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The sigma factor σ^B is required for the development of the growth phase- and temperature-dependent increases in thermoresistance and membrane rigidity in *Staphylococcus aureus*

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ABSTRACT

The aim of this study is to determine the role that σ^B factor plays in the development of growth phase- and temperature-dependent thermoresistance in *Staphylococcus aureus* cells and its possible relationship with membrane fluidity. Cells of *S. aureus* strain Newman and its isogenic *ΔsigB* mutant (strain IK184) were grown at different temperatures to exponential and stationary growth phases, and the relationship between σ^B activity (estimated by RT-qPCR), heat resistance, and membrane fluidity (fatty acid profiles and DPH fluorescence anisotropy) was assessed. σ^B was required for the development of the growth phase- and temperature-dependent increases in thermoresistance and membrane rigidity (up to 37 °C), although a direct correlation between σ^B activity and heat resistance or membrane fluidity in *S. aureus* could not be established. Results obtained also revealed the existence of σ^B-independent growth phase and temperature (>37 °C) heat adaptation mechanisms. Moreover, it was demonstrated that σ^B does not control the fatty acid synthesis in *S. aureus*. Results obtained in this work contribute to a deeper knowledge of *S. aureus* physiology and to the development and design of more effective processes used by the food industry for microbial inactivation.

1. Introduction

Bacteria are able to develop different resistance mechanisms in order to adapt to adverse conditions and survive. According to Abee and Wouters (1999), the existence of several recognition subunits, called σ factors, of the RNA polymerase constitutes in numerous bacterial genera the most relevant regulation system of gene expression. Thus, depending on which σ factor is bound to the core enzyme the set of transcribed genes will vary. In parallel, the particular σ factor bound to the RNA polymerase at a specific moment will depend on the cell growth phase and on certain external stimuli, among other factors (Giachino et al., 2001). Thereby, for a given environmental conditions, the greater or lower transcription of the genes regulated by a specific σ factor will depend on its concentration with respect to the rest of σ factors and on its affinity for the core enzyme (Maeda, 2000). Notwithstanding, one should bear in mind that the concentration of each σ factor is regulated at a transcriptional and posttranscriptional level and that the regulation networks are extremely complex (Senn et al., 2005).

The alternative general stress sigma factor of Gram-positive bacteria, σ^B, controls the transcription of specific regulons under particular physiological and environmental conditions, leading to a greater capacity of microbial cells to survive. Its role in the development of stress responses has been widely studied in microorganisms such as *Bacillus subtilis* and *Listeria monocytogenes* (Hecker et al., 2007), and has also been documented in *Staphylococcus aureus* (Cebrián, Sagarzazu, et al., 2009; Giachino et al., 2001; Pané-Farré et al., 2006; Senn et al., 2005). However, in the particular case of this latter microorganism, deeper research is required to fully understand the physiological role of the σ^B factor regarding cell survival to different stressing agents, including heat.

S. aureus is a Gram-positive foodborne pathogen commonly found in the skin and mucous membranes. It is responsible for causing diverse infections and intoxications, for instance, food poisoning, superficial skin infections, and even more severe diseases, such as pneumonia, endocarditis, osteomyelitis, meningitis, septicemia, and sepsis (Lowy, 1998). Food poisoning caused by *S. aureus* is due to the ingestion of enterotoxins, previously produced by the microorganism inside the food. 24 different enterotoxins are known so far, and they are highly

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resistant to heat, freezing and enzymatic digestion (Fisher et al., 2018). Enterotoxin poisoning of *S. aureus* is characterized by presenting a short incubation period and a symptomatology that includes nausea, vomiting, abdominal pain, and diarrhoea (Baird-Parker, 2000). It is one of the food diseases with the highest number of communications and one of the main microbial causes of food poisoning in Europe (EFSA & ECDC, 2023). The ability of *S. aureus* to survive against numerous stresses such as the limitation of macro and/or micro nutrients, variations in pH and temperature, or desiccation is remarkable (Baird-Parker, 2000). In this microorganism, σ^B regulates the synthesis of more than 200 proteins (Bischoff et al., 2004), including among them approximately 150 proteins related to the environmental stress response (Gertz et al., 2000; Senn et al., 2005). In addition, the *S. aureus* σ^B factor controls several metabolic processes, namely transport through the membrane, envelope composition, adhesion to different tissues or host cells, antibiotic resistance, and virulence expression (Müller et al., 2014; Pané-Farré et al., 2006).

Recently, the possible relationship between membrane fluidity and heat resistance has been proved with more evidence in *S. aureus* (Cebrián et al., 2019). Given the role of σ^B in *S. aureus* membrane physiology, it is reasonable to hypothesize that the increase in heat resistance provoked by its activation (Cebrián, Sagarzazu, et al., 2009), might be related to changes in membrane structure, composition, or behaviour. Notwithstanding, further research is required to verify this hypothesis. The aim of this work was to determine the role that the σ^B factor plays in the development of growth phase- and temperature-dependent thermoresistance in *S. aureus* cells and its possible relationship with membrane fluidity.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains employed in this study were *S. aureus* strain Newman (σ^B +) and its isogenic Δ*rsbUVW-sigB* mutant, strain IK184 (σ^B -). They were kindly provided by Brigitte Berger-Bächi from the Institute of Medical Microbiology, University of Zurich. Cultures were maintained frozen at −80 °C in cryovials. Stationary and exponential growth phase cultures were prepared by inoculating 10 mL of tryptone soya broth (Biolife, Milan, Italy) supplemented with 0.6 % yeast extract (Biolife; TSB-YE) with a loopful of growth from tryptone soy agar supplemented with 0.6 % yeast extract (Biolife; TSA-YE) and incubating the resulting culture for 12 h at 37 °C in a shaking incubator. 50 μL of this culture was then inoculated into 50 mL of fresh TSB-YE at 10, 20, 30, 37 and 42 °C. For each culture temperature, samples were taken after different incubation times to construct growth curves and to determine the time required to reach the stationary growth phase (containing approximately $8 \times 10^8 - 1 \times 10^9$ cells/mL). The required time was of 344, 48, 24, 24 and 12 h at 10, 20, 30, 37 and 42 °C, respectively. For exponential growth-phase assays, cultures were grown until the suspensions reached approximately $8 \times 10^7 - 1 \times 10^8$ cells/mL.

2.2. Heat treatments

Heat treatments were carried out in a specially designed resistometer (Condón et al., 1993). Briefly, this instrument consists of a 400-mL vessel provided with an electrical heater for thermostation, an agitation device to ensure inoculum distribution and temperature homogeneity, and ports for the injection of the microbial suspension and for the extraction of samples. Once treatment temperature (54–62 °C) had attained stability (±0.1 °C), 0.2 mL of an appropriately diluted or concentrated cell suspension was injected into the main chamber containing the treatment medium, McIlvaine citrate-phosphate buffer (pH 7.0). After inoculation, 0.25- mL samples were collected at different treatment times and immediately pour-plated (with TSA-YE) and incubated (24 h at 37 °C) for survival counting.

2.3. Resistance parameters

Survival curves were obtained by plotting the logarithm of the fraction of survivors *versus* the treatment time. Curves were fitted by linear regression using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, California, U.S.A.). The lethality of heat treatments was measured by the decimal reduction time value (D_t value), which is defined as the time (min) of treatment required at constant temperature for the number of survivors to drop one log cycle. Decimal reduction times curves (DRTC) were represented by plotting the log of the D_t values *versus* the treatment temperature, and z values (increase of temperature (in °C) necessary to reduce the D_t value one log cycle) were also calculated.

2.4. Sample preparation for membrane fatty acid analysis

Analysis of *S. aureus* membrane fatty acid composition was carried out at the Spanish Type Culture Collection (STCC) using the MIDI Inc.'s Sherlock® Microbial Identification System (Kunitsky et al., 2006). For this purpose, cultures in exponential and stationary growth phase were obtained at all the incubation temperatures (10, 20, 30, 37 and 42 °C), centrifuged twice (1766 *g*; 10 min) and finally stored at −80 °C until the analysis.

2.5. Quantitative PCR assays

To perform the RNA extraction, bacterial suspensions were centrifuged, and pellets were resuspended in 1.5 mL of TRIzol (Sigma-Aldrich, Saint Louis, U.S.A.). Afterwards, cells were lysed in the Mini bead beater (Biospec), incubated for 5 min at room temperature and the liquid fraction obtained was mixed with 0.3 mL of trichloromethane. After shaking the samples for 15 s and incubating them for 3 min at room temperature, they were centrifuged at refrigeration temperature for 15 min at 12,000 *g*. The supernatant was transferred into a clean eppendorf and the RNA was purified with the RNeasy Protect Bacteria kit (Qiagen, Hilden, Germany), adding RLT buffer containing β-mercaptoethanol to this supernatant and following the subsequent manufacturer instructions. The concentration and quality of the isolated RNA was measured by means of a spectrophotometer (Biochrom, Cambridge, U.K.).

Once purified, the RNA samples were treated with DNase using the Rapidout DNA Removal Kit (Thermo Fisher Scientific, Massachusetts, U.S.A.), and cDNA was synthesized using the SuperScript IV Reverse Transcriptase Kit (Invitrogen, Carlsbad, U.S.A.). The obtained cDNA was stored at −20 °C until its amplification by qPCR.

All the reagents required for the amplification were mixed (10 μL of Nuclease-Free Water, 10 μL of GoTaq qPCR Master Mix (Promega, Madison, U.S.A.) including the Taq polymerase enzyme, 0.4 μL of each primer and 2.25 μL of sample). *asp23* was used as the reporter gene for σ^B expression (Gertz et al., 2000; Pané-Farré et al., 2006), and *gmk* was chosen as housekeeping or reference gene as indicated in Tuchscherr et al. (2015). Table 1 shows the primers selected to conduct this assay and their sequences.

The amplification process was monitored with a real time thermocycler (Bio-Rad Laboratories, Hercules, U.S.A.) connected to a computer with specific software (Bio-Rad CFX Manager 2.1) to process and analyse the results. This software calculates the normalized expression $\Delta\Delta C_T$ (relative quantity of genes of interest is normalized to relative quantity of the reference genes across samples).

2.6. Measurement of fluorescence anisotropy

Changes in membrane fluidity were monitored by means of the fluorescence anisotropy technique using DPH (1,6-diphenyl-1,3,5-hexatriene; Sigma-Aldrich) as a lipophilic marker probe, according to the protocol described by Aricha et al. (2004). Anisotropy values are inversely related to membrane fluidity (Shinitzky, 1984).

Table 1
Selected primers for the qPCR assay (Tuchscherr et al., 2015).

Gene	Forward primer sequence	Reverse primer sequence
asp23	AAAGCAAAACAAGCATACGACAATC	AGCGATACCAGCAATTTTTTCAAC
gmk	AAGGTGCAAAGCAAGTTAGAA	CTTTACGCGCTTCGTTAATAC

Briefly, samples of bacterial cultures were diluted, if necessary, to obtain an OD_{600} = 0.80, washed twice with PBS containing 0.25 % formaldehyde (pH 7.4) for fixation, and then incubated for 45 min at 37 °C with 5×10^{-6} M DPH (Sigma-Aldrich; added as a 10^{-4} M solution in tetrahydrofuran) for probe insertion in the membrane. Steady-state fluorescence anisotropy was measured at the different established temperatures (54–62 °C) with a Cary-Eclipse spectrofluorometer provided with a thermostatized multicell holder and a manual polarizer accessory (Varian Inc., Mulgrave, Australia) with excitation at 355 nm and emission at 425 nm, 2.5- and 2.5-nm slits, respectively, and a 3-s integration time.

Anisotropy values (r) were calculated according to Shinitzky (1984), as follows:

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2.G.I_{VH}} \; with \; G = \frac{I_{HV}}{I_{HH}}$$

$_V$ and $_H$ stand for polarisation direction (vertical and horizontal directions), while I represents the corrected fluorescence intensity obtained by:

$$I_{HH} = I_{(L)HH} - I_{(BUFFER+CELL)HH} - I_{(BUFFER+PROBE)HH} + I_{(BUFFER)HH}$$

$I_{(L)}$: fluorescence intensity of probe-labelled cell suspension; $I_{(BUFFER + CELL)}$: fluorescence intensity of non-labelled cell suspension; $I_{(BUFFER + PROBE)}$: fluorescence intensity of the buffer incubated with the DPH probe; $I_{(BUFFER)}$: fluorescence intensity of the sole buffer. This calculation was repeated for the other three signals (I_{HV}, I_{VV}, I_{VH}).

2.7. Statistical analysis

The results obtained after conducting the experiments were statistically analysed using GraphPad Prism 8. The experimental design and results of the statistical analyses that have been conducted are shown in Tables S1–S7 (Supplementary Material). All the experiments included in this document were carried out at least by triplicate (except in the case of fatty acid analysis) and error bars indicate the standard deviation of the means.

3. Results

3.1. Influence of growth temperature and phase on S. aureus σ^B activity

The influence of the growth temperature on the σ^B activity of *S. aureus* cells in different growth phase was studied by means of RT-qPCR. For this purpose, *asp23* transcription was chosen as indicator of σ^B expression, as described in Gertz et al. (2000) and *gmk* was chosen as a housekeeping gene, as indicated in Tuchscherr et al. (2015). Fig. 1 shows the influence of both, growth temperature and phase, on the σ^B expression of *S. aureus* Newman cells. In this figure, the relative transcription of *asp23* at the different temperatures studied, and for exponential and stationary growth phase cells, is presented. The normalized Ct value of exponential phase cells grown at 37 °C was established as the reference value (1) for calculating *asp23* relative expression.

As can be observed in the figure, *S. aureus* Newman exponential growth phase cells cultivated at 20 and 42 °C displayed a significantly lower ($p < 0.05$) *asp23* transcription (*i.e.* a decreased σ^B activity), than those grown at 10, 30 and 37 °C. The entry into the stationary phase of growth resulted in an increase in σ^B activity. However, the magnitude of this increase varied depending on the growth temperature. Thus, this

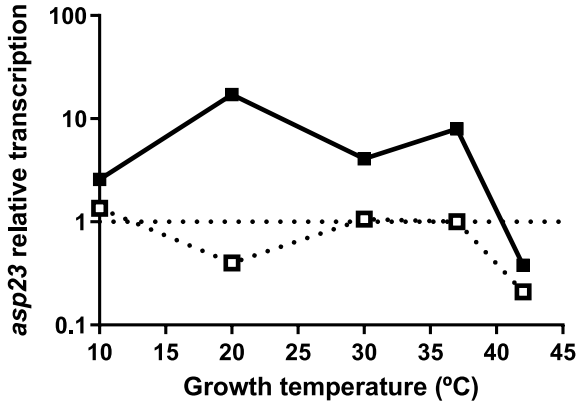

Fig. 1. Influence of growth temperature (10–42 °C) on the *asp23* relative expression in *S. aureus* strain Newman exponential (□) and stationary (■) growth phase cells. Exponential growth phase cells obtained at 37 °C were established as the reference value (1). Error bars correspond to the standard deviations.

increase was remarkably higher for the cells grown at 20 °C (more than 40-fold) than for the cells grown at 10 or 42 °C (approx. 2-fold). This resulted in *S. aureus* Newman stationary growth phase cells obtained at 20 °C showing the highest σ^B activity, whereas stationary growth phase cells grown at 42 °C displayed the lowest σ^B activity, even lower than that of exponential growth phase cells grown under most of the conditions assayed, except those grown at 42 °C.

3.2. Influence of growth temperature and phase on S. aureus heat resistance

In parallel to the study of σ^B expression, the heat resistance of *S. aureus* cells grown at different temperatures (10–42 °C) and in different growth phases was evaluated. *S. aureus* Newman and IK184 strains, which differ only in the presence and absence (IK184) of the *sigB* operon, were used for this purpose. After obtaining the survival curves to heat at 58 °C of both strains, the D_t values were calculated as described above.

The influence of growth phase and temperature on the D_{58} values calculated for each strain is depicted in Fig. 2. As can be observed in this figure, the entry into stationary growth phase also resulted in an increase in heat resistance for both strains, although this increase was particularly pronounced for the Newman strain.

Regarding the effect of growth temperature on the heat resistance of exponential growth phase cells, no significant ($p > 0.05$) differences among the D_{58} values were found, when comparing cells grown between 10 and 37 °C, for the two strains. By contrast, cells grown at 42 °C were significantly ($p < 0.05$) more resistant than those grown between 10 and 37 °C (3.5-fold increase for strain Newman and 2.7-fold increase for strain IK184).

S. aureus IK184 stationary growth phase cells displayed a similar behaviour (in relation to growth temperature) to those cells in exponential growth phase since statistical differences ($p < 0.05$) in heat resistance were only observed after increasing the growth temperature from 37 to 42 °C. By contrast, the heat resistance of *S. aureus* Newman

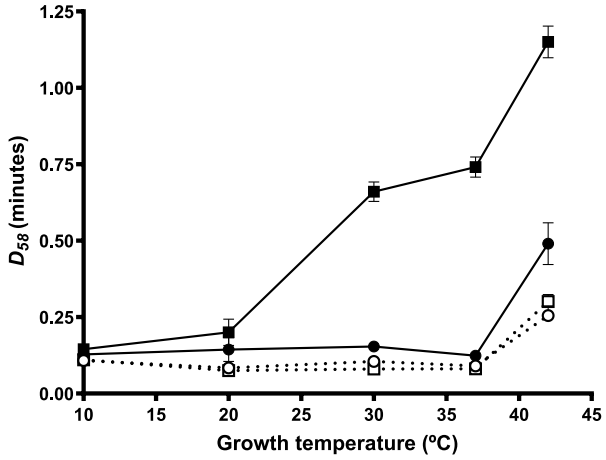

Fig. 2. Influence of growth temperature (10–42 °C) on the D_{58} values calculated for *S. aureus* Newman (squares) and IK184 (circles) cells in exponential (empty symbols, discontinuous line) and stationary (full symbols, continuous line) growth phase. Error bars correspond to the standard deviations.

stationary growth phase cells increased progressively as the growth temperature was raised from 20 to 42 °C -no significant differences ($p > 0.05$) were found between the D_{58} values of cells grown at 10 and 20 °C-.

It should be noted that similar trends were obtained regardless of the treatment temperature (54–62 °C) studied (data not shown).

3.3. Influence of growth temperature and phase on S. aureus membrane fluidity

The membrane fluidity of *S. aureus* cells grown at different temperatures and in different growth phases was studied by means of the DPH fluorescence anisotropy technique. Fig. 3 displays the anisotropy values (*r*) obtained at 58 °C for *S. aureus* exponential and stationary growth phase cells grown at different temperatures. Anisotropy values are inversely related to membrane fluidity (Shinitzky, 1984). As shown in this figure, cells of both strains (Newman and IK184) in exponential growth phase and grown between 10 and 37 °C showed similar fluorescence anisotropy values, whereas for those cells grown at 42 °C the anisotropy values were higher, which means that their membrane was more rigid. Regarding stationary growth phase cells, those grown at higher temperatures presented higher anisotropy values and thus, a more rigid membrane than those cells grown at lower temperatures. It should be taken into consideration that in all cases the entry into stationary growth phase resulted in a decrease in membrane fluidity of *S. aureus* cells. Nevertheless, this decrease was considerably lower in IK184 cells, lacking the σ^B factor.

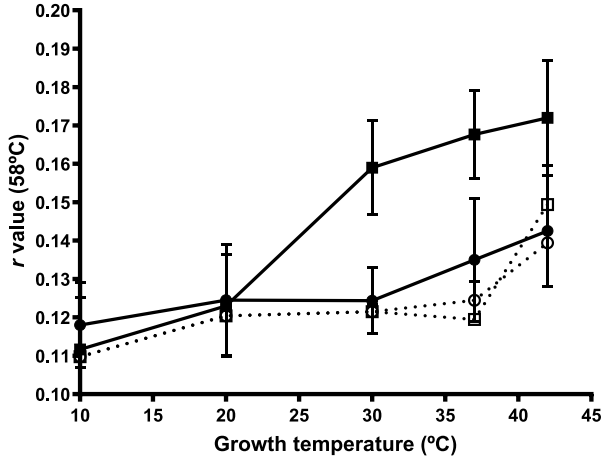

Fig. 3. Influence of growth temperature (10–42 °C) on the *r* values calculated at 58 °C for *S. aureus* Newman (squares) and IK184 (circles) cells in exponential (empty symbols, discontinuous line) and stationary (full symbols, continuous line) phase of growth. Error bars correspond to the standard deviations.

The fatty acid composition of *S. aureus* Newman and IK184 stationary and exponential phase cells grown at different temperatures was also determined, and as can be observed in Tables 2 and 3, it widely varied depending on the growth temperature and phase. The fluidity index (FI) (Casadei et al., 2002), determined through the fatty acid composition (FI = % unsaturated + branched fatty acids/% saturated fatty acids), was used to establish comparisons. Hence, in Fig. 4A, it can be observed that the membrane FI of the stationary phase cells of both strains decreased with the growth temperature, *i.e.*, the proportion of branched and unsaturated fatty acids decreased as the incubation temperature was raised. This trend was similar to that obtained by DPH fluorescence anisotropy (Fig. 3). However, it should be noted that no significant differences ($p > 0.05$) were found between the FI of the two strains, regardless of the growth temperature, whereas a clear trend indicating higher *r* values for the Newman strain when cells were grown at 30 °C or above can be observed.

As far as exponential growth phase cells are concerned, results obtained indicate that, as described for stationary growth phase cells, the increase in the growth temperature also resulted in a decrease in the FI for cells of both strains, although this decrease was only significant ($p < 0.05$) for the Newman strain (Fig. 4B). This behaviour differs, to a certain extent, from the results obtained by means of fluorescence anisotropy, since no significant differences ($p > 0.05$) among the *r* values of the Newman and IK184 exponential phase cells grown at the different temperatures were found (except for those grown at 42 °C).

Finally, the comparison of the fatty acid composition of exponential and stationary growth phase cells revealed that the former presented a significantly lower proportion of unsaturated and branched fatty acids than the stationary ones – when grown at the same temperature-so their membrane would be, *a priori*, more rigid according to the FI parameter.

3.4. Relationship between σ^B activity, heat resistance and membrane fluidity

From all the data obtained it can be deduced that the σ^B factor expression would be necessary for the development of the growth phase- and temperature-dependent increase in thermoresistance in *S. aureus* cells, since cells lacking the *sigB* gene (IK184 strain) were not able to develop this increase except, partially, those grown at 42 °C. At this temperature, the increase in heat resistance would be due to both, σ^B-dependent and independent mechanisms. Nevertheless, results obtained also indicate that there would be no significant correlation ($p > 0.05$; Pearson r = 0.025; Spearman r = 0.164) between σ^B activity and heat resistance, suggesting that, perhaps, the expression of the σ^B factor and its regulon would not be the ultimate and only cause determining the magnitude of the increase in thermotolerance observed as growth temperature raised (Fig. 5A).

Neither could a significant correlation ($p > 0.05$; Pearson r = 0.023; Spearman r = −0.018) be established between σ^B activity and fluorescence anisotropy values despite the differences in membrane fluidity found between the Newman and the IK184 strain, lacking the σ^B factor (Fig. 5B). Therefore, although results obtained also reveal the relevance of the σ^B factor on *S. aureus* membrane fluidity, it seems that its level of expression would not be the sole determinant of the growth temperature- and phase-dependent rigidification of *S. aureus* membranes.

Results obtained also indicate that σ^B expression would not have an impact on the fatty acid composition of *S. aureus* cells, given that no significant ($p > 0.05$) differences were found between the FI of the parental and mutant strain, lacking *sigB*. Moreover, a significant

Table 2
Results (mean (%) ± standard deviation) obtained in the analysis of the cytoplasmic membrane fatty acids of *S. aureus* Newman depending on the growth phase and temperature.

Fatty acid	Stationary phase					Exponential phase	
	10 °C	20 °C	30 °C	37 °C	42 °C	20 °C	37 °C
10:0	–	–	0.05 ± 0.04	0.11 ± 0.11	0.12 ± 0.10	0.70 ± 0.37	0.75 ± 0.65
12:0	–	0.02 ± 0.03	0.04 ± 0.07	–	0.19 ± 0.32	–	0.27 ± 0.33
13:0	–	–	–	–	0.05 ± 0.09	–	–
13:0 *iso*	–	0.05 ± 0.07	0.12 ± 0.12	0.12 ± 0.10	–	0.47 ± 0.03	0.39 ± 0.36
13:0 *anteiso*	–	–	–	–	–	0.07 ± 0.12	–
14:0 *iso*	0.09 ± 0.13	0.80 ± 0.30	4.08 ± 1.25	6.48 ± 0.48	9.98 ± 2.88	2.87 ± 0.51	5.48 ± 0.28
14:0	0.02 ± 0.03	0.13 ± 0.01	0.36 ± 0.08	0.53 ± 0.15	1.52 ± 0.49	0.94 ± 0.17	2.98 ± 0.76
15:1 *iso* G	–	–	–	–	0.05 ± 0.09	–	–
15:0 *iso*	2.70 ± 0.01	7.30 ± 1.11	9.72 ± 1.62	8.46 ± 2.32	5.04 ± 0.09	15.19 ± 0.56	10.60 ± 1.30
15:0 *anteiso*	46.53 ± 5.11	45.54 ± 8.08	40.33 ± 3.89	36.59 ± 6.76	29.68 ± 1.98	36.29 ± 1.76	29.11 ± 1.97
15:0	–	0.02 ± 0.04	–	–	0.10 ± 0.17	–	0.19 ± 0.32
15:0 2OH	–	–	–	–	–	0.12 ± 0.21	–
16:0 *iso*	0.75 ± 0.43	1.46 ± 0.26	3.24 ± 0.27	3.76 ± 0.20	7.95 ± 0.84	2.47 ± 0.11	2.88 ± 0.30
16:0	0.76 ± 0.27	1.64 ± 0.28	2.71 ± 0.43	2.34 ± 0.34	3.35 ± 0.31	5.51 ± 0.58	6.65 ± 1.74
16:1	0.06 ± 0.09	–	0.13 ± 0.23	–	–	–	–
17:0 *iso*	4.56 ± 1.19	6.52 ± 0.50	3.28 ± 0.49	2.50 ± 0.23	1.47 ± 0.28	4.24 ± 0.61	1.61 ± 0.18
17:0 *anteiso*	21.79 ± 8.37	12.77 ± 3.20	6.97 ± 1.78	6.57 ± 0.84	4.77 ± 0.98	5.62 ± 0.72	2.79 ± 0.52
17:0	0.05 ± 0.06	0.29 ± 0.12	0.63 ± 0.04	0.53 ± 0.03	0.97 ± 0.09	1.36 ± 0.16	1.17 ± 0.36
18:0 *iso*	0.61 ± 0.02	0.98 ± 0.62	1.89 ± 0.41	2.08 ± 0.31	3.85 ± 0.70	0.41 ± 0.35	0.51 ± 0.52
18:1 w9c	0.14 ± 0.20	0.13 ± 0.04	0.09 ± 0.07	0.70 ± 0.97	0.43 ± 0.31	0.50 ± 0.27	0.53 ± 0.14
18:0	3.75 ± 0.12	6.70 ± 0.62	10.64 ± 1.28	10.69 ± 1.01	12.50 ± 1.38	12.62 ± 0.73	17.88 ± 1.33
19:0 *iso*	3.40 ± 2.92	3.08 ± 1.00	1.73 ± 0.39	1.62 ± 0.31	0.75 ± 0.19	0.69 ± 0.59	0.34 ± 0.58
19:0 *anteiso*	6.78 ± 2.98	3.56 ± 0.57	2.17 ± 0.44	2.55 ± 0.78	2.48 ± 0.36	0.37 ± 0.32	0.34 ± 0.59
19:0	0.44 ± 0.08	0.84 ± 0.79	1.83 ± 0.67	2.23 ± 0.70	2.71 ± 0.65	2.21 ± 0.17	2.92 ± 1.68
20:0 *iso*	0.15 ± 0.21	0.21 ± 0.28	0.51 ± 0.20	0.69 ± 0.37	0.96 ± 0.22	–	0.09 ± 0.16
20:0	5.66 ± 3.87	7.41 ± 5.66	8.19 ± 1.10	10.78 ± 4.06	9.97 ± 1.54	7.20 ± 0.36	12.54 ± 1.89
20:1 w9c	–	–	–	–	–	0.06 ± 0.10	–

Table 3
Results (mean (%) ± standard deviation) obtained in the analysis of the cytoplasmic membrane fatty acids of *S. aureus* IK184 depending on the growth phase and temperature.

Fatty acid	Stationary phase					Exponential phase	
	10 °C	20 °C	30 °C	37 °C	42 °C	20 °C	37 °C
10:0	0.05 ± 0.06	–	0.05 ± 0.07	–	–	0.48 ± 0.01	0.18 ± 0.25
12:0	–	0.06 ± 0.09	0.04 ± 0.05	–	0.24 ± 0.33	–	0.06 ± 0.09
13:0	–	–	–	–	0.07 ± 0.10	–	–
13:0 *iso*	–	0.13 ± 0.00	0.13 ± 0.18	0.06 ± 0.08	–	0.55 ± 0.17	0.17 ± 0.23
13:0 *anteiso*	0.09 ± 0.13	0.03 ± 0.04	0.08 ± 0.11	0.05 ± 0.06	–	0.10 ± 0.14	–
14:0 *iso*	0.38 ± 0.09	1.10 ± 0.23	4.28 ± 4.75	5.52 ± 3.70	7.47 ± 3.85	3.38 ± 0.30	5.34 ± 1.71
14:0	0.18 ± 0.03	0.17 ± 0.04	0.38 ± 0.18	0.60 ± 0.18	0.89 ± 0.15	1.27 ± 0.13	3.01 ± 1.17
15:1 *iso* G	–	–	–	–	–	–	–
15:0 *iso*	5.66 ± 0.11	7.82 ± 1.33	8.21 ± 3.29	6.63 ± 0.38	4.55 ± 0.22	14.49 ± 1.46	8.54 ± 0.54
15:0 *anteiso*	48.02 ± 5.59	46.59 ± 11.47	37.13 ± 9.29	34.40 ± 6.54	28.86 ± 0.23	37.50 ± 4.53	27.97 ± 1.34
15:0	–	–	0.04 ± 0.05	–	0.08 ± 0.12	–	0.28 ± 0.40
15:0 2OH	–	0.04 ± 0.05	–	–	–	0.40 ± 0.02	–
16:0 *iso*	1.27 ± 0.70	1.62 ± 0.77	2.80 ± 2.32	4.75 ± 1.06	7.61 ± 3.20	2.48 ± 0.15	2.83 ± 0.26
16:0	1.68 ± 0.33	1.85 ± 0.24	4.26 ± 1.53	2.88 ± 1.47	3.87 ± 0.92	6.58 ± 0.13	7.29 ± 2.60
16:1	–	0.14 ± 0.20	0.07 ± 0.10	–	0.32 ± 0.45	–	–
17:0 *iso*	5.31 ± 0.71	5.71 ± 0.87	3.14 ± 0.95	2.72 ± 1.13	1.86 ± 0.27	3.93 ± 0.01	1.59 ± 0.64
17:0 *anteiso*	19.67 ± 3.10	10.62 ± 2.38	7.20 ± 2.43	7.84 ± 2.08	5.92 ± 1.02	5.34 ± 0.25	3.00 ± 1.35
17:0	0.29 ± 0.06	0.42 ± 0.08	0.68 ± 0.37	0.79 ± 0.42	1.00 ± 0.47	1.52 ± 0.04	1.60 ± 0.17
18:0 *iso*	0.52 ± 0.02	0.96 ± 0.30	1.49 ± 0.32	2.85 ± 1.11	4.76 ± 0.39	0.52 ± 0.06	0.77 ± 0.28
18:1 w9c	0.17 ± 0.08	–	2.60 ± 3.60	0.53 ± 0.62	1.07 ± 1.51	0.14 ± 0.19	0.53 ± 0.11
18:0	5.10 ± 0.48	6.65 ± 2.19	8.59 ± 1.77	11.64 ± 1.87	12.82 ± 1.95	12.77 ± 2.60	20.09 ± 4.01
19:0 *iso*	2.12 ± 1.69	2.79 ± 2.01	1.95 ± 1.85	2.01 ± 2.05	0.90 ± 0.27	0.78 ± 0.28	0.59 ± 0.43
19:0 *anteiso*	3.19 ± 1.68	2.98 ± 1.92	2.90 ± 3.13	2.96 ± 2.74	2.34 ± 0.61	0.45 ± 0.15	0.62 ± 0.88
19:0	0.62 ± 0.29	1.24 ± 1.15	2.04 ± 0.13	2.71 ± 0.11	2.82 ± 0.67	1.85 ± 0.75	3.40 ± 1.51
20:0 *iso*	0.07 ± 0.10	0.27 ± 0.28	0.45 ± 0.29	0.99 ± 0.82	1.15 ± 0.13	–	0.12 ± 0.17
20:0	4.77 ± 3.91	7.05 ± 6.75	7.44 ± 3.05	9.20 ± 4.09	8.01 ± 0.18	5.52 ± 3.54	10.43 ± 0.78
20:1 w9c	–	–	–	–	–	–	–

correlation between σ^B activity and membrane fluidity, determined through the fatty acid composition, was not observed ($p > 0.05$; Pearson r = 0.435; Spearman r = 0.643) (Fig. 5C).

In addition, it should be noted that an inverse relationship between heat resistance (D_{58} values) and membrane fluidity, measured through fluorescence anisotropy, could be established ($p < 0.05$; Pearson r = 0.902****; Spearman r = 0.716***) (Fig. 6A). By contrast, this inverse relationship could not be found using the FI (calculated based on the fatty acid composition) to determine membrane fluidity ($p > 0.05$; Pearson r = −0.229; Spearman r = 0.222) (Fig. 6B).

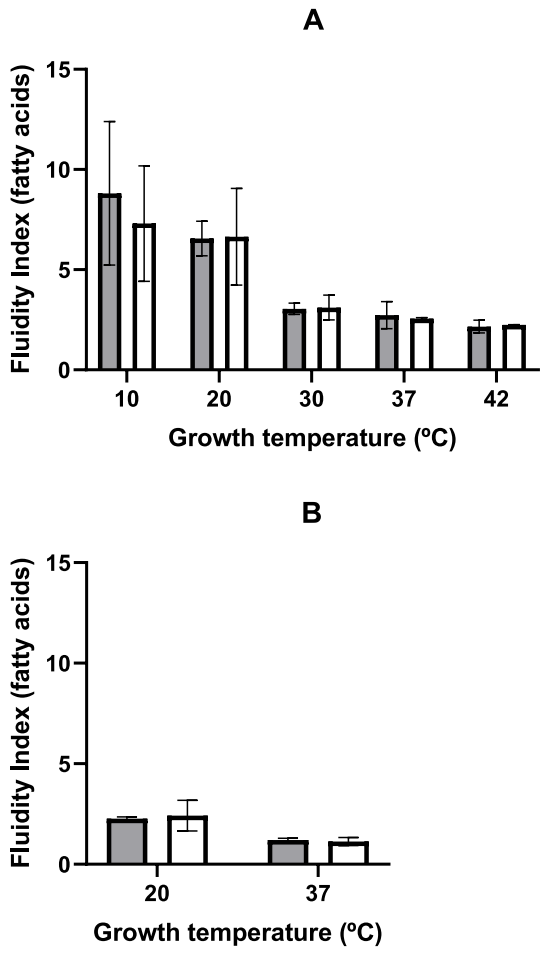

Fig. 4. Influence of growth temperature (10–42 °C) on the fluidity index (% unsaturated and branched fatty acids/% saturated fatty acids) of the cytoplasmic membrane, in stationary (A) and exponential (B) growth phase cells of *S. aureus* Newman (grey) and IK184 (white). Error bars correspond to the standard deviations.

4. Discussion

In order to survive to adverse conditions, bacteria are able to develop several stress response mechanisms, for instance, the increase in the expression of the alternative sigma factors. The alternative general stress sigma factor in Gram-positive bacteria σ^B is induced in *S. aureus* by different conditions, namely, heat or starvation (Senn et al., 2005) and upon entry into stationary growth phase (Giachino et al., 2001; Kullik & Giachino, 1997; Pané-Farré et al., 2006). However, to the best of our knowledge, the effect of growth temperature on σ^B activity had only been explored before in *L. monocytogenes* (Utratna et al., 2014). In the present study, the influence of the growth temperature and growth phase on the σ^B activity of *S. aureus* cells was investigated by means of the RT-qPCR method.

Our results are in accordance with those previously obtained since they indicate that the entry into stationary phase of growth entailed an increase in σ^B activity for cells grown at all the studied temperatures. Regarding the influence of growth temperature on σ^B activity, results here obtained are slightly different from those reported by Utratna et al. (2014), with *L. monocytogenes* cells grown at two temperatures, 4 and 37 °C. Thus, for instance, in *S. aureus* stationary growth phase cells, those grown at 20–37 °C showed a higher σ^B activity than those grown at 10 or 42 °C. By contrast, Utratna et al. (2014) observed a similar σ^B activation pattern when cells were grown at 37 °C and at 4 °C and concluded that in *L. monocytogenes* σ^B expression would be more

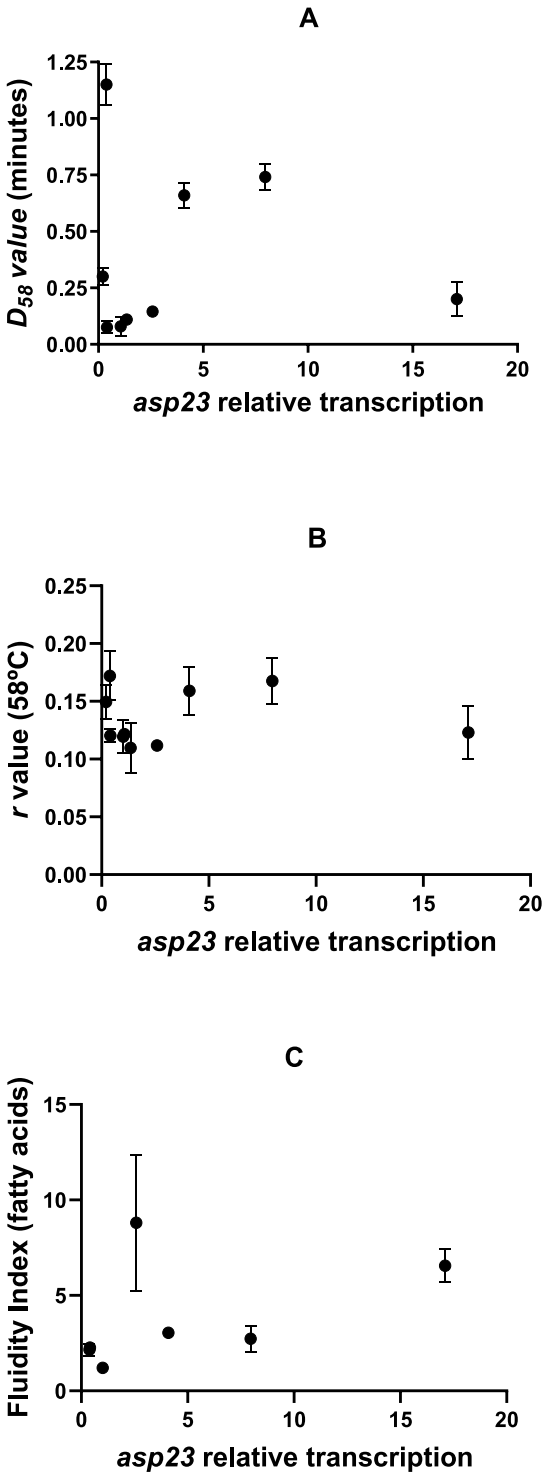

Fig. 5. Correlation between σ^B activity, expressed as the *asp23* relative transcription, and (A) heat resistance, expressed as D_{58} value, (B) membrane fluidity, expressed as *r* value, (C) membrane fluidity, expressed as fluidity index (% unsaturated and branched fatty acids/% saturated fatty acids), for *S. aureus* Newman exponential and stationary growth phase cells, grown at different temperatures (10–42 °C). Error bars correspond to the standard deviations.

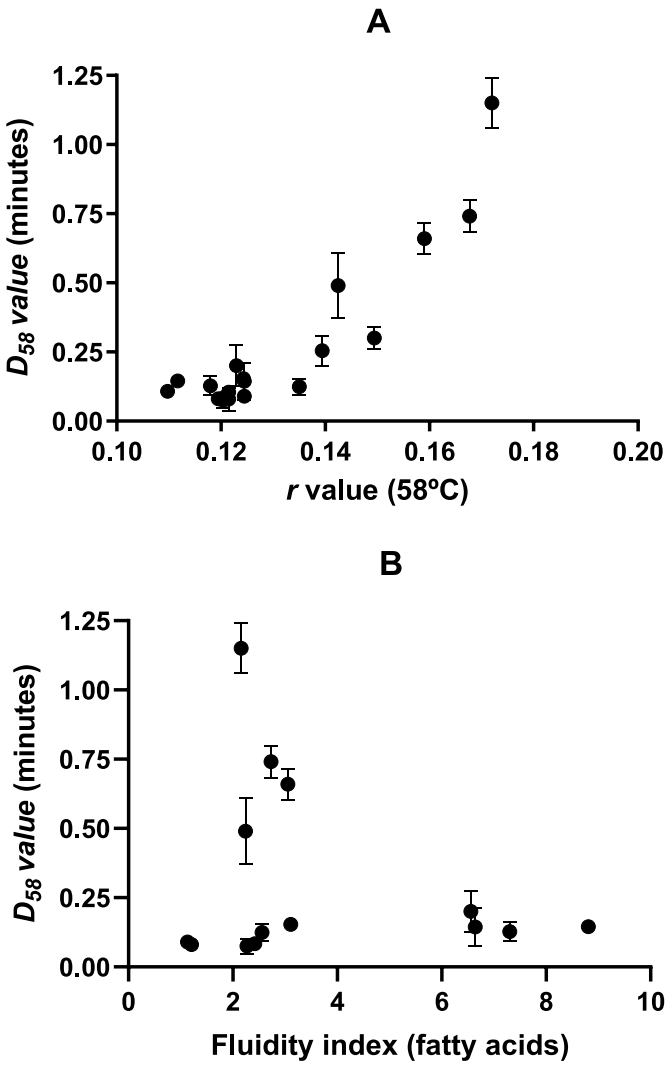

Fig. 6. Correlation between heat resistance, expressed as D_{58} value (minutes), and (A) membrane fluidity, expressed as *r* value, (B) membrane fluidity, expressed as fluidity index (% unsaturated and branched fatty acids/% saturated fatty acids), for *S. aureus* Newman and IK184 exponential and stationary growth phase cells, grown at different temperatures (10–42 °C). Error bars correspond to the standard deviations.

influenced by the phase of growth than by the growth temperature. These discrepancies might be due to the different microorganisms and growth conditions studied. In any case, our results also seem to indicate that, except for cells grown at 42 °C, σ^B activity in *S. aureus* would be more influenced by the growth phase than by the growth temperature.

The heat resistance of *S. aureus* strain Newman and its isogenic Δ*sigB* mutant strain IK184 when grown between 10 and 42 °C was also determined for exponential and stationary growth phase cells. Results obtained show that the entry into stationary growth phase resulted in an increase in heat resistance, although it is relevant to highlight that this increase was notably higher in the case of the Newman strain. Differences in heat resistance between the Newman and the IK184 strain, as well as between exponential and stationary growth phase cells of both strains, had been already described for cells grown at 37 °C (Cebrián, Condón, & Mañas, 2009; Cebrián, Sagarzazu, et al., 2009). It is generally acknowledged that exponential growth phase cells display a lower resistance against heat than those that are in the stationary phase (Cebrián et al., 2017; Smelt & Brul, 2014), something that in the case of *S. aureus* has been attributed to an increase in the σ^B activity (Cebrián, Sagarzazu, et al., 2009).

Regarding the effect of growth temperature on microbial heat resistance, only results obtained for stationary growth phase cells of the Newman strain are consistent with the general assumption that cells grown at higher temperatures present a higher heat resistance (Mañas et al., 2003; Pagán et al., 1999) since for *S. aureus* Newman exponential growth phase cells and for *S. aureus* IK184 exponential and stationary phase cells a significant increase in heat resistance was only observed when the growth temperature raised up to 42 °C. These results demonstrate that σ^B is required for the development of the growth phase- and temperature- (up to 37 °C) dependent increase in heat resistance in *S. aureus*. Nevertheless, as described in the results section, no direct quantitative relationship between the σ^B activity and the heat resistance of *S. aureus* cells could be established. This strongly suggests that σ^B would not ultimately control the cellular changes responsible for the increase in heat resistance observed as the growth temperature of *S. aureus* cells is raised. Thus, it can be hypothesized that σ^B might be required for inducing the synthesis of one or various cellular components that are critical to enable cells to develop other heat resistance responses. However, these components would not be responsible for the increase in heat resistance itself. Further research would be required to verify this hypothesis and fully elucidate the role of σ^B in *S. aureus* heat resistance.

At this point it should be noted that, conversely to other microorganisms in which σ^B plays an essential role in the development of stress resistance, in the case of *S. aureus* it has been suggested that its function would be more related to basic cellular processes such as transport through the membrane, cell envelope composition, metabolism, post-transcriptional regulation, antibiotic resistance and expression of virulence factors (Bischoff et al., 2004; Gertz et al., 2000; Giachino et al., 2001; Palma et al., 2006; Pané-Farré et al., 2006; Price et al., 2002). Regarding the expression of virulence factors, it should be remarked that σ^B contributes to the transcription of the *sar* locus coding for one of the major regulators of virulence associated genes, such as *sarA* (Bischoff et al., 2004; Pané-Farré et al., 2006). In addition, it has been reported that *S. aureus* is able to develop stress resistance by means of σ^B-independent mechanisms. In the particular case of heat resistance, heat shock proteins (HSPs) seem to play a major role. Thus, according to Singh et al. (2012), the production of HSPs is essential to guarantee the survival of *S. aureus* cells at higher temperatures.

HSPs are mostly chaperones and proteases which contribute to protein folding, assembly, transport, repairing processes and prevention of protein misfolding and aggregation (Schumann, 2007). Four classes of HSPs are associated with Gram-positive bacteria, being only one of these classes σ^B-dependent. Class I HSPs are encoded by genes of the *groESL* and *dnaK* operons and controlled by HrcA, class II are part of the σ^B regulon, class III are under the control of CtsR, and class IV are regulated by other systems (Chastanet et al., 2003). Among all these HSPs, in bacteria it is important to highlight the role of the GroESL and DnaK HSP systems (Muga & Moro, 2008). The relevance of the DnaK heat shock system for the *S. aureus* stress response in certain conditions has been widely demonstrated (Singh et al., 2007, 2012).

On the other hand, it is a well-known fact that the cytoplasmic membrane of prokaryotic cells is involved in multiple and critical processes for cell viability. The correct performance of the functions related to the membrane depends to a large extent on its physical condition. In this context, the relevance of the membrane fluidity, which is assumed to be largely determined by its fatty acid composition, should be noted (Denich et al., 2003). The cytoplasmic membrane is also considered to be one of the main targets of multiple preservation agents employed by the food industry and it has been suggested that its condition, composition, and fluidity could influence the microbial resistance against these agents, especially against heat (Cebrián et al., 2019; Mañas & Pagán, 2005).

In this work, the membrane fluidity of *S. aureus* cells was assessed through the quantification of membrane fatty acid composition (FI) and by means of DPH fluorescence anisotropy (*r* values). Regarding this

latter, results obtained in this investigation totally agree with those obtained in the study conducted by Cebrián et al. (2019) with *S. aureus* CECT 4459, indicating that the membrane fluidity of exponential growth phase cells is similar for those grown between 10 and 37 °C, and that it decreases in the case of cells grown at 42 °C, leading to stiffer membranes. The entry into stationary growth phase resulted in a decrease in membrane fluidity of *S. aureus* cells (higher *r* values), although this decrease was considerably less remarkable in the case of the IK184 strain, lacking the σ^B factor. Within the stationary growth phase cells, those grown at higher temperatures presented a more rigid membrane (higher *r* values). This growth temperature-dependent rigidification of *S. aureus* membranes determined by DPH fluorescence anisotropy is consistent with the changes observed in the fatty acid profiles and has also been demonstrated to occur with other Gram-positive bacteria, such as *L. monocytogenes* (Najjar et al., 2007; Pan et al., 2020), and with Gram-negative bacteria, such as *Salmonella* (Yun et al., 2016) or *Escherichia coli* (Liu et al., 2017).

In order to easily compare all the growth conditions studied, an index that was able to reflect the global changes in the membrane fatty acid composition of *S. aureus* cells (FI) was selected. Other indexes such as the average length of the fatty acid chains or the relationship between the percentages of branched fatty acids in *iso* position against *anteiso* position were calculated. Nevertheless, they did not reflect so clearly the changes produced in the membrane fatty acids due to variations in temperature. It is noteworthy that the exponential growth phase cells presented a significantly lower proportion of unsaturated and branched fatty acids than the stationary ones. This is a surprising fact, given that, in general, it is assumed that stationary growth phase cells present stiffer membranes than those in exponential phase (Cebrián et al., 2017; Smelt & Brul, 2014), and therefore it would be expected that stationary phase cells displayed a lower proportion of unsaturated and/or branched fatty acids. Nevertheless, apart from fatty acids, other components that can have a large impact on membrane fluidity should also be taken into consideration (Denich et al., 2003). For instance, carotenoids, that act as fluidity modulators (Chamberlain et al., 1991), and proteins, such as the HSPs, that possess stabilising and repairing functions of different microbial structures (Schumann, 2007). It should also be noted that, despite having a similar fatty acid composition (similar FI), the membranes of stationary phase *S. aureus* IK184 cells were less rigid (lower *r* values) than those of the Newman strain. The possible contribution of these components aforementioned to bacterial membrane fluidity cannot be detected through the quantification of membrane fatty acid composition, whereas DPH fluorescence anisotropy considers the contribution of all the components, besides fatty acids, that could impact the membrane mechanical properties. For this reason, in our opinion, measurements conducted with membrane-inserted fluorescence probes give more information in terms of mechanical behaviour, than fatty acid composition analyses. However, in the present study, both approaches were employed in order to acquire a deeper knowledge of *S. aureus* physiology and it should be noted that this experimental design has scarcely been employed in bacteria.

Altogether these data seem to indicate that the rigidification of the membrane of *S. aureus* cells upon entry into stationary growth phase would rely on mechanisms other than the change in fatty acid composition and that σ^B would play a relevant role in determining the membrane fluidity of *S. aureus* cells, although not through the modulation of the fatty acid composition. In this sense, it has already been described that the σ^B factor regulates the synthesis of carotenoids and several membrane proteins (Bischoff et al., 2004; Pané-Farré et al., 2006), which might contribute to regulate membrane fluidity, as has been described for some HSPs (Coucheney et al., 2005; Lin & Chou, 2004; Török et al., 1997; Tsvetkova et al., 2002). In any case, no relationship between the level of σ^B activity and the membrane fluidity of *S. aureus* cells was observed. Thus, although being critical for the growth temperature-dependent stiffening of *S. aureus* membranes, as described for heat resistance, σ^B would probably not regulate the mechanisms responsible for the rigidification process itself. Nevertheless, as it was previously indicated, σ^B is involved in the synthesis/regulation of class II HSPs and it has been demonstrated that some HSPs are able to interact with the lipid fraction of the membranes, leading to membrane stabilization and thus, to a decrease in membrane fluidity (Coucheney et al., 2005; Török et al., 1997; Tsvetkova et al., 2002). Therefore, this mechanism could be responsible for causing the growth temperature-dependent rigidification of *S. aureus* membranes. However, it is important to bear in mind that, as described above, σ^B regulates only the synthesis of one type of HSPs and that there exist many other systems of regulation and factors involved in HSP production.

Finally, and as pointed out in the results section, a relationship between membrane fluidity (determined through fluorescence anisotropy, not through the fatty acid profile) and heat resistance was observed. This correlation has already been observed by several authors (Beuchat & Worthington, 1976; Cebrián et al., 2019; Shigapova et al., 2005; Török et al., 1997; Yang et al., 2014). The reasons why a more rigid membrane might provide a higher heat resistance to *S. aureus* cells have been discussed in detail in Cebrián et al. (2019). In this sense, this work provides further evidence of the existence of this relationship and indicates that factors other than σ^B would play a major role in the synthesis/regulation of the components responsible for this phenomenon.

5. Conclusion

Results obtained in this investigation showed that the entry into stationary phase of growth implies an increase in the σ^B activity of *S. aureus* cells, regardless of the growth temperature. On the other hand, although the σ^B factor expression is necessary for the development of the growth phase- and temperature-dependent increases in thermoresistance and membrane rigidity in *S. aureus* cells (up to 37 °C), a direct correlation between the σ^B activity level and the magnitude of these increases could not be established. Our results also indicate that σ^B does not control the fatty acid synthesis in *S. aureus* and reveal the existence of σ^B-independent growth phase and temperature (>37 °C) heat adaptation mechanisms. Further experiments would be required to fully elucidate the role of σ^B in *S. aureus* heat resistance and membrane rigidification (particularly considering the possible involvement of HSPs in these processes) as well as to acquire a deeper knowledge on these σ^B-independent heat adaptation mechanisms. A relationship between heat resistance and membrane fluidity of *S. aureus* cells, measured through fluorescence anisotropy, was found. Conversely, this correlation could not be established when the fatty acid profile was employed to determine membrane fluidity. Results obtained in this work contribute to a deeper knowledge of *S. aureus* physiology and to the development and design of more effective processes used by the food industry for microbial inactivation.

CRediT authorship contribution statement

Laura Nadal: Writing – original draft, Investigation, Formal analysis. **Pilar Mañas:** Writing – review & editing, Supervision, Conceptualization. **Guillermo Cebrián:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116814.

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