but the plates were incubated at 37°C or 39°C. The MIC was determined following the instructions.

H_2O_2 sensitivity was evaluated in agar plates as previously (66). Briefly, cultures grown as described in Figure S1 were seeded in a lawn, and a sterile Whatman no. 1 filter disc (6 mm) impregnated with 5 µM of 30% (v/v) H_2O_2 (Merck) was placed on the seeded plate. Plates were incubated only at 37°C since the H_2O_2 effect is almost instant. Inhibition zones were measured using the ImageJ software.

Biofilm and motility assays

SA monocultures were seeded on Red Congo plates and then incubated overnight at 37°C or 39°C to detect exopolysaccharide production. Black-colored colonies were analyzed. Biofilm formation was analyzed in 96-multiwell polystyrene plates. Briefly,

SA monocultures were inoculated at an initial OD_{600nm} of 0.025 in TSB supplemented with 0.5% KNO_3. Incubation was carried out at 37°C or 39°C for 24 h. Planktonic cells were collected and OD_{595nm} was measured. Biofilm was stained with crystal violet as described before (67) and the attached biomass was determined at 550 nm in a plate reader (DR - 200Bs).

For motility assays, the PA culture was adjusted to an OD_{600nm} of 1 (for co-cultures assay, the proportion was 1:1 for each bacterium) and a 5-µl drop was placed in a motility plate containing 8 g/l nutritive broth, 0.5% agar and supplemented with 0.5% glucose(68). Plates were incubated overnight at 37°C or 39°C. The entire movement was measured using the ImageJ software (65).

Invasion assays on A549 cells

For invasion assays, A549 cells (a human adenocarcinoma epithelial cell line) were cultured at 37°C under 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 1% penicillin-streptomycin (Pen-Strep, Gibco) and 10% fetal bovine serum (FBS, Gibco).A549 cells were seeded in 24-well microtiter plates at 2.5 x 10^5 cells per well and then incubated for 48 h (37°C, 5% CO_2) in DMEM supplemented with 10% FBS and 1% Pen-Strep. Cells were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS – Gibco) to remove antibiotics and then 1 ml DMEM supplemented with 10% FBS. Cells were infected at a multiplicity of infection (MOI) of 30 for SA monocultures and of 20 for PA monocultures. For invasion experiments with co-cultures, after determining the CFU/ml to the OD_{600} ratio of co-cultures, the OD_{600nm} was adjusted to 1. Then, 100 µl of this adjusted suspension was added to each well, which resulted in a MOI of 14 for SA and of 21 for PA. Bacteria and host cells were incubated for 1.5 h and then washed twice with DPBS. To kill extracellular bacteria,

infected cells were incubated for 1.5 h in DMEM supplemented with 2.5 μg ml^{-1} lysostaphin (Sigma) for SA or gentamicin 200 μg ml^{-1} (Sigma) for PA infection or both for co-infections. Cells were washed again with DPBS and lysed with 0.1% Triton X-100, 0.5% trypsin, and 0.3 mg x ml^{-1} DNase (Sigma) in DPBS. Serial dilutions of cell lysates were plated in TSA to quantify the internalized bacteria for monocultures. In the case of co-infected cells, the lysates were plated on TSA-NaCl to detect SA and in cetrimide agar to detect PA. Results are presented as a percentage of the internalized bacterial inoculum.

Cytokine measurements

For cytokine measurements, A549 cells were seeded in 96-well microtiter plates at a concentration of $5x10^4$ cells per well for 24 h (37°C, 5% CO_2) in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% Pen-Strep (Gibco). After that, cells were washed with DPBS, and then 198μl of fresh DMEM without antibiotics was added. Cells were stimulated by the addition of 2μl of bacterial cultures (OD_{600nm} adjusted to 0.1). After 24 h of incubation (37°C, 5% CO_2), plates were centrifuged, and the supernatants were used for cytokine determinations. Human cytokine secretion was measured using the Invitrogen enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8 according to the manufacturer´s instructions.

Bacterial inoculum for mouse lung infection experiments

SA and PA were cultured as previously described. Cells from monocultures and co-cultures were incubated at 37ºC or 39ºC and then harvested by centrifugation at 8000 rpm for 5 min, washed twice using sterile PBS and resuspended in PBS. The resuspended cells were then used to infect mice intranasally. For SA, mice were

infected with $2x10^8$ CFU per individual, whereas for PA infection, mice were infected with $1x10^7$ CFU per individual. For co-cultures, the resuspension was adjusted to an OD_{600} of 0.1 (corresponding to a concentration of 1.4 10^7 CFU/ml for SA and 2.1 10^7 CFU ml^{-1} for PA) and each mouse was infected with 200 µL.

Mouse survival assay

Female DDY mice (6-8 weeks; 10 per group) were infected with SA monocultures at 37ºC and 39 ºC, PA monocultures preincubated at 37 ºC and 39ºC, or SA and PA co-cultures at 37 ºC and 39 ºC. Mice infected with sterile PBS were used as negative control. Mouse survival was monitored daily for 10 days. All experiments were carried out following the ethics guidelines and approved by the Institutional Ethics Committee of the University of Surabaya, Indonesia.

Bacterial burden assay

For the bacterial burden assay, six mice per group were infected using the same bacterial growth conditions as in the mouse survival assays. Mice were sacrificed 24 h after infection, and the lungs, kidney and liver were aseptically recovered. These organs were then homogenized in sterile PBS containing 0.1% Tween 20. The bacterial load was determined by diluting the homogenized organ accordingly and plating on Mannitol Salt Agar (Merck) to count SA and Cetrimide Agar (Merck) to count PA. The CFU/ml were determined after 24 h incubation at 37 ºC.

Bacterial count in the presence of human serum albumin (HSA)

Bacterial count was performed in TSB medium supplemented or not with HSA (3.5%) following the same scheme described above. Cultures were plated in TSA+NaCl and cetrimide agar plates for SA and PA determinations, respectively.

Statistical analysis

One- or two-way ANOVA or non-parametric test was performed depending on the experiment, using GraphPad software (https://www.graphpad.com/). PCA was performed through the Singular Value Decomposition method on the normalized read counts. For multifactorial test applied to the entire RNAseq data set, two-way ANOVAs were carried out for each expression feature (genes) with factors: culture temperature (37º and 39º C), type (mono and co-culture), and interaction. The p-values of the interaction terms were adjusted for multiple testing with the Benjamini-Hochberg method, the same used by Rockhopper, which will be subsequently called q-values for consistency with this tool. PCA and multiple ANOVAs were carried out in R programming language (69).

Ethics statement

Mouse experiments using Female DDY mice (6-8 weeks) were carried out with the approval of the Institutional Ethics Committee, University of Surabaya, Indonesia, no. 108/KE/IX/2022, following all the ethics guidelines.

Author contributions: ESV (under the PMT and FG supervision) and PMT performed the conceptualization. ESV, MMR, MBG, SAR, DEE and PMT performed most of the investigation. AB and THM (under the supervision of AL) conducted the mice studies. NB and JE (under the supervision of MSR) carried out the HSA protection experiments. SN performed formal multivariant analysis. FS, MMR and DDP carried out data

curation. PMT and ESV wrote the manuscript. FG, AL and MMR review and edited the manuscript. MMR, PMT and ESV carried out the visualization. PMT performed mostly the project administration and funding acquisition. MSR, DDP, AL and FG contributed with resources.

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Legends

Figure 1: Survival and competence assays. SA and PA were cultured under microaerobic conditions either at 37ºC or 39ºC following the scheme showed in Fig. S1, in mono- or co-culture, and survival was measured as CFU/ml on TSA+NaCl plates (**A**) or cetrimide plates (**B**). Competence test in agar plates performed as described in Materials and Methods (**C**).

Figure 2: A. Schematic overview of arginine biosynthesis and degradation metabolism in SA. Genes that were upregulated at 39°C are represented with a dark green arrow. Light green and orange arrows represent genes with a non-significant increase or decrease in their expression at 39°C, respectively. Genes without change are represented by black arrows. **B.** SA survival in CDM medium lacking arginine and proline and supplemented with 0.5% KNO_3. Cultures were incubated under microaerobiosis for 2 h at 37°C and further split and incubated at 37°C or 39°C for 2 h. CFU/ml was determined on TSA agar plates. Asterisk (*) denotes $P< 0.05$ (Student t-Test).

Figure 3: A. Schematic overview of SA carbon central metabolic pathways for monocultures 39°C vs 37°C and co-cultures 39°C vs 37°C. Upregulated genes (*P* and *Q*< 0.05) are colored in green, whereas those with an expression ratio above 1.5 but without significant differences are shown in light green. In the same fashion, downregulated genes at 39°C are colored in red (*P* and *Q*< 0.05), whereas those with an expression ratio below 0.66 but without significant differences are shown in light red. Unchanged genes are colored in black (ratio between 0.67 and 1.49). **B.** PA carbon central metabolic pathways for monocultures 39°C vs 37°C and co-cultures 39°C vs 37°C. Upregulated genes (*P* and *Q*< 0.05) are colored in green, whereas those with an expression ratio above 1.5 but without significant differences are shown in light green.

In the same fashion, downregulated genes at 39°C are colored in red (*P* and *Q*< 0.05), whereas those with an expression ratio below 0.66 but without significant differences are shown in light red. Unchanged genes are colored in black (ratio between 0.66 and 1.5). **C.** Nitrite production in SA and PA monocultures and co-cultures at 37°C and 39°C as described in Fig.S1. Nitrite concentration in the supernatant was determined using the colorimetric Griess assay. **D.** Organic acid content in the culture supernatant was determined by HPLC in CDM cultures supplemented with glucose and 0.5% of $KNO_{3.}$ Cultures were incubated following the same scheme shown in Fig.S1. **E.** Denitrification assay using Durham tubes. Monocultures and co-cultures were performed in TSB+ KNO_3 (0.5%) and incubated at 37°C or 39°C for 48 hours and bubble size was observed.

Figure 4: Virulence traits in PA and SA in monocultures or co-cultures incubated at 37°C or 39°C. A. Heat map representing the gene expression profile of SA cultured in different conditions and temperatures. Upregulated genes are colored in dark green while genes with a fold-change higher than 1.5 but with *P*>0.05 are shown in light green. Downregulated genes are presented in red and genes with a fold-change lower than 0.66 P>0.05 are colored in light red. Genes with no change in their expression (Fold change between 0.67 and 1.49) are shown in black. Grey indicates that expression of the gene was not detected in one or both conditions. **B.** Heat map representing the gene expression profile of PA cultured under different conditions. **C.** Staphyloxanthin production after incubating SA in monoculture following the scheme described in Fig. SA1. Methanol-extracted pigment from supernatants was measured at 462nm. **D.** Extracellular DNAse activity for SA or PA monocultures was assayed in DNA-agar plates incubated overnight at 37° C or 39° C. The DNA-degradation halo and colony diameter were further determined**. E.** SA extracellular hemolytic activity was tested in

sheep blood-agar plates inoculated with a 5-µl drop of SA monocultures and incubated overnight at 37° C or 39° C. Hemolysis was determined by measuring the clear halo and the colony diameter. **F.** Biofilm formation was analyzed using multiwell polystyrene plates with SA monoculture and incubated overnight at 37° C or 39°. Biofilms were revealed using the crystal violet (CV) technique and the biofilm formation index was calculated as CV $\square_{570\ nm}$/planktonic cells $\square_{595\ nm}$ for each replicate. **G.** Sensitivity of the SA and PA monocultures to H_2O_2 was analyzed in an agar test. Lawns of SA, PA or co-cultures were incubated as described in Fig.S1. Cultures were adjusted to an OD_{600nm} value of 1 and seeded in TSA plates, and then a 5-µl drop was placed in a sterile paper disk and further incubated at 37° C or 39° C. **H.** Rhamnolipid production in PA monocultures was determined by the hemolytic halo in sheep-blood agar plates incubated at 37° C or 39°C. **I.** Lipase extracellular activity in egg-yolk (5%)-supplemented TSA plates incubated at 37°C or 39°C. Clear zones or white precipitates were determined. **J.** Protease extracellular activity in milk (5%)-supplemented TSA plates incubated at 37° C or 39°C. Afterwards, the clear zone and colony diameter were determined. **K.** Motility of PA mono- and co-cultures was analyzed in motility plates incubated at 37° C or 39° C. All diameter measurements were performed with the ImageJ software. Individual values for each measurement are shown. Asterisk (*) denotes significant differences for t-test while different letters mean significant differences in a 2-way ANOVA.

Figure 5: A. Production of IL-8 in the A549 epithelial lung cell line was measured as described in Material and Methods. **B**. Production of IL-6 in the A549 epithelial lung cell line was measured as described in Material and Methods **C.** Invasion was performed using bacterial cultures incubated in mono- or co-cultures following the same scheme shown in Fig.S1 and as detailed in the Material and Method section. Results are

presented as percentage of bacterial inoculum that was internalized. **D.** Mouse survival was evaluated using 10 individuals for each group intranasally inoculated with mono- or co-cultures incubated as described in Fig.S1. Sterile PBS was used as a negative control. Mouse survival was monitored daily for 10 days. **E.** Bacterial burden in the liver. **F.** Bacterial burden in the lungs. **G**. Bacterial burden in the kidney. **H**. Effect of human serum albumin on SA and PA survival at 37°C or 39°C. When it corresponded, a two-way ANOVA was performed. Different letters denote P<0.05. Log scale in the graph was used for bacterial burden for better visualization, and zero values obtained in the bacterial count are not shown. Zero values were obtained for control (N=6) for all organs. Regarding the bacterial burden, PA mono 37 presented zero values in N=1 in the liver; zero values in N=1 in the lungs, and zero values in N=5 in the kidney. PA mono 39° C presented zero values in N=1.

Figure 6: Summary of the relevant effects of temperature on *S. aureus* USA300 and *P. aeruginosa* PAO monocultures and co-cultures.

Supplementary Legends

S1: Experimental scheme to analyze the effect of fever-like temperatures on the physiology and interaction of *S. aureus* USA300 (SA) and *P. aeruginosa* PAO1 (PA).

Fig S2: A. Number of differentially expressed genes detected by RNAseq. **B.** Cytochrome expression for SA. Upregulated genes are depicted in dark green. Genes with a fold-change higher than 1.5 but with P>0.05 are colored in light green. Downregulated genes are presented in red. Genes with a fold-change lower than 0.66 P>0.05 are colored in light red. Genes with fold changes between 0.67 and 1.49 are shown in black. Grey indicates that expression of the gene was not detected in one or both conditions. **C.** Cytochrome expression for PA. Upregulated genes: dark green. Genes with a fold-change higher than 1.5 but with P>0.05 are colored in light green.

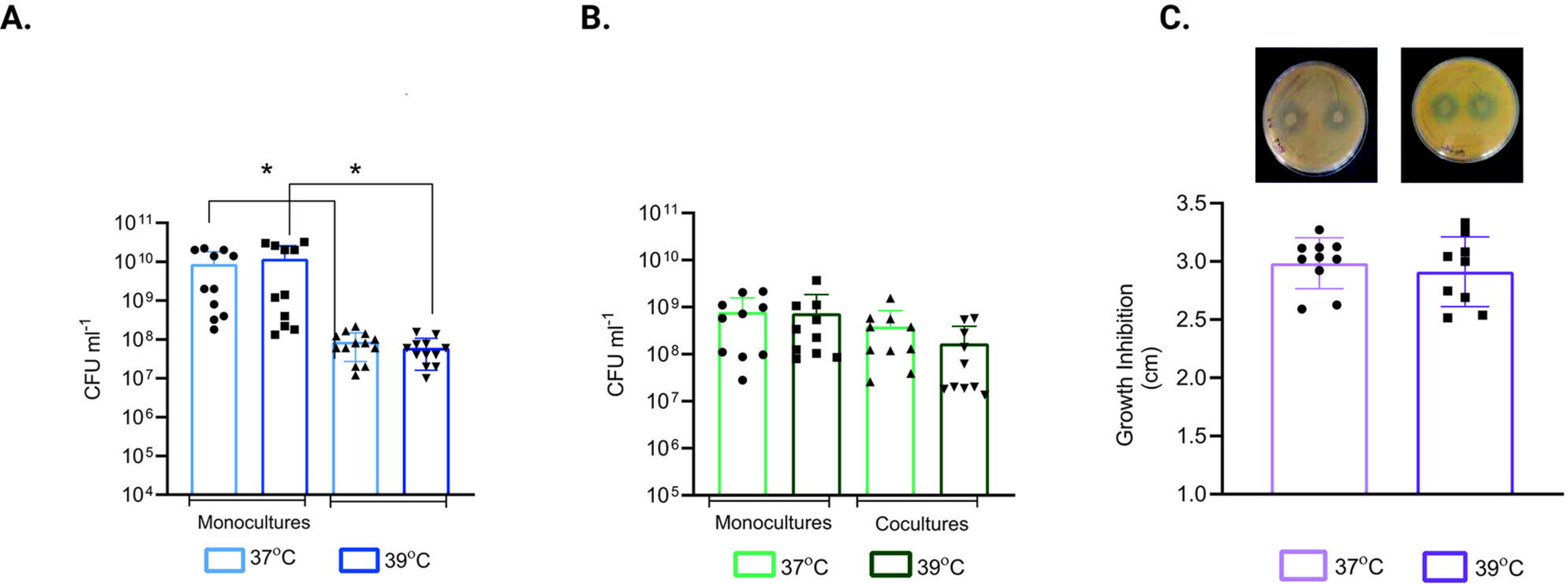
Downregulated genes are presented in red. Genes with a fold-change lower than 0.66 P>0.05 are colored in light red. Genes with fold changes between 0.67 and 1.49 are shown in black. Grey indicates that expression of the gene was not detected in one or both conditions.

Fig. S3: Functional classification of genes of SA incubated under different culture conditions as described in Fig.S1. Upregulated genes are represented in green while downregulated genes are shown in red.

Fig. S4: Functional classification of genes of PA incubated under different culture conditions as described in Fig.S1. Upregulated genes are represented in green while downregulated genes are shown in red.

Fig. S 5: **A.** Principal Component Analysis for SA expression profile. **B.** Principal Component Analysis for PA expression profile. **C**. Interaction ANOVA for SA expressed genes **D**. Interaction ANOVA for PA expressed genes. Analysis was performed using R language.

Fig. S6: A. SA, PA or co-cultures were incubated for 24 h at 37°C or 39°C. Pigment production in co-cultures at 39° is marked with an arrow. **B**. SA CFU/ml counting in monocultures and co-cultures incubated at 37°C or 39°C for 24 h. **C**. PA CFU/ml counting in monocultures and co-cultures at 37°C or 39°C for 24 h. Asterisk (*) denotes P<0.05 in a paired T-test.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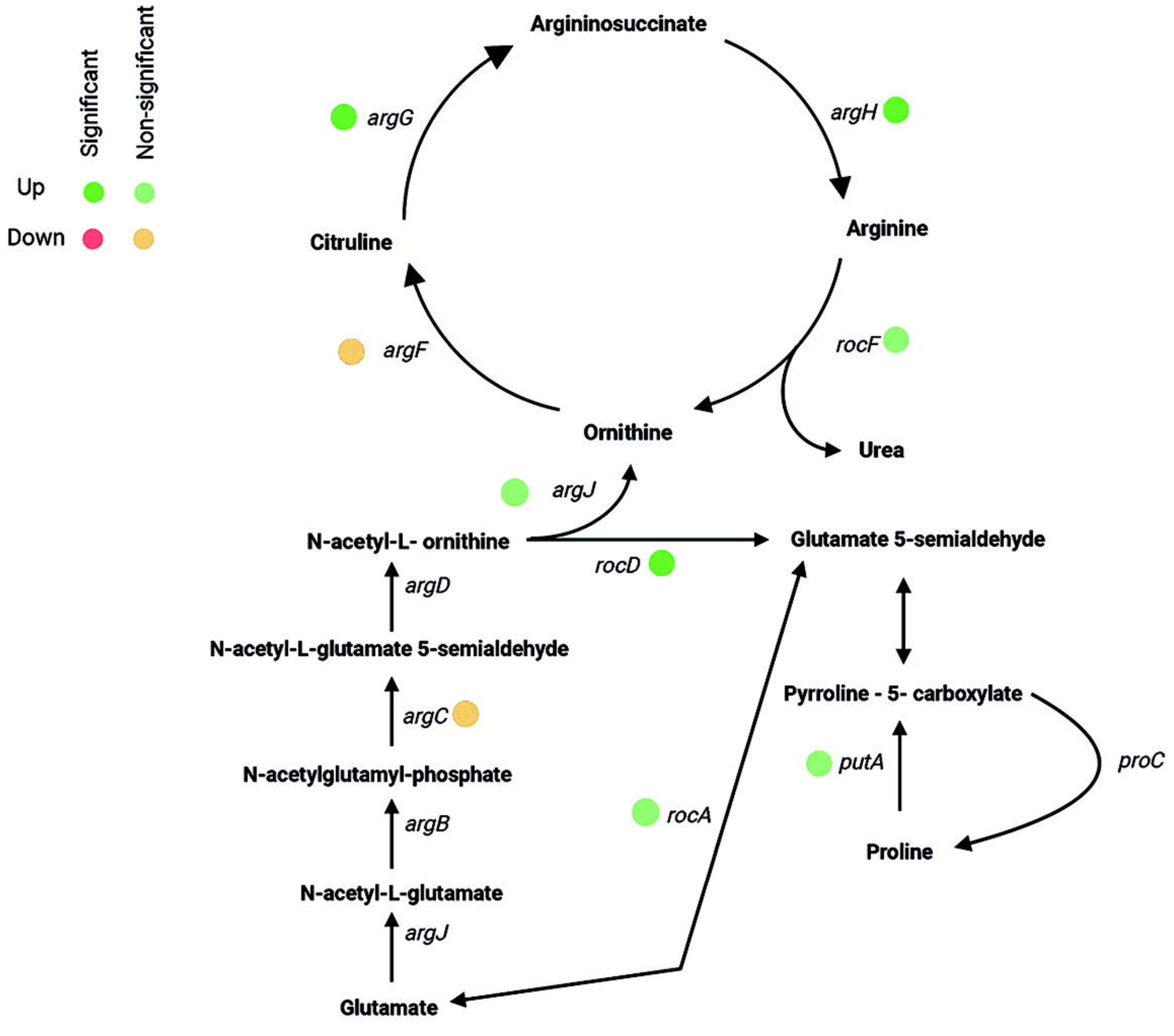
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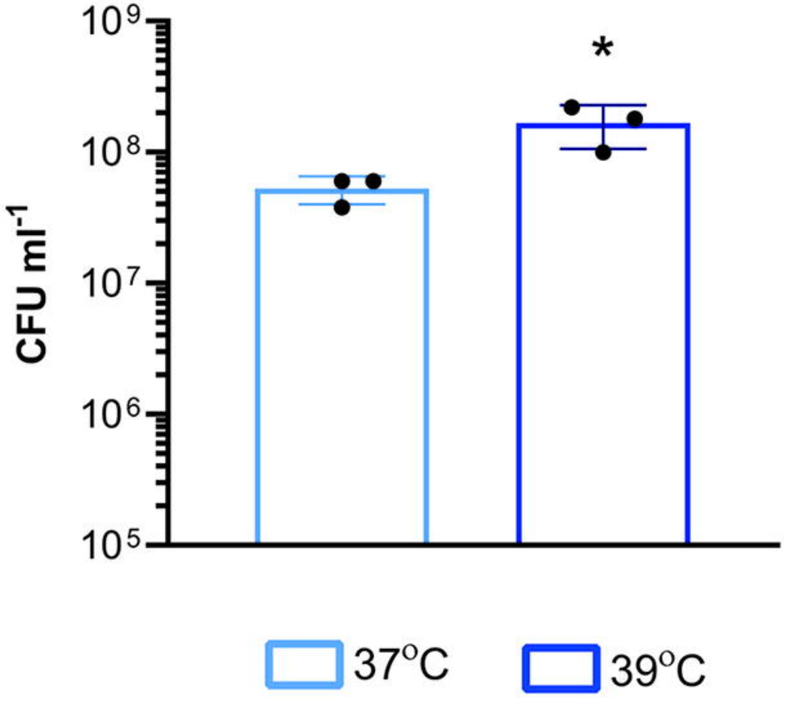
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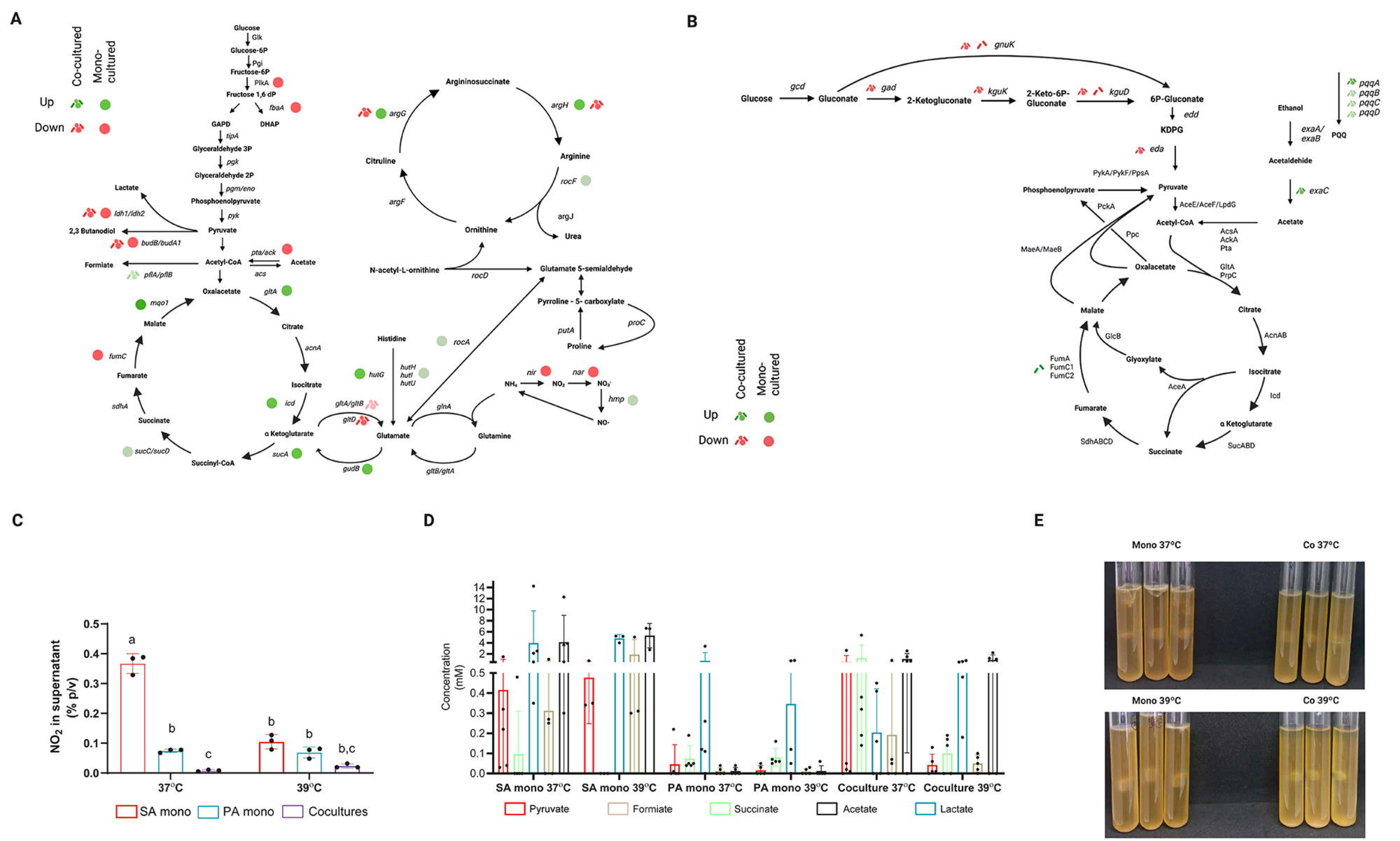
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Urea
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rocD
Glutamate 5-semialdehyde
Pyrroline - 5- carboxylate
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proC
Proline
Histidine
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Pta
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Glyoxylate
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FumC1
FumC2
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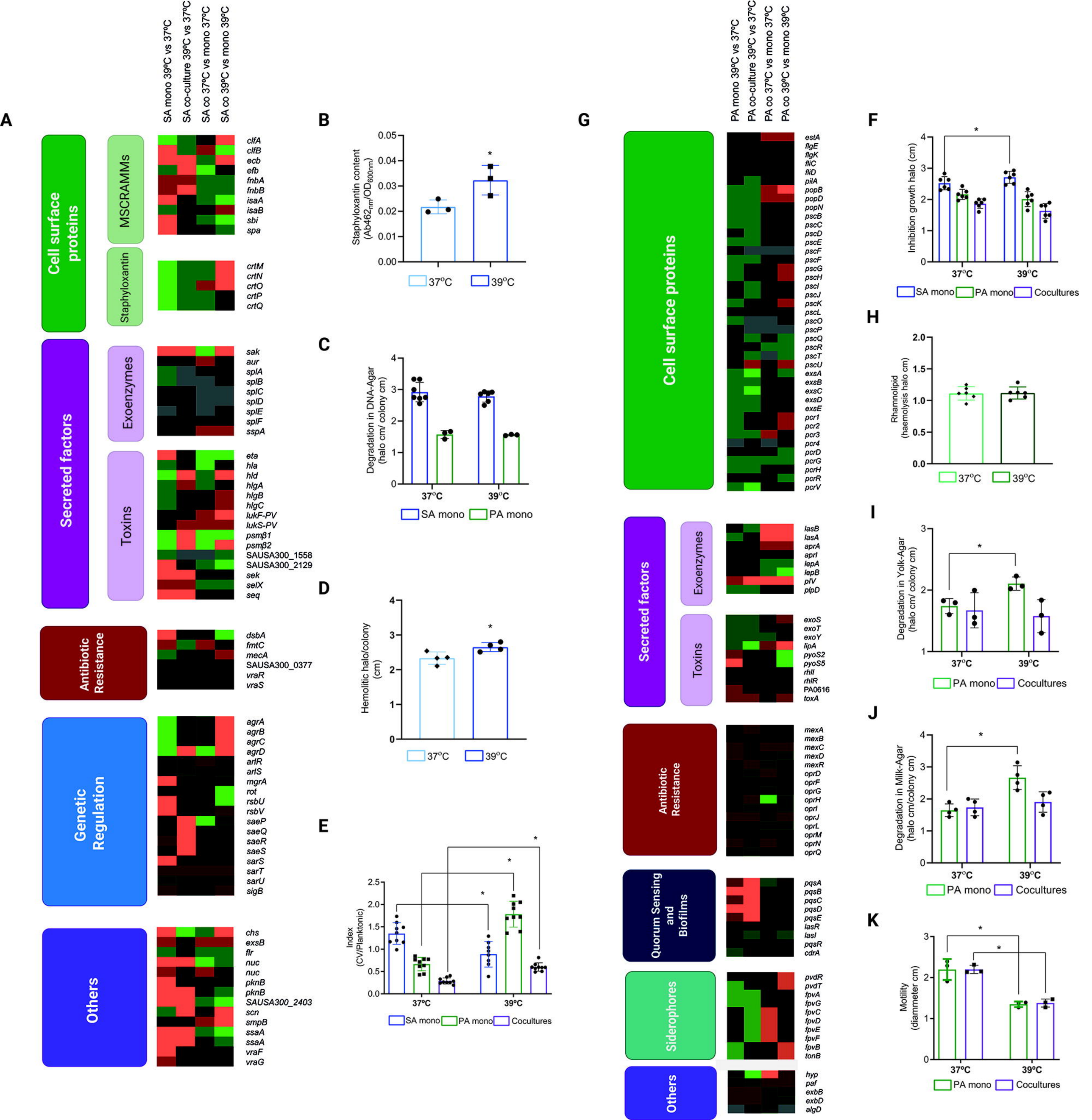
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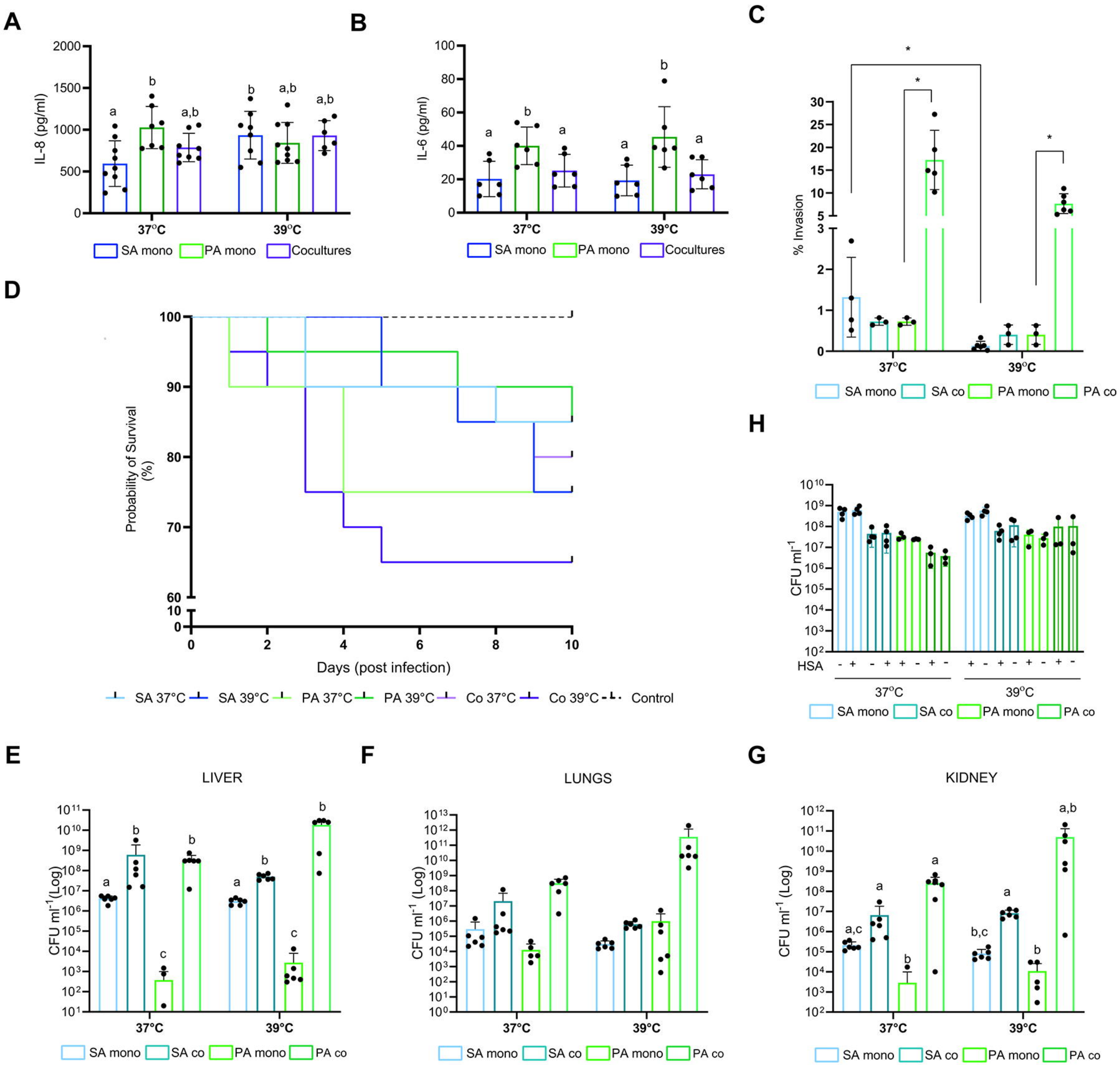
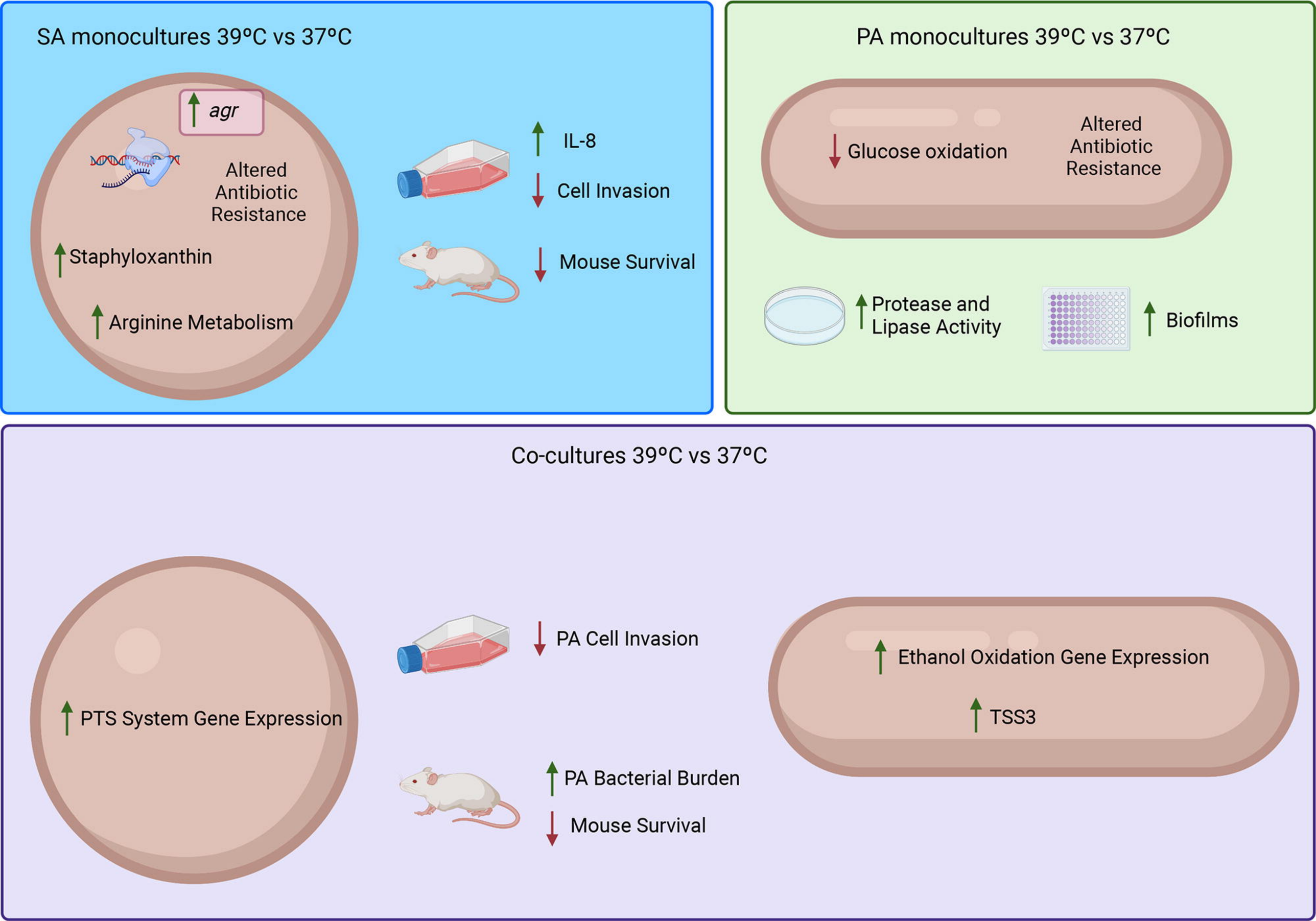
vraS
Genetic Regulation
agrA
agrB
agrC
agrD
arlR
arlS
mgrA
rot
rsbU
rsbV
saeP
saeQ
saeR
saeS
sarS
sarT
sarU
sigB
Others
chs
exsB
flr
nuc
nuc
pknB
pknB
SAUSA300_2403
scn
smpB
ssaA
ssaA
vraF
vraG
B
Staphyloxantin content (Ab462nm/OD600nm)
0.05
0.04
0.03
0.02
0.01
0.00
*
37ºC
39ºC
C
Degradation in DNA-Agar (halo cm/ colony cm)
4
3
2
1
0
37ºC
39ºC
SA mono
PA mono
D
Hemolitic halo/colony (cm)
4
3
2
1
0
*
37ºC
39ºC
E
Index (CV/Planktonic)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
37ºC
39ºC
SA mono
PA mono
Cocultures
G
PA mono 39ºC vs 37ºC
PA co-culture 39ºC vs 37ºC
PA co 37ºC vs mono 37ºC
PA co 39ºC vs mono 39ºC
Cell surface proteins
estA
flgE
flgK
fliC
fliD
pilA
popB
popD
popN
pscB
pscC
pscD
pscE
pscF
pscF
pscG
pscH
pscI
pscJ
pscK
pscL
pscO
pscP
pscQ
pscR
pscT
pscU
exsA
exsB
exsC
exsD
exsE
pcr1
pcr2
pcr3
pcr4
pcrD
pcrG
pcrH
pcrR
pcrV
Secreted factors
Exoenzymes
lasB
lasA
aprA
aprI
lepA
lepB
plV
plpD
Toxins
exoS
exoT
exoY
lipA
pyoS2
pyoS5
rhlI
rhlR
PA0616
toxA
Antibiotic Resistance
mexA
mexB
mexC
mexD
mexR
oprD
oprF
oprG
oprH
oprI
oprJ
oprL
oprM
oprN
oprQ
Quorum Sensing and Biofilms
pqsA
pqsB
pqsC
pqsD
pqsE
lasR
lasI
pqsR
cdrA
Siderophores
pvdR
pvdT
fpvA
fpvG
fpvC
fpvD
fpvE
fpvF
fpvB
tonB
Others
hyp
paf
exbB
exbD
algD
F
Inhibition growth halo (cm)
4
3
2
1
0
*
37ºC
39ºC
SA mono
PA mono
Cocultures
H
Rhamnolipid (haemolysis halo cm)
2.0
1.5
1.0
0.5
0.0
37ºC
39ºC
I
Degradation in Yolk-Agar (halo cm/ colony cm)
3
2
1
*
37ºC
39ºC
PA mono
Cocultures
J
Degradation in Milk-Agar (halo cm/colony cm)
4
3
2
1
0
*
37ºC
39ºC
PA mono
Cocultures
K
Motility (diammeter cm)
3
2
1
0
*
*
37ºC
39ºC
PA mono
Cocultures


A
IL-8 (pg/ml)
37°C
39°C
SA mono
PA mono
Cocultures
B
IL-6 (pg/ml)
37°C
39°C
SA mono
PA mono
Cocultures
C
% Invasion
37°C
39°C
SA mono
SA co
PA mono
PA co
D
Probability of Survival (%)
Days (post infection)
SA 37°C
SA 39°C
PA 37°C
PA 39°C
Co 37°C
Co 39°C
Control
H
CFU ml-1
HSA
37°C
39°C
SA mono
SA co
PA mono
PA co
E
LIVER
CFU ml-1 (Log)
37°C
39°C
SA mono
SA co
PA mono
PA co
F
LUNGS
CFU ml-1 (Log)
37°C
39°C
SA mono
SA co
PA mono
PA co
G
KIDNEY
CFU ml-1 (Log)
37°C
39°C
SA mono
SA co
PA mono
PA co


SA monocultures 39ºC vs 37ºC
agr
Altered Antibiotic Resistance
Staphyloxanthin
Arginine Metabolism
IL-8
Cell Invasion
Mouse Survival
PA monocultures 39ºC vs 37ºC
Glucose oxidation
Altered Antibiotic Resistance
Protease and Lipase Activity
Biofilms
Co-cultures 39ºC vs 37ºC
PTS System Gene Expression
PA Cell Invasion
PA Bacterial Burden
Mouse Survival
Ethanol Oxidation Gene Expression
TSS3