Research article

Fever temperatures impair hemolysis caused by strains of *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Hemolysis modulates susceptibility to bacterial infections and predicts poor sepsis outcome. Hemolytic bacteria use hemolysins to induce erythrocyte lysis and obtain the heme that is essential for bacterial growth. Hemolysins are however potent immunogens and infections with hemolytic bacteria may cause a reversible fever response from the host that will aid in pathogen clearance. We hypothesized that fever temperatures impact the growth and infectivity of two hemolytic bacteria that are known to evoke fever in patients. To that end, we used high-sensitivity microcalorimetry to measure the evolution of heat production in fever-inducing strains of *Escherichia coli* and *Staphylococcus aureus,* under different temperature conditions. We determined specific bacterial aggregation profiles at temperatures equal to or exceeding 38.5 °C. Two melting temperatures peaks ranged from 38 °C to 43 °C for either species, a feature that we assigned to the formation of hemolysin aggregates of different oligomerization order. In order to measure the role of fever temperatures on hemolysis, we incubated the pathogens on blood agar plates at relevant temperatures, measuring the presence of hemolysis at 37 °C and its absence at 40.5 °C, respectively. We conclude that fever temperatures affect the kinetics of hemolysin pore formation and subsequently the hemolysis of red blood cells *in vitro*. We reveal the potential of microcalorimetry to monitor heat response from fever inducing bacterial species. Furthermore, these results help establish an additional positive role of febrile temperatures in modulating the immune response to infections, through the abolishment of hemolysis.

1. Introduction

Fever following infections is an adaptive, acute-phase response to the presence of pathogens. Transient increase in core body temperature has been associated with improved survival in sepsis and enhanced resolution of many infections, with 1 °C rise leading to a decrease in the odds of death by 15% [1]. However, reversible changes in baseline temperatures do not normally pass a threshold of around 40 °C [2], suggesting the existence of an optimal range for the febrile response [3]. To this end, differential scanning calorimetry (DSC) is uniquely suited to monitor heat processes resulting from thermally stressed live pathogens [4]. For antigens from fever-causing pathogens, calorimetric techniques have proved essential to detect changes in immune complex formation with their monoclonal antibodies, under physiological and pathological thermal conditions [5]. In hemolytic infections, extensive hemolysis leads to release of the heme moiety and to increased rate of bacterial co-infections, as most pathogens depend on environmental iron for growth, while heme itself can suppress phagocyte functions [6]. We hypothesized that bacterial growth and/or infectivity with respect to hemolysis is affected at the fever temperatures they induce. In order to test this assumption, we used DSC to scan the bacterial response to increasing heat stimuli, with a focus on the physiologically relevant range of 37 °C–42 °C. We then used isothermal calorimetry to measure the decay in heat signals at fixed temperatures relevant for fever production. To confirm our findings, we incubated at different pertinent temperatures, strains of *Escherichia coli* and *Staphylococcus aureus* on blood agar plates, in order to detect the presence or absence of hemolysis.

2. Results and discussion

Bacterial viability was unaffected by the thermal treatments in this study, as evidenced by robust re-growth of bacteria from solutions drawn

out of the calorimetric cells (Supplementary Figure 1). As such, we have herein focused strictly on how temperature affected bacterial hemolysis, and in particular on the responsible hemolytic factors. Both bacterial species under investigation exhibited large exothermic peaks in the temperature range of physiological interest of 36 °C–41 °C indicating aggregation (Figure 1), as previously measured in other bacteria [7]. A sharp endothermic peak was observed at 38.4 °C for the *S. aureus* 92 thermogram during aggregation, indicative of a denaturation process following protein unfolding.

Thermodynamic parameters derived from the differential scanning thermograms indicate that the melting temperature (T_m) values for both bacterial strains ranged from 38 °C to 43 °C. For *E. coli* 508, two T_m values were measured at 38.7 °C and 40.6 °C; for *S. aureus* 92, T_m values were present at 39.3 °C and 43.1 °C. The presence for each species of two T_m values may correspond to formation of aggregates of different oligomerization order (Figure 1 A, B). All isothermal calorimetry measurements at fever temperatures presented initial negative heat flows (Figure 1 C), corresponding to exothermic processes [8]. In *in vitro* isothermal titration calorimetry studies of pore formation in model lipid bilayers, exothermic heat is directly related to the formation of pores [9]. We used a single exponential decay function to model the isothermal data, assuming that aggregation rate is much higher than the denaturation rate, and that a single apparent rate constant characterizes the aggregation process [8]. We modeled the decrease in heat signals observed in the isotherms using the exponential decay function:

$$y = y_o + A \exp(-x/T) \tag{1}$$

with y_0 = offset, A = amplitude, T = time constant parameters were varied until best fits were obtained. Adjusted R-square coefficients ranged from 0.95 to 0.97. We derived, from the time constant parameter, the decay rate: $k = 1/t_1$ and half-life: $\tau = t_1 * \ln(2)$ parameters. Fitting parameters for all curves are summarized in Supplementary Table 1.

Kinetics of pore-forming is strongly affected by increasing temperature (4 °C–37 °C range), as observed with hemolytic cytolysin from *G. vaginalis* [10], although fever temperatures were not used in that study. The number and position of pore-forming bacterial aggregates can vary during conditions of mild heat stress, such that short-lived aggregates can dissolve before completion of a single bacterial cell cycle, which may affect hemolysis [11]. The decay rate parameter obtained from Eq. (1) is a measure of protein aggregation rate that is affected by both temperature and toxin monomer concentration. The disruption of pores was experimentally measured as endothermic heat spikes in isothermal calorimetry studies [9]. Furthermore, the half-life parameter obtained from Eq. (1) provides the average duration for the formation of protein aggregates which is proportional to increasing temperature [12]. For *S. aureus* 92, similar decay rates obtained in the 37 °C–39.5 °C range are comparatively lower by an average factor of 5 than the heat dissipation rates obtained at 40.5 °C, indicative of aggregation at this temperature. However, compared to other temperatures, higher decay rate by an average factor of 4 was observed for *E. coli* 508 at 38.5 °C. These values could relate to the double T_m peaks observed in differential scanning calorimetry and reinforce the notion of aggregates of distinct oligomerization order formed at dissimilar temperatures, with different thermal decay rates. Furthermore, the half-lives of the heat signals at the
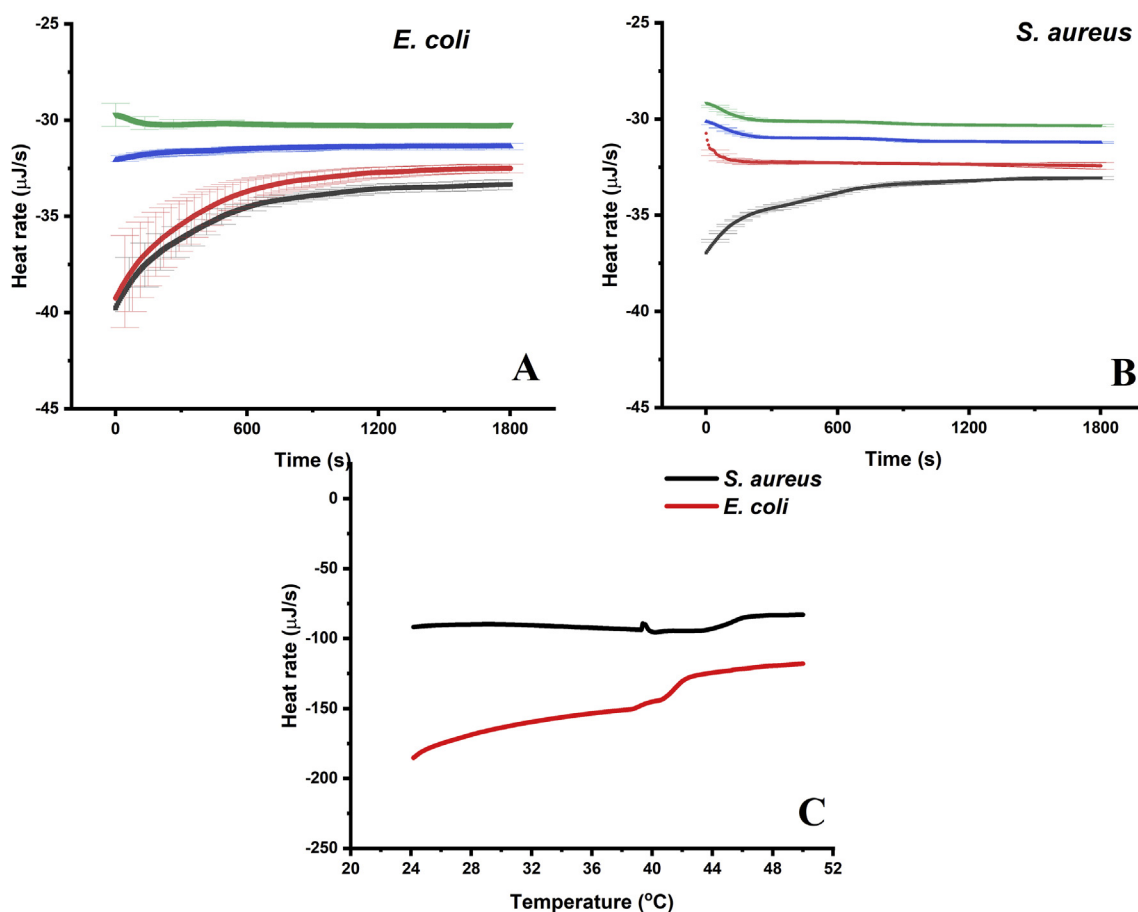

Figure 1. A, B. Averages with SD of isothermal calorimetry thermograms at the fever temperatures used in this study. C. Examples of differential scanning thermograms of live *S. aureus* and *E. coli*. All isothermal scans were obtained in succession starting from 37 °C, similar to the temperature changes during the fever response.

highest temperature are reduced by an average factor of 8 compared to the value at 37 °C for *S. aureus* 92, and by an average factor of 2–5 for *E. coli* 508, respectively, suggestive of lower duration for the formation of temperature-sensitive protein aggregates. We note that temperature-induced generation of kinetically trapped cytolysin species has been measured that resulted in either partial or full pore formation, depending on initial protein concentration and thermal stimulus used [13].

Cultured plates at fever temperatures indicated absence of hemolysis at 40.5 °C and differential absence of hemolysis at other temperatures (Figure 2 and Supplementary Figure 2).

Previous studies evaluating the thermal inactivation of *E. coli* using DSC identified key thermal endothermal peaks: at ∼56 °C, 70°C–80 °C, 94 °C, 102.5 °C and 115 °C corresponding to denaturation of the 30S ribosomal elements, of ribosomes of greater size, the fusion of the DNA, the initial denaturation of the cell wall and the full denaturation of the outer cell wall, respectively [14]. We have instead focused exclusively on the thermal response of these fever-inducing bacteria in a physiologically relevant range. Importantly, hemolysis of plated red blood cells alone, strictly achieved through temperature, is not present until a 45 °C threshold is reached after 24 h of incubation. At that temperature, a lipid multilayer forms that induces hemolysis in the presence of temperature stress alone [15]. Bacterial hemolysis is mediated by pore-forming oligomer toxins that are important immunogens and virulence factors. While the monomers are themselves heat-stable, pore formation on the surface of red blood cell is a kinetic process that depends critically on temperature and monomer concentration [13]. In *E. coli*, key pore-forming cytolysin A (ClyA) slowly aggregates into two distinct oligomer species, when incubated overnight at 37 °C. These aggregates are irreversible, off-pathway products of pore complex assembly, with only about 1% hemolytic activity compared with identical mass concentrations of

freshly prepared ClyA monomers [16]. In *S. aureus,* oligomerization of the immunogen hemolytic agent streptolysin O on erythrocyte membranes is optimal in the 34°C–37 °C range and decreases at 40 °C [17]. Furthermore, clumping factor B from *S. aureus* that promotes, among other roles, bacterial adhesion to host tissues and hemolysis, is composed of three subdomains of which the N3 domain unfolds at 38.5 ± 1.4 °C, adversely affecting activity [18]. We propose that the calorimetric features we measured are associated mainly with the exothermic aggregation of pore-forming bacterial toxins. Importantly, thermal stress did not impair bacterial survival, as viability assays subsequent to DSC measurements showed normal bacterial growth (Supplementary Figure 1). We note that in conditions such as the hemolytic-uremic syndrome caused by *E. coli*, fever is an important initial symptom [19] and moderate hyperthermia therapy (38 °C–42 °C) is currently used in an adjuvant setting [20].

3. Conclusions

This study describes an *in vitro* role of fever temperatures on bacterial hemolysis, and the potential to monitor bacterial heat response using microcalorimetry. We hypothesized that fever temperatures will affect the infectivity in two clinically relevant bacterial species that cause hemolysis. We revealed thermal aggregation peaks for these species at physiologically and pathologically relevant temperatures, ranging from 38 °C to 43 °C. We fitted the isothermal curves of the heat decaying signals to an equation that allowed for the establishing of heat decay rates and half-lives. For either bacterial species, we obtained different thermal decay rates at different fever temperatures that we attributed to aggregates of distinct oligomerization order. We noted half-lives of the heat signals to be shortest at the highest fever temperatures used in this study, indicating fast formation of temperature-sensitive hemolysin aggregates. We also incubated these bacteria under physiological or fever
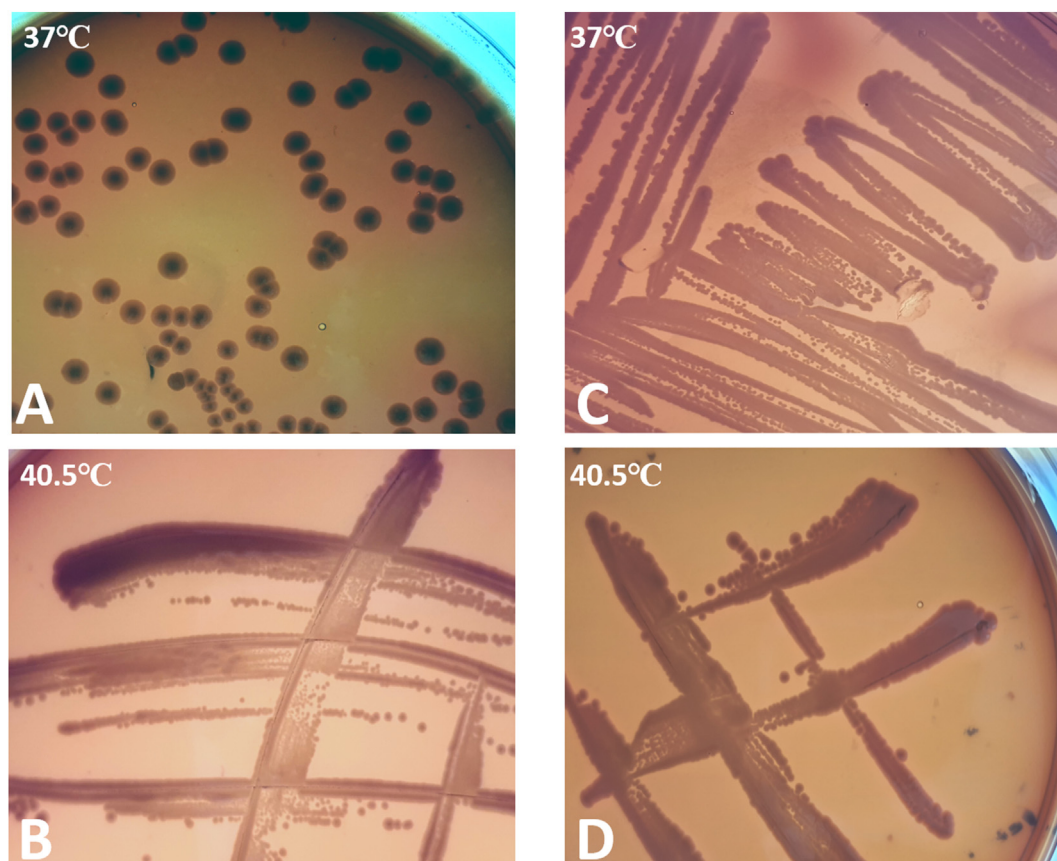

Figure 2. Alpha and gamma-hemolysis of *E. coli* 508 (A and B, respectively). Beta and gamma-hemolysis of *S. aureus* 92 (C and D, respectively). Plates were incubated for 24 h at indicated temperatures.

conditions on blood agar plates and measured the presence or the absence, respectively, of hemolysis. Because the pathogenic bacterial species here investigated are fever-inducing and hemolytic in clinical conditions, further study of similar bacteria is warranted. Additionally, using our protocol, the presence of thermal aggregation peaks may serve as a complementary and fast bacterial identification tool in clinical microbiology. Lastly, these results may contribute to the growing clinical interest in the management of fever as an important adjuvant for treatment of relevant bacterial infectious diseases.

4. Methods

4.1. Sample preparation

Stocks of our own clinical isolates of *Escherichia coli* strain 508 and *Staphylococcus aureus* strain 92 were stored at $-80\,^{\circ}$C in 30% (v/v) sterile glycerol until use. Columbia sheep blood agar plate were seeded and incubated overnight in aerobic conditions at 37 °C. Single colonies were thereafter incubated on Luria Bertani agar plates for 24 h incubation aerobic atmosphere at 37 °C. Cells were washed with 5 ml phosphate-buffer saline (PBS, 150 mM, pH 7.4). McFarland density values were determined with a DEN-1 densitometer (Biosan, USA). For calorimetry studies, samples were diluted in PBS and used immediately thereafter. For hemolysis assays on plates, 100 µL of each bacterial suspension was incubated at physiological (37 °C) and fever temperatures (38.5 °C, 39.5 °C and 40.5 °C), on Columbia sheep blood agar plates for 24 h. Images of plates were processed using ImageJ (NIH, USA). Colony forming units counting was performed using OpenCFU 3.8.

4.2. Calorimetric investigations

DSC measurements were performed with a NanoDSC (TA Instruments, USA). Solutions of live cells were kept at 4 °C and degassed for 10 min before loading into the sample cell (300 µL active volume). Reference cell contained PBS. Equilibration time was 10 min before starting the scans at 20 °C until 50 °C, with 1 °C/min. For the isothermal calorimetry measurements, thermal ramping was started at 20 °C until the 37 °C value, followed by subsequent increases to fever temperatures (38.5 °C, 39.5 °C and 40.5 °C), with an equilibration step of 10 min before every consequent isotherm. Data in triplicates was analyzed with OriginPro 2020b (OriginLab, USA), and averages with SD (standard deviations) were obtained.

5. Ethics approval and consent to participate

Not applicable.

6. Consent for publication

All authors approved of this submission.

Declarations

Author contribution statement

Razvan C. Stan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Elena Diana Giol: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Mihaela Palela: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Andreia Amzuta: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Oxana G. Ologu: Performed the experiments.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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