Participation of the $arcR_{ACME}$ protein in self-activation of the $arc$ operon located in the arginine catabolism mobile element in pandemic clone USA300

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Staphylococcus aureus pandemic clone USA300 has, in addition to its constitutive arginine catabolism ($arc$) gene cluster, an arginine catabolism mobile element (ACME) carrying another such cluster, which gives this clone advantages in colonisation and infection. Gene $arcR$, which encodes an oxygen-sensitive transcriptional regulator, is inside ACME and downstream of the constitutive $arc$ gene cluster, and this situation may have an impact on its activation. Different relative expression behaviours are proven here for $arcR_{ACME}$ and the $arcR_{ACME}$ operon compared to the constitutive ones. We also show that the artificially expressed recombinant ArcR$_{ACME}$ protein binds to the promoter region of the $arc_{ACME}$ operon; this mechanism can be related to a positive feedback model, which may be responsible for increased anaerobic survival of the USA300 clone during infection-related processes.

Key words: USA300 clone - ACME - transcriptional activator

Staphylococcus aureus USA300 clone is characterised by its resistance to most β-lactam antibiotics, virulence, global spread, and association with invasive diseases (Diep et al. 2006). Under stressful conditions such as oxygen depletion, USA300 ensures its survival by glucose fermentation or by means of nitrates as alternative electron acceptors. However, in the absence of glucose or nitrates in the medium, arginine becomes an important alternative energy source. S. aureus has a constitutive arginine deaminase (ADI) pathway encoded by the arginine catabolism operon ($arc_{can}$) (Makhlin et al. 2007). This pathway has been identified in some facultative or absolute anaerobic eukaryotes (Saccharomyces cerevisiae and Giardia intestinalis) and has also broadly spread into prokaryotes, mainly facultative anaerobes such as Streptococcus spp., Bacillus spp., and Staphylococcus spp. among others (Barcelona-Andres et al. 2002, Zúñiga et al. 2002, Makhlin et al. 2007, Lindgren et al. 2014).

Sequencing of the USA300 clone genome led to identification of an alternative $arc$ operon, which is transported in the arginine catabolism mobile element (ACME) (Diep et al. 2006). Although the $arc$ operon in ACME ($arc_{ACME}$) and constitutive $arc$ operon ($arc_{can}$) have the same structural genes ($arcABDC$), they have different genetic arrangements (Fig. 1). These genes have sequence identity ranging from 56.7% to 75.5%, and their proteins have sequence identity and similarity ranging from 40.1% to 81.3% and 63.1% to 89.9%, respectively (Urushibara et al. 2012, Thurlow et al. 2013). In addition, the $arcR_{ACME}$ operon has a hypothetical open reading frame (ORF) of 690 bp inserted into its central region coding for a putative ArcR protein, as opposed to $arcR$ in the constitutive operon; that is located downstream and has an independent promoter region (Diep et al. 2006) (Fig. 1). This ORF encodes a protein of 229 amino acid (aa) residues that belongs to the family of cAMP-CR receptor proteins just as its homologous constitutive protein ArcR (234 aa) from $arc_{can}$ (Tonon et al. 2001, Ibarra et al. 2013). The biological implications of this rearrangement have not been assessed. We hypothesised that the rearrangement of the $arcR$ gene inside the $arc_{ACME}$ operon is energetically favourable for its self-activation, contributing to the adaptive nature of pandemic clone USA300.

In order to assess the role of $arcR$ in transcription of the $arc_{ACME}$ operon, total RNA was extracted using the TRIzol method from anaerobic trypticase soy broth (TSB) cultures (using the GENbag microaer system, BioMérieux® and Resazurin, Oxoid®, to verify the anaerobic environment during the experiment) supplemented (or not) with arginine (50 mM, pH 7.2) and incubated at 37°C for 20 h. Prior to all the experiments, RNA was treated with DNase (Promega), and the presence of genomic DNA contamination was ruled out because no gyrB gene amplification was detected by polymerase chain reaction (PCR) with the RNA as template. Integrity of the (DNA-free) RNA was assessed by agarose gel electrophoresis. From these RNA samples, cDNAs were synthesised using reverse transcriptase (MMLV RT, 200 U/μL), a 1:4 ratio of random hexamers to RNA, 0.5 mM dNTPs, and 1X buffer [50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, and 10 mM dithiothreitol (DTT)] in a final volume of 25 μL [adjusted with

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This reaction was allowed to proceed for 1 h at 37°C. Quantification of relative expression of constitutive \[ \text{arcR} \] and \[ \text{arcC} \] genes (\[ \text{arcC}_{\text{cons}} \] and \[ \text{arcR}_{\text{cons}} \]) and the genes from ACME (\[ \text{arcR}_{\text{ACME}} \], \[ \text{arcC}_{\text{ACME}} \]) was determined by quantitative PCR (qPCR) to treated (50 mM arginine) and untreated (without arginine) \[ S. aureus \] strains (as a control of basal expression of the constitutive genes) strains in TSB cultures (Supplementary data, Tables I-II and methods). Relative amounts were calculated according to the method proposed by Schefe et al. (2006), with normalisation to housekeeping gene \[ \text{gyrB} \] (Fig. 1C) (Schefe et al. 2006). Additionally, to test whether the ArcR\[ \text{ACME} \] protein binds to the regulatory region of the \[ \text{arc}_{\text{ACME}} \] operon, \[ r\text{ArcR}_{\text{ACME}} \] (recombinant ArcR\[ \text{ACME} \] protein) was produced and purified from \[ E. coli \] BL21 (DE3) cells following the manufacturer recommendations (Thermo Scientific) (Supplementary data, Table I and methods). Protein expression was confirmed by western blotting using the monoclonal anti-6x-His tag antibody (Abcam; Fig. 2). A 123-bp fragment of the \[ \text{arc}_{\text{ACME}} \] operon promoter was synthesised by PCR and labelled using biotin-11-dUTP (biotinylated probe; Supplementary data, Table II). The possible binding of \[ r\text{ArcR}_{\text{ACME}} \] to this biotinylated DNA was evaluated by means of electrophoretic mobility shift assay (EMSA), and formation of the protein-probe complex was detected by chemiluminescence. Specificity of the binding was assessed using specific and non-specific unbiotinylated competitors. In addition, the biotinylated probe and recombinant protein were cross-linked by mixing them followed by irradiation with UV light for 10 min. This product was separated in duplicate by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) and nylon membranes for western blotting and chemiluminescence assays, respectively.

The ADI metabolic pathway is regulated by the ArgR and ArcR proteins acting as a repressor and an activator, respectively (Makhlin et al. 2007, Thurlow et al. 2013). The putative ArcR\[ \text{ACME} \] protein (GenBank accession number: WP_000272781.1), showed 40% sequence similarity with ArcR\[ \text{cons} \] from the constitutive operon (GenBank accession number: WP_000138214.1) in \[ S. aureus \] and 99.9% identity with the orthologous protein identified in the \[ S. epidermidis \] ATCC 12228 (GenBank accession number: NP_763659.1). Additionally, bioinformatics analysis of the ArcR\[ \text{ACME} \] protein identified a cAMP-binding domain from the CRP family (Conserved Domains Database accession number: COG0664) and a C-terminal helix-turn-helix DNA-binding motif (Conserved Domains Database accession number: cl21459),
which are reported as defining characteristics of CRP/ FNR transcription factors (Maghnouj et al. 2000, Körner et al. 2003, Gruening et al. 2006, Plane et al. 2013). To determine whether the arcR<sub>ACME</sub> gene activation responds to the same stimuli as the arc<sub>ACME</sub> operon does, we tried to find simultaneous co-transcripts from the remaining genes in the operon and arcR<sub>ACME</sub> in the cDNA by means of PCR. Using the arcD-arc<sup>C</sup> primers and the arcD-arc<sup>R</sup>ACME primers (Fig. 1A), we found that the arcR<sub>ACME</sub> gene is co-transcribed with the other arc<sub>ACME</sub> operon genes (Fig. 1A-B). This result suggests that when arc<sub>ACME</sub> is activated, the Arc<sub>ACME</sub> protein is expressed along with the arc<sub>ACME</sub> proteins. This means that the arc<sub>ACME</sub> gene responds to the same activating stimuli as the arc<sub>ACME</sub> operon does, contrary to the activation of the arc<sub>C</sub> operon and arc<sub>R</sub> operon, which respond to different stimuli, because the arc<sub>H</sub> operon has an independent promoter region (Makhlin et al. 2007). In order to determine the possible impact of this differential activation of the two arc operons, a qPCR analysis was carried out as mentioned above. It was found that under anaerobic conditions, the arc<sub>ACME</sub> operon in strain USA300 was transcribed 90-fold more abundantly than the arc<sub>C</sub> operon was, as inferred from expression of the arc<sub>C</sub> gene (Fig. 1C). Additionally, the arcR<sub>ACME</sub> gene was transcribed 83-fold more abundantly than the arc<sub>R</sub> operon was (Fig. 1C). These results suggest that Arc<sub>ACME</sub> may have a positive feedback effect on the arc<sub>ACME</sub> operon because minimal quantities of this transcriptional regulator can increase transcription of the whole operon and in turn, also increase its own production. Furthermore, it is possible that the increase in the transcription of arcR<sub>ACME</sub> has an additional effect upon the constitutive arc operon because an increase in its transcription above its basal expression level is also observed in the NCTC8325 strain without ACME (a sevenfold smaller increase, Fig. 1C). However, the influence of additional unexplored factors could not be ruled out in the analysis of the differences in transcription observed between these two strains.

Alignments of the promoter sequences of the two arc operons in strain USA300 showed relatively low nucleotide identity (43.9%). However, transcription basic elements and a putative binding site for CRP-regulatory proteins (TTGGA-N<sub>-</sub>TCACA) were found to be conserved (Fig. 3C) (Makhlin et al. 2007, Ibarra et al. 2013, Matsui et al. 2013). EMSA experiments with a biotinylated 123-bp double-stranded DNA fragment corresponding to a part of the ACME promoter, encompassing the TTGGA-N<sub>-</sub>TCACA hypothetical ArcR-binding site, revealed gel retardation by arcR<sub>ACME</sub>. Additionally, this arcR<sub>ACME</sub> electrophoretic shift was prevented by a specific competitor (the same probe without biotin; Figs 1A, 3A) but not by a non-specific competitor (91-bp gyrB fragment). These results confirmed the specific binding of arcR<sub>ACME</sub> to the promoter region of the arc<sub>ACME</sub> operon. Furthermore, arcR<sub>ACME</sub> incubated with the promoter region probe (Fig. 3B) and later UV-cross-linked so that it covalently binds to the DNA interacting with the recombinant protein, showed a single signal in the western blot (corresponding to the recombinant protein) and three signals in the chemiluminescence assay, one of which co-localised with the arcR<sub>ACME</sub> protein. Therefore, when we used a total protein extract, it was impossible to detect formation of this protein-probe complex, possibly because of a low concentration of the native protein in this total extract or because in the total extract, the native protein was already blocked by some remnant DNA (Fig. 3B). The highly active transcription of the arc<sub>ACME</sub> operon, its co-transcription with the arc<sub>R</sub>ACME gene, and the ability of arcR<sub>ACME</sub> to bind to the promoter region of the arc<sub>ACME</sub> operon support the role of this protein in the activation of this important operon in the <i>S. aureus</i> USA300 clone. Moreover, the Arc<sub>ACME</sub> protein belongs to the CRP family, known for its ability to activate RNA polymerase and to facilitate the transcription process of some genes under its control (Blake et al. 2002, Akyol & Çömlekçıoğlu 2009, Shimada et al. 2011).
Currently, the influence of ADI on the pathogenicity of the USA300 clone (in the context of colonisation and infection) is unclear. Nevertheless, several studies suggest that the pathogenicity of this clone is mediated by its increased virulence (up-regulation of some virulence genes), which is best viewed as an adaptation to the hostile environment of the host and host’s antibacterial defences. For this reason, changes in concentration of oxygen in the medium can control virulence factor expression and the capacity for colonisation in hostile environments (Malachowa & DeLeo 2010, Lindgren et al. 2014). The reorganisation of the arc\textsubscript{ACME} operon and the inclusion of regulator ArcR\textsubscript{ACME} possibly allow for faster use of arginine and a better response to adverse environmental conditions (e.g., acidity, polyamines) through the self-activation related to a positive feedback model. This mechanism can be a contributing factor of the successful adaptation of pandemic clone USA300.

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**AUTHORS’ CONTRIBUTION**

NVG and JEP - Designed the study; ZLCR - conducted the experiments; RAMO - performed the bioinformatics analysis; BEC - contributed to the microbiological experiments; ZLCR, RAMO and JEP - wrote the paper. All the authors analysed data, read and approved the final manuscript. The authors declare that they have no conflicts of interest.

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