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Staphylococcus aureus ClpC is involved in protection of carbon-metabolizing enzymes from carbonylation during stationary growth phase

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ABSTRACT

The ability of *Staphylococcus aureus* to adapt to various conditions of stress is the result of a complex regulatory response. Among them, ClpC, belonging to the Hsp100/Clp ATPase family, seems to play an important role. For instance, we previously demonstrated that a functional *clpC* deletion resulted in enhanced survival in the late stationary phase (death phase period) compared to the parental *S. aureus* strain. However, the mechanisms for the enhanced survival of a *S. aureus clpC* mutant during the death phase period are still elusive. In *Escherichia coli*, among the factors that might lead to bacterial cell death during stationary phase, the amount of protein aggregates and/or oxidized proteins appears to be of major importance. Thus, in the present study, we have evaluated protein aggregates and carbonylated protein (as a marker of protein oxidation) contents both in the wild type and in an *S. aureus clpC* mutant during the exponential growth phase and the death phase. Whereas at all time points the tested *clpC* mutant exhibits the same amount of protein aggregates as the WT strain, the total amount of carbonylated proteins appears to be lower in the *clpC* mutant. Moreover, we observed that at the entrance of the death phase carbon-metabolizing enzymes [such as the TCA cycle enzymes Mqo2 (malate: quinone oxidoreductase) and FumC/CitG (fumarate hydratase)] albeit not the bulk proteins are carbonylated to a larger extent in the *clpC* mutant. Reduced activity of the TCA cycle due to specific carbonylation of these proteins will result in a decrease of endogenous oxidative stress which in turn might confer enhanced survival of the *clpC* mutant during the death phase period thus contributing to bacterial longevity and chronic infection.

Introduction

Staphylococcus aureus is a major human pathogen (Lowy, 1998) that causes a broad spectrum of invasive diseases and is a significant cause of morbidity and mortality. A common pathological feature is the organism's ability to colonize biological and artificial surfaces (Herrmann, 2003; Lew et al., 2001) by producing a structured community of bacterial cells enclosed in a biofilm. *S. aureus* is notorious for its resistance to untoward environmental conditions (Chan et al., 1998). The ability to tolerate stress and to readily switch between planktonic and sessile growth phases requires extensive gene regulatory and metabolic adaptation allowing the pathogen efficient adjustment to various living conditions.

An important element of the *S. aureus* stress response system is the Hsp100/Clp ATPase family (including ClpC, ClpB, ClpL, ClpX, and ClpP) (Frees et al., 2004, 2005; Michel et al., 2006; Chatterjee et al., 2010). We previously demonstrated that a *clpC* deletion leads to an enhanced survival in the late stationary phase (death phase period). Whereas the *S. aureus* WT strain entered into the death phase after 3 days of culture (10^9 cfu/ml at 3 days and 10^7 cfu/ml at 7 days), a *clpC* mutant remained totally culturable for up to 7 days of growth (10^9 cfu/ml at 3 and 7 days) (Chatterjee et al., 2005). More recently, we have shown that ClpC is involved in regulating the expression of genes and/or proteins of gluconeogenesis, the pentose-phosphate pathway, pyruvate metabolism, the electron transport chain, nucleotide metabolism, oxidative stress, metal ion homeostasis, stringent response, and programmed cell death (Chatterjee et al., 2009). Thus, one major function of ClpC is balancing late-growth-phase carbon metabolism. Furthermore, these changes in carbon metabolism (TCA cycle) result in alterations of the intracellular concentration of free NADH, the amount of cell-associated iron, and fatty acid metabolism (Chatterjee et al., 2009). However, mechanisms for the enhanced survival

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of a *S. aureus clpC* mutant during the death phase are still elusive.

Among the factors that might lead to cell death during the stationary phase, the amount of protein aggregates and/or oxidized proteins appears to be of major importance (Lindner et al., 2008; Maisonneuve et al., 2008b,c; Nyström, 2007). Within the area of protein oxidation, protein carbonylation is extensively used to monitor protein oxidative damage due to its irreversible and irreparable nature and is currently used to detect oxidative damages due to the development of sensitive immunochemical methods permitting their detection (Dukan and Nyström, 1998; Levine, 2002). Carbonyl derivatives are essentially formed by direct metal-catalyzed oxidative attacks on the amino-acid side chains of arginine, lysine, threonine, and proline residues. As previously demonstrated in *E. coli* by our group, a correlation between the bacterial die-off rate during stationary phase and the increased carbonyl content per milligram of protein was established, strongly suggesting that the amount of carbonylated proteins is an important aging factor (Desnues et al., 2003; Maisonneuve et al., 2008c). Moreover, in *E. coli*, we have recently reported that protein aggregates which overrepresent abnormal proteins represent one aging factor affecting not only sibling-specific fitness, but also bacterial cell death during stationary phase (Maisonneuve et al., 2008b).

Previously, we observed that *clpC* inactivation increased *S. aureus* survival relative to the isogenic wild-type (WT) strain. ClpC is an ATPase that is involved in the regulated proteolysis of damaged or unneeded proteins. Because protein aggregates are involved in the aging and death of *E. coli* and nothing is known about this in *S. aureus*, we chose to examine the carbonylation status and aggregation of *S. aureus* proteins during the exponential growth phase and the death phase. In addition, the function of ClpC in controlling protein aggregation was also examined.

Materials and methods

Bacterial strains and medium

S. aureus DSM20231 (corresponding ATCC12600) and its *clpC* derivative PBM001 (Chatterjee et al., 2005) were inoculated from an overnight culture, diluted to an OD at 600 nm (OD_{600}) of 0.1 into BHI (brain-heart infusion) medium, and incubated at 37 °C in rotary shaker (230 rpm) to ensure aerobic growth (flask-to-volume ratio, 10:1).

Protein preparation

Protein preparation and analysis of fractions was performed as previously described (Maisonneuve et al., 2008b,c) with slight modifications. Exponential (2 h), and death phase-grown cells (72 and 96 h) (Chatterjee et al., 2005) were harvested, then washed twice with phosphate buffer (pH 7, 0.05 M, 4 °C) by centrifugation at 5500 × *g* for 20 min at 4 °C. Cells were resuspended to an OD_{600} of 12 in phosphate buffer containing 12 μg/ml lysostaphin and then incubated for 30 min at 37 °C. Cells were then lysed by 4 cycles of French press yielding crude extract (CE) containing both soluble and aggregated proteins. Next, all samples were treated with 0.2 mg/ml DNase and 50 μg/ml RNase. Immediately afterwards, CE was centrifuged at 18,000 × *g* for 30 min at 4 °C to obtain supernatants (SN) and pellets. The protein concentration of SN_{30} was determined using the BCA protein assay kit (Pierce). Subsequently, pellets were resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH 8) with Triton-X100 1% and incubated at 4 °C for 3 h. This centrifugation step was repeated twice following resuspension of the pellet in buffer A containing 0.5% Triton-X100 and buffer A without Triton-X100, respectively. After the third round of centrifugation, the pellet consisted of aggregate proteins (Maisonneuve et al., 2008c). In order to analyze these protein aggregates, they were solubilized in a rehydration buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 100 mM dithiothreitol [DTT]).

2D SDS-PAGE and carbonylation assays

Protein samples (100 μg) in rehydration buffer [as above plus 0.2% (v/v) ampholyte 3–10 (Bio-Rad) and 0.01% (w/v) bromophenol blue] were adsorbed onto 17-cm immobilized pH gradient (IPG) strips (pH 4–7 linear). After isoelectric focusing, the strips were (i) subjected to equilibration for 20 min in 60 mM Tris-base containing 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercapto-ethanol, and 0.1% (w/v) bromophenol blue, or (ii) first dinitrophenyl (DNP)-derivatized (Conrad et al., 2001), then subjected to equilibration. Molecular weight separation was achieved on 10% acrylamide gel using the PROTEAN II XL Multi-Cells slab gel SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) system (Bio-Rad). Proteins were either stained by silver nitrate for mass spectrometry analysis (Amersham) or transferred onto a PVDF membrane (Western blots and dot-blot chamber).

Carbonylation assays

Carbonylated proteins were detected and quantified as previously described (Maisonneuve et al., 2008c). Briefly, the carbonyl groups in the protein side chains were derivatized using the OxyBlot kit (Chemicon) to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNP derivatization). The DNP-derivatized CE or SN material was dot-blotted onto a PVDF membrane. To detect specific carbonylated proteins, either (i) DNP-derivatized proteins were separated by one dimensional SDS-PAGE and transferred subsequently to PVDF membranes by using a semidry blotting system, or (ii) proteins were submitted to 2D SDS-PAGE and derivatized after the first dimension using the in-strip derivatization method as described by Conrad (Conrad et al., 2001; Maisonneuve et al., 2008c). The membranes were then incubated with primary antibody, specific to the DNP moiety of the proteins, and subsequently incubated with a secondary (goat anti-rabbit) horseradish peroxidase-antibody conjugate directed against the primary antibody. Membranes were subsequently treated with the chemiluminescence-blotting substrate (ECL+, Amersham) for detection.

Identification of proteins and carbonylated proteins by mass spectrometry

Excised silver-stained spots were destained using the ProteoSilver™ destainer kit (Sigma) and digested with trypsin (Promega, Madison, WI) as previously described (Shevchenko et al., 1996). Proteomic analysis was performed by liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry (LC nano-ESI MS/MS) as previously described (Maisonneuve et al., 2008a, 2009) with the following modification: Oxidation modifications on turboSEQUEST search parameters were used on residues of the amino acids methionine, proline, threonine, arginine, and lysine (M-P-T-R-K), with mass variation of +16, +16, −2, −43, and −1 Da, respectively.