INTRODUCTION

Genomes encode several different types of protein paralogs that can be explained by gene duplication events and/or gene acquisition through horizontal gene transfer. Once a new duplicated gene is obtained, functional diversity might occur through mutations (Conrad & Antonarakis, 2007). In addition, the generation of different transcriptional and posttranscriptional regulatory mechanisms that
control the expression of paralogs is a strategy that has enabled organisms to rapidly diversify their regulatory capacities (Zallot, Harrison, Kolaczkowski, & Crécy-Lagard, 2016).

Bacterial cold shock proteins (CSPs) are small RNA chaperones of about 7.4 kDa that are often found alongside several paralogs in bacterial genomes. Their number is variable depending on the species, for example, the chromosome and plasmids of *Bacillus megaterium* encode 18 CSPs whereas in *Escherichia coli*, *Bacillus subtilis* or *Streptococcus pyogenes* nine, three or just one variant of these proteins are found, respectively. The structure of CSPs is determined by the Cold Shock Domain (CSD), which consists of five antiparallel β-strands that form a β-barrel (Newkirk et al., 1994; Schindelin, Jiang, Inouye, & Heinemann, 1994). The CSD is a universal domain that is present in proteins from all kingdoms of life, including the eukaryotic Y-box proteins (YPBs), the mammalian Unr proteins, a fraction of the plant glycine-rich protein family (GRPs), the nematode LIN-28 proteins and the bacterial CSPs. This particular domain is usually involved in the regulation of gene expression at different levels thanks to its ability to interact with RNAs (Graumann & Marahiel, 1998; Mihailovich, Miliitti, Gabaldón, & Gebauer, 2010). The aromatic residues present within the RNA-binding motifs of CSPs, RNAP1 and RNAP2, enable interactions with the RNA chain (Lee et al., 2013; Newkirk et al., 1994). Several studies showed how mutating residues from the aromatic cluster of *E. coli* CspA and *B. subtilis* CspB impaired their nucleic acid-binding capacity (Hillier, Rodriguez, & Gregoret, 1998; Rennella et al., 2017; Schröder, Graumann, Schnuchel, Holak, & Marahiel, 1995). Additional works on the structural features of this protein domain revealed key amino acids involved in the protein-nucleic acid interaction. According to them, the RNA-binding and RNA-melting activities seem to be carried out by different amino acids. (Phadtare, Tyagi, Inouye, & Severinov, 2002; Sachs, Max, Heinemann, & Balbach, 2012; Schröder et al., 1995; Zeeb et al., 2006). To re-arrange RNA secondary structures, CSPs are thought to bind single-stranded RNA more strongly than double-stranded structures (Herschlag, 1995; Woodson, Panja, & Santiago-Frangos, 2018).

Since mutagenesis experiments in *E. coli*, *Yersinia enterocolitica* and *Salmonella* show that one or more CSPs can be functionally compensated by the remaining non-mutated homologs, it is thought that CSPs might have redundant roles. However, not all CSPs can totally restore the function of their mutated paralogs, suggesting a certain degree of specificity among them (Eshwar, Guldimann, Oevermann, & Tasara, 2017; Michaux et al., 2017; Neuhaus, Rapposch, Francis, & Scherer, 2000; Xia, Ke, & Inouye, 2001).

The *Staphylococcus aureus* genome contains three CSP paralogs (CspA, CspB and CspC) with a protein identity higher than 70%. *S. aureus* is one of the most important pathogens worldwide due to its capacity to cause both nosocomial and community-acquired infections, ranging from minor skin abscesses to life-threatening illnesses (e.g. endocarditis, osteomyelitis, biofilm-associated infections of medical devices or sepsis). Moreover, the emergence of methicillin-resistant *S. aureus* (MRSA) strains is a major concern from a healthcare perspective (Lakhundi & Zhang, 2018; Moellering, 2011).

In a recent study, we showed that the deletion of cspA in *S. aureus* had an effect on the expression of hundreds of genes. As a consequence, several relevant phenotypes, including biofilm formation and staphyloxanthin (STX) production, were affected in a cspA mutant (Caballero et al., 2018). Staphyloxanthin is a golden pigment carotenoid that gives *S. aureus* its characteristic color and also works as an important virulence factor that promotes resistance to oxidative stress and neutrophil-mediated killing (Liu et al., 2005). Therefore, STX has been proposed as a potential target when fighting MRSA infections (Liu et al., 2008). In addition, CspB has also been implicated in STX production (Donegan, Manna, Tseng, Liu, & Cheung, 2019; Duval, Mathew, Satola, & Shafer, 2010), suggesting a putative functional redundancy of CSPs in *S. aureus*. However, whether a particular *S. aureus* CSP may be able to compensate for the lack of expression of its counterparts remains unknown. In this study, we aimed at investigating the functional redundancy and/or divergence among CSP paralogs in *S. aureus*. To that end, we used STX biosynthesis as an in vivo reporter of CSP functionality. We demonstrated that STX production is specifically modulated by CspA since expression of CspB and CspC in a ΔcspA strain could not restore the function of CspA. In addition, we provided evidence that CspA specificity is determined by one amino acid, proline 58 (Pro58). This indicates that a few evolutionary changes in specific amino acid positions might lead to functional diversification among different bacterial CSP paralogs.

## Results

### 2.1 *S. aureus* CspA specifically modulates STX production

*S. aureus* CSP paralogs (CspA, CspB and CspC) show a high degree of identity between them. When comparing the protein sequence of CspA with those of CspB and CspC, 20 and 13 out of 66 amino acid (aa) differences are found, respectively (Figure 1). Since STX levels are easily measured by spectrophotometry after pigment extraction (Liu et al., 2005, 2008; Lan, Cheng, Dunman, Missiakas, & He, 2010), we chose STX production as an in vivo reporter to evaluate if *S. aureus* CSP paralogs play redundant roles. First, we measured the pigment levels from csp deletion mutants, which were generated by recombination using an engineered pMAD plasmid (Arnaud, Chastanet, & Debarbouille, 2004). Figure 2a shows that the elimination of cspB or cspC did not reduce STX production, as opposed to deletion of cspA. This indicated that out of the three CSP paralogs, CspA was solely responsible for promoting STX synthesis (Figure 2a). Previous studies showed that the deletion of *cspB* also affected STX production (Donegan et al., 2019; Duval et al., 2010). Due to differences in the strains, culture media and bacterial growth conditions between said studies, the expression levels of CSPs might not be comparable. Therefore, and to avoid any putative transcriptional bias, we complemented the ΔcspA strain with plasmids expressing each csp mRNA under the control of a heterologous promoter. To do so, we first determined the
**FIGURE 1**  *S. aureus* CSP paralogs share a high sequence identity. Comparison of CSPs (CspA, CspB and CspC) amino acid sequence. Identical amino acids are shown as dots. The RNP1 and RNP2 motifs are indicated. The secondary structure layout is shown along with the conserved aromatic residues across CSP paralogs [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 2**  CspA specifically modulates staphyloxanthin (STX) production. (a) Quantification of STX production in the *S. aureus* 15981 wild type (WT) and its isogenic ΔcspA, ΔcspB and ΔcspC mutant strains. The STX pigment was extracted with ethanol from normalized bacterial pellets after growth for 15 hr at 37°C. The pigment was quantified by measuring the absorbance from the ethanol solutions at 460 nm. The production level of STX for each strain is expressed in %, considering the WT STX levels as a 100%. Column and error bars represent the mean and the standard deviation from three independent biological replicates. (b, d and f). Schematic representation of the pCN51 plasmid constructs harboring the different csp mRNA sequences that were transformed into the ΔcspA strain. Restriction enzymes used for their cloning are indicated. (c) and (h) Quantification of STX production in the ΔcspA strain complemented with the plasmids shown in (b) and (f), respectively. The WT and ΔcspA strains harboring the pCN51 plasmid were used as positive and negative controls, respectively. (e) and (g) Western blot showing the levels of the 3xFLAG-tagged CspA, CspB and CspC proteins in the ΔcspA complemented strains, which are expressed from the native csp mRNAs or from the chimeric cspA mRNAs that encode CspB or CspC. Total protein extraction was performed after growth at 37°C and 200 rpm for approximately 15 hr. Samples were run in 12% polyacrylamide gels and transferred to Nitrocellulose membranes. Tagged proteins were developed using peroxidase conjugated anti-FLAG antibodies and a bioluminescence kit. Coomassie stained gel portions show sample preparation controls.
boundaries of the csp mRNA sequences using previously reported transcriptome maps (Koch et al., 2014; Las et al., 2011; Figure S1) and then cloned the csp mRNA sequences into the pCN51 plasmid, which carried a heterologous cadmium inducible Pcad-CadC module (Charpentier et al., 2004; Endo & Silver, 1995). Next, we transformed the ΔcspA strain with the resulting constructs (Figure 2b). We also introduced the empty pCN51 plasmid into the wild type (WT) and ΔcspA strains to generate the appropriate positive and negative controls, respectively. After growing the complemented strains for 15 hr (late stationary phase) in the presence of 1 µM CdCl₂, we extracted the STX pigment and quantified it. The results revealed that the cspA mutant strains expressing CspB or CspC showed similar pigment levels to those found in the ΔcspA pCN51 strain (Figure 2c). Since all CSP paralogs were expressed under the same promoter, this result initially suggested that only CspA was able to modulate STX production. However, it was already known that the 5'UTRs of csp mRNAs contain posttranscriptional regulatory elements that affect protein expression (Caballero et al., 2018; Giuliiodori et al., 2010). To evaluate whether the complemented ΔcspA mutant strains produced comparable CSP levels, we constructed an additional set of ΔcspA strains that expressed 3xFLAG-tagged CSPs (Figure 2d). Western blot analyses revealed that the expression of the csp mRNAs from the Pcad promoter led to much lower CspB⁴xF and CspC⁴xF protein yields than those of CspA⁴xF (Figure 2e). This indicated that different posttranscriptional events may modulate the expression of CSPs in S. aureus. In order to minimize the effect of such posttranscriptional control, we sought an alternative strategy that consisted in constructing pCN51 plasmids carrying the cspA mRNA with the fewest nucleotide changes that made it encode CspB⁴xF or CspC⁴xF instead of CspA⁴xF (Figures 2f and S2). Figure S2 shows the nucleotide substitutions performed in each codon to change the necessary amino acids. The resulting plasmids were used to transform the ΔcspA strain and total protein and pigment extractions were made. Western blot analysis confirmed that the new chimeric mRNAs expressed similar levels of CspA⁴xF, CspB⁴xF and CspC⁴xF (Figure 2g). However, CspA⁴xF remained as the only CSP able to restore STX in a ΔcspA strain (Figure 2h). Altogether, these data demonstrated that CspA specifically modulates STX biosynthesis and suggested the existence of specific roles for CSPs in S. aureus.

2.2 The RNP-connecting loop is not responsible for CspA specificity

The lack of functional redundancy between CspA and CspB/CspC made us wonder which were the residues responsible for the specificity of these proteins in S. aureus. It is widely accepted that the RNA-binding capacity of CSPs mainly resides in the RNP1 and RNP2 motifs, which are formed by the β2- and β3-strands, respectively. These amino acids are highly conserved in most organisms, including S. aureus (Figure 1). Some studies suggest that the different residues flanking the RNP1 and RNP2 motifs or the non-conserved amino acidic loop 2, which connects RNP1 and RNP2, could determine target specificity (Figure 1; Kenan, Query, & Keene, 1991; Phadatre & Inouye, 1999; Yamanaka, Fang, & Inouye, 1998). The amino acid differences in loop 2 of S. aureus CSPs are relatively high (four out of six amino acid changes) compared to the overall protein sequence variation (Figure 1). For this reason, we decided to examine whether the RNP-connecting loop 2 was responsible for target specificity in S. aureus CSPs. We began by replacing loop 2 of the 3xFLAG-tagged CspA with its CspB or CspC equivalent. For this purpose, we performed the following amino acid substitutions: V20Q, G22N, E23G and N24G to generate the CspA_L2_B⁴xF protein, and V20R, G22D, E23G and N24S to generate the CspA_L2_C⁴xF variant (Figure S2 and 3a). The resulting plasmids were used to transform the ΔcspA strain. Western blot analyses revealed that amino acid substitutions did not affect Csp⁴xF levels (Figure 3b). Interestingly, both chimeric proteins restored STX production, indicating that the specificity in regulating STX production is not determined by the RNP-connecting loop (Figure 3c).

2.3 One amino acid at the C-terminal end of CspA drives the specific control of STX production

Amino acids located at the C-terminal half, specifically at loop 3 and β5-strand, might also be important for RNA interaction by increasing the protein surface that makes contact with nucleic acids (Max, Zeeb, Bienert, Balbach, & Heinemann, 2006, 2007; Sachs et al., 2012). To evaluate the functional consequence of amino acid variations located at the C-terminal half of the protein, we focused on the differences between CspA and CspC since the identity between their protein sequences was the highest (Figure 1). We constructed plasmids expressing chimeric 3xFLAG-tagged CSPs, which combined the N-terminal half, including the RNP1 and RNP2 motifs, of one of the two CSPs with the C-terminal half of the other (Figure S3a). For this purpose, we followed the codon substitution criteria indicated in Figure S2, which resulted in modified cspA mRNAs that encoded the chimeric CspAC⁴xF and CspCA⁴xF proteins (Figure S3a). On the one hand, Western blot analysis revealed that these chimeric CSPs were expressed at comparable levels to those of CspA⁴xF and CspC⁴xF (Figure S3b). On the other hand, the analysis of STX production in ΔcspA strains complemented with the different chimeras showed that the pigment levels were not restored by CspAC⁴xF. In contrast, the cspA mutant complemented with the CspCA⁴xF chimera (C-terminal half of CspA), produced similar STX levels to those of the same strain complemented with CspA⁴xF (Figure S3c). These results suggested that amino acid differences located at the C-terminal half of CSPs might be responsible for determining functional specificity. When comparing the C-terminal half of CspA and CspC, only seven aa differences made one distinguishable from the other. Specifically, there were two aa variations between CspA and CspC in loop 3 (N34A; Q35E), three in β4-strand (A44K, E50D and V51I) and two in β5-strand (P58E; L66M), respectively (Figure 1). The effect of these amino acids on CSP functional specificity was studied by expressing three CspA⁴xF variants that included the amino acid differences.
Following the codon substitution criteria shown in Figure S2, we constructed plasmids that expressed chimeras CspA_L3_C3xF (N34A; Q35E), CspA_p4_C3xF (A46K; E50D; M66L) and CspA_p5_C3xF (P58E; L66M) (Figure 4a). We used these constructs to complement the cspA mutant and their expression was checked by Western blot (Figure 4b). Next, we measured STX production and found that the strains expressing the CspA_L3_C3xF and CspA_p4_C3xF chimeras produced pigment levels similar or slightly higher than those of the WT strain (Figure 4c). In contrast, complementation of the cspA mutant with CspA_p5_C3xF did not restore the STX synthesis, suggesting that CspA functional specificity might be comprised in β5-strand (Figure 4c). To study this in detail, we created two different plasmids that expressed CspC_L3p4_A3xF (carrying loop 3 and β4-strand of CspA) and CspC_p5_A3xF (carrying the E58P and M66L substitutions in β5-strand; Figure 4d). The expression levels of these chimeric proteins were again found to be similar to those of CspA3xF (Figure 4e). Interestingly, the strain expressing the chimeric CspC3xF protein that carried CspA β5-strand produced STX, while the strain expressing CspC_L3p4_A3xF showed an absence of pigment production (Figure 4f). These results restricted the putative CspA functional specificity to only two amino acid variations within β5-strand (positions 58 and 66 in Figure 1). Previous structural and in vitro binding experiments with B. subtilis CspB and an hexa-nucleotide RNA suggested that position 58 could participate in target binding (Sachs et al., 2012). To explore this idea, we expressed a new chimeric CspA3xF protein carrying the orthologous P58E substitution, which is naturally present in CspC (Figure 5a). After verifying by Western blot that protein levels were similar (Figure 5b), we measured STX production and observed that the STX levels in the ΔcspA strain expressing the CspA3xF_P58E protein were below those in the WT strain (Figure 5c). Notably, when doing the opposite, that is substituting CspC Glu58 by CspA Pro58 in CspC (chimeric CspC3xF_E58P; Figure 5a), STX production was restored to WT levels in a ΔcspA strain (Figure 5c). This gain of function in the chimeric CspC3xF_E58P protein confirmed that Proline 58 is critical for modulating STX biosynthesis. We also analyzed the consequences of substituting CspB Asp58 by CspA Pro58. When the chimeric CspB3xF_D58P was expressed in a ΔcspA strain, STX production was not complemented, suggesting that additional amino acids might participate in target discrimination (Figure S4). From these experiments, we concluded that just one amino acid was enough to create functional divergence between two CSPs (e.g. CspA and CspC).

To evaluate how amino acid changes in position 58 impact CSP functionality, we performed structural CSP modeling (Figure 6a). Superposition of CspA and CspC models (alpha-carbon superposition, root mean squared XYZ displacement: 0.340 Å) showed that there were no major differences between their RNP motifs (Figure 6b). Specifically, position 58 in CspA is occupied by a proline that is oriented to potentially enable a stronger interaction with the RNA target. Following this line of reasoning, the solvent exposed side chain of Glu58 in CspC would determine an alternative binding target with a different affinity. This might explain the specificity of CspA towards controlling STX production (Figure 6c).

### 2.4 Proline 58 leads to CspA specific control of σB activity

STX production is carried out by the proteins encoded in the crtOPQMN operon, which depends on the alternative sigma B factor...
Analogously, the activity of $\sigma^B$ is modulated by CspA (Caballero et al., 2018; Donegan et al., 2019; Katzif, Lee, Law, Tzeng, & Shafer, 2005). We previously showed that CspA directly interacts with the rsbV-rsbW-sigB operon, resulting in an improved $\sigma^B$ activity (Caballero et al., 2018). To confirm that the CspA-mediated specific control of STX production by Pro58 occurs through the modulation of $\sigma^B$ activity, we analyzed additional $\sigma^B$-associated phenotypes in cspA mutants complemented with CspA$_{3xF}$, CspC$_{3xF}$, CspA$_{3xF}$, P58E and CspC$_{3xF}$, E58P. First, we used the alkaline shock protein Asp23 as a natural reporter for testing $\sigma^B$ activity, since it was previously described to be exclusively activated by $\sigma^B$ (Gertz et al., 1999; Giachino, Engelmann, & Bischoff, 2001). Western blot analyses using specific anti-Asp23 antibodies showed that Asp23 expression levels could be restored in $\Delta$ cspA strains expressing CspA$_{3xF}$ and CspC$_{3xF}$, E58P. In contrast, $\Delta$ cspA strains expressing CspC$_{3xF}$ and CspA$_{3xF}$, P58E presented similar Asp23 levels to those of the cspA mutant (Figure 7 a). Second, since the production of the poly-N-acetylglucosamine exopolysaccharide (PIA/PNAG) and biofilm formation in S. aureus are inhibited by CspA and $\sigma^B$ (Caballero et al., 2018; Valle, Echeverz, & Lasa, 2019), we analyzed both phenotypes...
and found that they could only be restored when CspA\textsuperscript{3xF} or CspC\textsuperscript{3xF–E58P} proteins were expressed in the ΔcspA strains (Figure 7b and c). Altogether, these results indicated that Pro58 in CspA is required to specifically control σ\textsubscript{B} activity and subsequently modulate σ\textsubscript{B}–associated phenotypes, such as Asp23 expression, STX biosynthesis, PIA/PNAG production and biofilm formation in \textit{S. aureus}.

2.5 | Proline 58 is evolutionarily conserved among CspA orthologs in \textit{Staphylococcus} species

Previous analysis of the consensus sequence of thousands of proteins carrying the CSD (both in eukaryotic and bacterial species) revealed several highly conserved amino acids, which resulted critical for the molecular function of CSPs regardless of their evolutionary origin (Amir et al., 2018). In this study, position 58 appears as not conserved among the superfamily of CSPs. In \textit{S. aureus} CspB and CspC, position 58 is represented by Asp and Glu, respectively (Figure 1). We hypothesized that if Pro58 plays a relevant role in CspA specificity, it should be conserved among its orthologs. To test this idea, we analyzed 98 CSP sequences from 44 \textit{Staphylococcus} species. On the one hand, we performed multiple CSP sequence alignments using Clustal Omega (Madeira et al., 2019), which produced phylogenetic CSP trees (Figure S5). On the other hand, we classified the CSP sequences according to their PATRIC-assigned protein family group (Wattam et al., 2017). This classification was based on sequence similarity over the entire protein length and the conserved genomic context (Wattam et al., 2017). A total of three protein family groups: PLF\textsubscript{1279}_00001953 (blue) and PLF\textsubscript{1279}_00007034 (green) were assigned. Most of the CSPs that fell into the red category were clustered in the same tree branches, which could be associated to CspA orthologs. Interestingly, 52 out of 53 CspA orthologs carried a proline in position 58 (Figure S5). In contrast, when analyzing CspB and CspC orthologs, which are included in the blue and green groups, respectively, several other amino acids were found in position 58 (Figure S5). Note that the number of CSPs for a given staphylococcal species is between one and four, with two being the most common number (Figure 8). CspA is present in all species and in at least five of them it is duplicated. Interestingly, when CspA was accompanied by a CspC ortholog, the latter usually included a glutamate, aspartate or alanine in position 58, depending on the species. In the few cases in which the CspB variant coexisted with CspA, an aspartic acid was found in such position (Figures 8 and S5). The fact that Pro58 is highly conserved among CspA orthologs highlighted the relevance of this particular amino acid in CspA specificity. At the same time, the substitutions in position 58 among CSP paralogs suggested functional divergence among them.

3 | DISCUSSION

The identification, annotation and classification of paralogs in a genome is currently determined by comparative genomics, based on sequence similarity and conserved genomic contexts across closely related genomes (Zallot et al., 2016). On average, protein paralogs represent approximately 24% of bacterial proteomes (Gevers, Vandepoele, Simillion, & Peer, 2004). When looking at a specific paralogous family, like CSPs, all members share the same molecular function, for example, the capacity to bind nucleic acids. However, it is almost impossible to infer from protein sequences (unless they share 100% of identity) if two paralogs play the same biological...
function, for example, control of $\sigma^B$ activity. Likewise, it is not easy to address what amino acid differences between paralogs correspond to silent changes, not having an influence on the biological function, and what amino acids might provide, if any, a specific role. Since protein specificity resides at the level of molecular interaction with their corresponding target, such discrimination in vivo is difficult without having a robust phenotype or reporter system. Here, we used the staphyloxanthin production as a natural in vivo reporter to demonstrate that interchanging just one orthologous amino acid is sufficient to transfer CspA functionality to its paralog CspC in *S. aureus*. Specifically, when Pro58 of CspA was substituted by Glu58 of CspC, CspA lost the capacity to modulate $\sigma^B$ activity and its associated phenotypes. In contrast, a chimeric CspC protein carrying a Pro instead of a Glu in position 58 gained the ability to promote $\sigma^B$ activation (Figures 5c and 7). According to the protein structural models (Figure 6), Pro58 is located on the CspA surface, close to the RNA-binding motifs, suggesting that this amino acid might directly interact with the RNA to provide a specific target selection. This idea is in agreement with a previous work that showed how *B. subtilis* CspB establishes in vitro water-mediated contacts between the backbone carbonyl group of Pro58 and the O4 of the third uracil of an hexa-nucleotide RNA (Sachs et al., 2012). Thus, different amino acid substitutions in position 58 throughout evolution would contribute to the selection and/or allosteric exclusion of specific targets. The structural models also showed that Pro58 is in trans configuration (Figure 6a). In spite of this, it might be possible that Pro58 alternates between cis/trans isomerization in vivo. Proline isomerization by peptidyl-prolyl cis-trans isomerases (PPIases) has been proven relevant for virulence and antibiotic resistance in previous studies (Ünal & Steinert, 2014; Wiemels et al., 2017). Regardless of the possibility of PPIases targeting CSPs, a putative Pro58 isomerization from trans to cis configuration might rearrange the protein structure by causing a reorientation of loop 4 (Figure 6a). The cis configuration in CspA Pro58 could impact the binding interface with the target and add versatility for target recognition in comparison to other amino acids in such position. This also opens the door to two different target profiles being recognized by the same protein depending on its Pro58 isomerization state. Addressing whether just one or both of the CspA configurations exist in vivo and what PPIases would be responsible for such proline isomerization requires further investigations.
that this specific amino acid position might be relevant for determining RNA target specificity (Dabrazhynetskaya et al., 2009). Amino acid variations in position 215 of PhoP cause changes in its activity and have probably contributed to an increased virulence in Y. pestis (Fukuto et al., 2018).

Similarly, evolutionary substitutions of amino acids in the vicinity of the DNA-binding interface of transcription factors has been shown to provide divergent DNA specificity among paralogs (Hudson et al., 2016). Our results also indicate that biological function specificity may exist outside the main RNA-binding motifs, RNP1 and RNP2. Since the efficiency of the molecular function should be preserved during evolution, it seems logical that amino acids surrounding the active site are selected to determine target specificity among paralogs. Regardless, we have shown that this cannot be easily predicted by just analyzing the protein sequence. In addition, since minor changes in the protein surface can be sufficient to discriminate or recognize a DNA/RNA target, structural models of real ribonucleoprotein complexes would be essential in order to establish their specific molecular interactions. The CspA RNA partner region participating in the control of $\sigma^B$ activity has not been elucidated yet. According to previous results from our group, we would expect CspA to interact with certain regions of the $rsbV-rsbW-sigB$ operon (Caballero et al., 2018), providing $\sigma^B$ activation and, as a consequence, controlling the $\sigma^B$-associated phenotypes. Further investigations are required to determine the precise molecular interactions between Pro58 of CspA and this operon. Moreover, it cannot be excluded that different and/or additional amino acids might also be required for the interaction of CspA with this or other mRNA targets. In fact, the lack of complementation in the $\Delta$cspA strain expressing the chimeric CspB$_3$F_D58P (Figure S4) suggested that target recognition is not exclusively determined by position 58 and that other amino acid differences may contribute to it.

An additional observation of this study, is that the expression of CSP paralogs is tightly controlled. It is often assumed that when performing parallel complementation experiments by expressing different proteins under the control of the same heterologous promoter, similar protein concentrations should be reached. However, our study showed that expressing the csp mRNAs from the same promoter lead to different CSP levels (Figure 2e). To unambiguously demonstrate CspA specificity in vivo, we engineered S. aureus strains that ensured comparable levels of expression for the three CSP paralogs. This was achieved by generating chimeric csp mRNAs that shared the same UTRs. The success of this strategy indicated that the differences found in the CSP levels should mainly be attributed to a differential post-transcriptional control. Noteworthy, different band migration patterns in the polyacrylamide gels were repeatedly observed for each of them. This could be interpreted as CSPs being affected at the posttranslational level (Figure 2). Similar variations in band migration had previously been shown for Salmonella CSPs (Michaux et al., 2017). A possible explanation might be protein conservation of Pro58 among CspA orthologs indicates that this specific amino acid position might be relevant for determining RNA target specificity (Figure 8 and S5). In agreement with our results, it has also been shown that single amino acid orthologous substitutions provide functional differences among DNA-binding proteins (Dabrazhynetskaya, Brendler, Ji, & Austin, 2009; Fukuto et al., 2018; Hudson et al., 2016; Treisman, Gönczy, Vashishtha, Harris, & Desplan, 1989). For example, a substitution of a single orthologous amino acid (Asp288) in the ParB protein that recognizes parS to promote segregation of the plasmid DNA to daughter cells, is sufficient to change or abolish plasmid specificity (Dabrazhynetskaya et al., 2009). Amino acid variations in position 215 of PhoP cause changes in its activity and have probably contributed to an increased virulence in Y. pestis (Fukuto et al., 2018).

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An additional observation of this study, is that the expression of CSP paralogs is tightly controlled. It is often assumed that when performing parallel complementation experiments by expressing different proteins under the control of the same heterologous promoter, similar protein concentrations should be reached. However, our study showed that expressing the csp mRNAs from the same promoter lead to different CSP levels (Figure 2e). To unambiguously demonstrate CspA specificity in vivo, we engineered S. aureus strains that ensured comparable levels of expression for the three CSP paralogs. This was achieved by generating chimeric csp mRNAs that shared the same UTRs. The success of this strategy indicated that the differences found in the CSP levels should mainly be attributed to a differential post-transcriptional control. Noteworthy, different band migration patterns in the polyacrylamide gels were repeatedly observed for each of them. This could be interpreted as CSPs being affected at the posttranslational level (Figure 2). Similar variations in band migration had previously been shown for Salmonella CSPs (Michaux et al., 2017). A possible explanation might be protein
modification but this possibility has not been found for CSPs yet (Donegan et al., 2019). This also applied to several of the chimeric CSPs, which migrated differently when compared to the WT CSPs. However, protein modification events alone cannot explain this. Furthermore, the different band patterns did not correlate with the capacity to produce STX. Therefore, although post-translational modifications cannot be fully ruled out, it is very likely that the abnormal migration of CSPs in polyacrylamide gels occurs due to the additional binding of SDS molecules to specific aa stretches, a phenomenon that is accentuated in small proteins like CSPs (Shi et al., 2012).

Considering that CSP sequences are highly conserved and that a tight expression control may also exist in other bacterial species, the genetic engineering strategies displayed here could be extrapolated to evaluate the putative functional divergence among CSP paralogs in bacteria. This would require the following steps: (a) the identification of a functional reporter such as a phenotype or a gene target whose expression depends on one of the CSP paralogs; and (b) the engineering of plasmids that bypass the putative differential posttranscriptional regulation for each of the CSPs and, thus, allow comparable protein levels.

Overall, our genetic strategy allowed us to unveil the key role played by one of the CspA amino acids, proline 58, in controlling $\sigma^B$ activity in S. aureus. Therefore, it may be proposed that a single amino acid change is sufficient to produce functional divergence among CSP paralogs.
4 | EXPERIMENTAL PROCEDURES

4.1 | Strains, plasmids, oligonucleotides and growth conditions

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Supporting Information Tables S1-S3 respectively. *Staphylococcus aureus* strains were grown in Mueller Hinton Broth (MH) (Sigma-Aldrich) or Tryptic Soy Broth (Prondanisa) supplemented with 0.25% of glucose, whereas *Escherichia coli* was grown in LB broth (Prondanisa). The B2 (casein hydrolyse, 10 g/L; yeast extract, 25 g/L; NaCl, 25 g/L; K2HPO4, 1 g/L; glucose, 5 g/L; pH 7.5) and SuperBroth (tryptone, 30 g/L; yeast extract, 20 g/L; MOPS, 10 g/L; pH 7) media were used to prepare *S. aureus* and *E. coli* competent cells, respectively. When selective bacterial growth was required, media were supplemented with the appropriate antibiotics at the following concentrations: Erythromycin (Erm), 1.5 µg/ml or 10 µg/ml; Ampicillin (Amp), 100 µg/ml. In addition, 1.0 µM of cadmium was added whenever activation of the Pcad promoter was required.

4.2 | Plasmid constructions

The plasmids used in this study were engineered as previously described (Caballero et al., 2018). All constructs were verified by Sanger sequencing. Specifically, the pmAD_ΔcspB and pmAD_ΔcspC plasmids (Table S2), which were required for chromosomal deletion of the cspB and cspC genes, were constructed by amplifying 400–500 nt of their flanking regions with the corresponding oligonucleotides (Table S3). The resulting PCR fragments were cloned and inserted in pmAD as previously described (Caballero et al., 2018).

The plasmids that expressed the native csp mRNAs under the control of the Pcad module (Table S2) were constructed by PCR amplification, using the chromosomal DNA of *S. aureus* 15981 strain as a template and the appropriate +1 and term oligonucleotides (Table S3). The resulting PCR fragments were cloned into the pCspA vector (Charpentier et al., 2004).

The pCspB3xF plasmid (Table S2), which expressed the 3xFLAG-tagged CspB protein under the control of the Pcad module, was generated by overlapping PCR using oligonucleotides CspB +1 BamHI, 3xFcsB_B, 3xFcsB_C, and CspB ter EcoRI (Table S3) and chromosomal DNA of *S. aureus* 15981 as template. The amplified PCR product was then cloned and ligated into pCN51.

The pmA_CspB3xF, pmA_CspC3xF, pmA_CspA_L2_B3xF, pmA_CspA_L2_C3xF and pmA_CspAC3xF plasmids (Table S2) expressed chimeric 3xFLAG-tagged CSPs from a modified cspA mRNA. Such modified cspA mRNA sequences were created by gene synthesis and cloned into pMA-T plasmids (Table S2) by GeneArt (Invitrogen, ThermoFisher Scientific). Next, the synthetic DNA fragments were subcloned into the pCN51 plasmid using the BamHI and EcoRI restriction sites.

The pmA_CspCA2xF, pmA_CspA_L3_C2xF and pmA_CspA_L4_C2xF plasmids were generated by overlapping PCR using the oligonucleotides listed in Table S3. Specifically, for construction of the pmA_CspCA2xF plasmid (Table S2), the PCR fragment amplified with the CspA_ovl_dcha and 3xF_Ncol_rv primers, using pCspA2xF as a template, and the PCR fragment amplified with the CspA +1 BamHI and CspCA_ovl_lqz primers, using pmA_CspC2xF as a template, were fused in a second PCR using the corresponding external primers. The resulting chimeric DNA fragment was cloned into the pCspA2xF construct using the BamHI and Ncol restriction sites. Similarly, the pmA_CspA_L3_C2xF and pmA_CspA_L4_C2xF plasmids (Table S2) were generated by overlapping PCR using pCspA2xF as a template and oligonucleotides CspA +1 BamHI and CspA_L3_C_lqz or CspA_L4_C_lqz to generate the first PCR fragment and CspA ter EcoRI and CspA_L3_C_dcha or CspA_L4_C_dcha to generate the second PCR fragment. These fragments were fused in a second PCR using the corresponding external primers and cloned into pCN51 using BamHI and EcoRI restriction sites.

The pmA_CspA β5_C2xF plasmid (Table S2) was constructed by digesting pCspA2xF with BamHI and Sacl and pmA_CspC3xF with SacII and EcoRI. The corresponding fragments were ligated into pCN51 using BamHI and EcoRI restriction sites. Analogously, pmA_CspC3xF_A2xF (Table S2) was generated by digesting pmA_CspCA2xF with BamHI and Sacl and pmA_CspC2xF with Sacl and EcoRI. The resulting DNA fragments were cloned into pCN51 digested with BamHI and EcoRI. The pmA_CspC β5_A2xF plasmid (Table S2) resulted from digesting pmA_CspC3xF with BamHI and Sacl and pCspA2xF with Sacl and EcoRI. Both fragments were then cloned into a BamHI and EcoRI digested pCN51. The pmA_CspA2xF_P58E and pmA_CspC2xF_ES58F plasmids (Table S2) were generated by digesting the pmA_CspA β5_C2xF and pmA_CspC β5_A2xF with BamHI, Accl and EcoRI restriction enzymes and combining the resulting DNA fragments accordingly to ligate them into a EcoRI and BamHI processed pCN51. The pmA_CspB2xF_DSP58 plasmid was constructed by PCR amplification using oligonucleotides CspA_+1_BamHI and CspB_DSP58. The resulting PCR fragment was cloned into pJET, digested by BamHI and Accl restriction enzymes and subcloned into pmA_CspB2xF, which was digested with the same enzymes.

4.3 | Generation of csp mutants by homologous recombination

The csp mutant strains were obtained by marker-less homologous recombination, using the pMDAD plasmid system (Arnaud et al., 2004). Briefly, the ΔcspB and ΔcspC strains were generated by a two-step procedure that replaces a gene of interest by the corresponding mutant allele, contained in the pMDAD plasmid (Valle et al., 2003). The resulting mutant strains were confirmed by PCR using the corresponding oligonucleotides, E and F (Table S3). In addition, the resulting PCR fragments were verified by Sanger sequencing.

4.4 | Bacterial cultures for total protein and staphyloxanthin extractions

Bacterial cultures were grown in 250 ml-Erlenmeyer flasks containing 50 ml of MH supplemented with 1 µM CdCl₂ and 10 µg/ml erythromycin. Media were inoculated (1:250) with normalized bacterial
aliquots, which were previously grown overnight. Bacterial cultures were incubated at 37°C and 200 rpm for approx. 15 hr. Each culture was divided into two aliquots, one of 2-ml for total protein extraction and another of the remaining volume for staphyloxanthin extraction.

### 4.5 | Total protein extraction and Western blotting

Eppendorf tubes containing 2-ml of cultures were centrifuged for 10 min at 21,000 g and 4°C. The resulting pellets were incubated at 4°C if protein extraction was performed immediately or frozen in liquid nitrogen and stored at −80°C until required. Pellets were washed once with PBS 1X and centrifuged for 10 min at 21,000 g. The resuspended bacteria were transferred to Lysing Matrix B tubes containing acid-washed 100 μm glass beads (MP Biomedicals) and mechanically lysed in a FastPrep-24 instrument (MP Biomedicals), for 45 s and speed 6, twice. Lysed bacteria were centrifuged for 10 min at 21,000 g and 4°C and supernatants containing total protein extracts transferred to 1.5-ml Eppendorf tubes. Total proteins were quantified using a Bradford protein assay kit (Bio-Rad) and samples prepared at the desired concentration in Laemmli buffer. Samples were stored at −20°C until needed.

Total protein sample stocks were prepared at the same concentration, loaded into 12% SDS-polyacrylamide gels that were prepared using the SureCast Handcast Station (Invitrogen, ThermoFisher Scientific), and run in Mini Gel Tanks (Life technologies). Resolved proteins were stained with Coomassie brilliant blue R250 (Sigma) to control sample preparation. Next, diluted sample aliquots were run into 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences). The WT and chimeric 3xFLAG-tagged CSPs were developed with anti-FLAG antibodies (Sigma) as previously described (Caballero et al., 2018). Asp23 protein was developed using polyclonal rabbit anti-Asp23 antibodies.

### 4.6 | Staphyloxanthin extraction and quantification

Staphyloxanthin extraction and quantification was performed as previously described, with slight modifications (Caballero et al., 2018). Pre-weighted 50 ml-Falcons containing the bacterial cultures were centrifuged for 10 min at 4,400 g. Bacterial pellets were washed with 50 ml of PBS, centrifuged again, and weighted after discarding the supernatant. The pellets were resuspended in a variable volume of PBS, proportional to their weight. Next, 700 μl of bacterial suspensions were transferred to Eppendorf tubes and centrifuged. Pellets were resuspended in 700 μl of ethanol 96% (Merck), incubated at 45°C for 1 hr and centrifuged for 10 min at 21,000 g. Finally, the STX concentration of the supernatants was determined by measuring their optical density at 460 nm. The STX extractions were repeated at least three

### 4.7 | Generation of Csp structure models

The 3D models of CspA and CspC were generated via templates and ab initio-based processes through the Phyre2 server (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015) using S. aureus CspA and CspC amino acid sequences. Ribbon representations of CspA and CspC models were generated with Pymol (Schrodinger, LLC, 2015).

### 4.8 | PIA-PNAG detection

PIA-PNAG production was quantified as previously described with slight modifications (Toledo-Arana et al., 2005). Two milliliters of overnight cultures in TSB supplemented with 0.25% of glucose were centrifuged. In order to obtain similar bacterial density, the pellets were washed and resuspended in a proportional quantity of 0.5 M EDTA (pH 8.0). Subsequently, 50 μl of the bacterial suspensions were boiled for 5 min and centrifuged. Supernatants (40 μl) were incubated with 10 μl of proteinase K (20 mg/ml; Sigma) for 30 min at 37°C. Next, 10 μl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue were added to each tube. Serial dilutions were performed and spotted on a nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). Membranes were blocked overnight with 5% skim milk in PBS 0.1% Tween 20. After three washes of 15 min with the same buffer, membranes were incubated for 2 hr with an anti-PNA antibody (1:25,000). After incubation, membranes were washed three times with PBS 0.1% Tween 20 and incubated with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (1:5,000). Finally, membranes were developed using the Amersham ECL Western blotting kit and a Chemi-Doc apparatus (Bio-Rad).

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### CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

### AUTHOR CONTRIBUTIONS

A. Catalan-Moreno, C. J. Caballero, N. Irurzun, S. Cuesta performed experiments. J. López-Sagaseta performed structure models. A. Catalan-Moreno, C. J. Caballero, J. López-Sagaseta and A. Toledo-Arana analyzed and interpreted the data. A. Toledo-Arana conceived, designed and supervised the research. C. J. Caballero and A. Toledo-Arana wrote the manuscript. All authors read, commented and approved the final version of the article.
DATA AVAILABILITY STATEMENT
The strains and plasmids used in this study, which are listed in Tables S1 and S2, respectively, are available from the corresponding author upon reasonable request.

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REFERENCES
Amir, M., Kumar, V., Dohare, R., Islam, A., Ahmad, F., & Hassan, M. I. (2018). Sequence, structure and evolutionary analysis of cold shock domain proteins, a member of OB fold family. Journal of Evolutionary Biology, 31, 1903–1917. https://doi.org/10.1111/jeb.13382
Arnaud, M., Chastanet, A., & Debarbouille, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Applied and Environmental Microbiology, 70, 6887–6891. https://doi.org/10.1128/AEM.70.11.6887-6891.2004
Caballeró, C. J., Menéndez-Gil, P., Catalan-Moreno, A., Vergara-Irigaray, M., García, B., Segura, V., ... Toledo-Arana, A. (2018). The regulon of the RNA chaperone CspA and its auto-regulation in Staphylococcus aureus. Nucleic Acids Research, 46, 1345–1361. https://doi.org/10.1093/nar/gkx1284
Charpentier, E., Anton, A. I., Barry, P., Alfonso, B., Fang, Y., & Novick, R. P. (2004). Novel cassette-based shuttle vector system for Gram-positive bacteria. Applied and Environmental Microbiology, 70, 6076–6085. https://doi.org/10.1128/AEM.70.10.6076-6085.2004
Conrad, B., & Antonarakis, S. E. (2007). Gene duplication: A drive for phenotypic diversity and cause of human disease. Annual Review of Genomics and Human Genetics, 8(1), 17–35. https://doi.org/10.1146/annurev.genom.8.021307.110233
Dabrzyhynetska, A., Brendler, T., Ji, X., & Austin, S. (2009). Switching protein-DNA recognition specificity by single-amino-acid substitutions in the P1 par family of plasmid partition elements. Journal of Bacteriology, 191, 1126–1131. https://doi.org/10.1128/JB.01358-08
Donegan, N. P., Manna, A. C., Tseng, C. W., Liu, G. Y., & Cheung, A. L. (2009). Switching protein-DNA recognition specificity by single-amino-acid substitution in two different Staphylococcus aureus strains. Molecular and General Genetics, 261, 558–566. https://doi.org/10.1007/s004380051001
Gevers, D., Vandepoele, K., Simillion, C., & Van de Peer, Y. (2004). Gene duplication and biased functional retention of paralogs in bacterial genomes. Trends in Microbiology, 12, 148–154. https://doi.org/10.1016/j.tim.2004.02.007
Giachino, P., Engelmann, S., & Bischoff, M. (2001). α-Specificity activity depends on RsbU in Staphylococcus aureus. Journal of Bacteriology, 183, 1843–1852. https://doi.org/10.1128/JB.183.6.1843-1852.2001
Giuliodori, A. M., Di Pietro, F., Marzi, S., Masquida, B., Wagner, R., Romby, P., ... Pon, C. L. (2010). The cspA mRNA is a thermosensor that modulates translation of the cold-shock protein CspA. Molecular Cell, 37, 21–33. https://doi.org/10.1016/j.molcel.2009.11.033
Graumann, P. L., & Marahiel, M. A. (1998). A superfamily of proteins that contain the cold-shock domain. Trends in Biochemical Sciences, 23, 286–290. https://doi.org/10.1016/S0968-0004(01)01255-9
Herschlag, D. (1995). RNA chaperones and the RNA folding problem. Journal of Biological Chemistry, 270, 20871–20874. https://doi.org/10.1074/jbc.270.36.20871
Hillier, B. J., Rodriguez, H. M., & Gregoret, L. M. (1998). Coupling protein stability and protein function in Escherichia coli CspA. Folding and Design, 3, 87–93. https://doi.org/10.1016/S1359-0278(98)00014-5
Hudson, W. H., Kossmann, B. R., de Vera, I. M. S., Chuo, S.-W., Weikum, E. R., Eick, G. N., ... Ortlund, E. A. (2016). Distal substitutions drive divergent DNA specificity among paralogous transcription factors through subdivision of conformational space. Proceedings of the National Academy of Sciences of the United States of America, 113, 326–331. https://doi.org/10.1073/pnas.1518690113
Katzf, S., Lee, E. H., Law, A. B., Tseng, Y. L., & Shafer, W. M. (2005). CspA regulates pigment production in Staphylococcus aureus through a SigB-dependent mechanism. Journal of Bacteriology, 187, 8181–8184. https://doi.org/10.1128/JB.187.23.8181-8184.2005
Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. Nature Protocols, 10, 845–858. https://doi.org/10.1038/nprot.2015.053
Kenan, D. J., Query, C. C., & Keene, J. D. (1991). RNA recognition: Towards identifying determinants of specificity. Trends in Biochemical Sciences, 16, 214–220. https://doi.org/10.1016/0968-0004(91)90088-D
Koch, G., Yepes, A., Förstner, K. U., Wermser, C., Stengel, S. T., Modamio, J., ... Lopez, D. (2014). Evolution of resistance to a last-resort antibiotic in Staphylococcus aureus via bacterial competition. Cell, 158, 1060–1071. https://doi.org/10.1016/j.cell.2014.06.046
Kumar, S., Stecher, G., Suleski, M., & Hedges, S. B. (2017). TimeTree: A resource for timelines, timetrees, and divergence times. Molecular Biology and Evolution, 34, 1812–1819. https://doi.org/10.1093/molbev/msx116
Lakhundi, S., & Zhang, K. (2018). Methicillin-resistant Staphylococcus aureus: Molecular characterization, evolution, and epidemiology. Clinical Microbiology Reviews, 31, e00020–e118. https://doi.org/10.1128/CMR.00020-18
Lan, L., Cheng, A., Dunman, P. M., Missiakas, D., & He, C. (2010). Golden pigment production and virulence gene expression are affected by metabolisms in Staphylococcus aureus. Journal of Bacteriology, 192, 3068–3077. https://doi.org/10.1128/JB.00928-09
Lasa, I., Toledo-Arana, A., Dobin, A., Villanueva, M., de los Mozos, I. R., Vergara-Irigaray, M., ... Gingeras, T. R. (2011). Genome-wide antisense transcription drives mRNA processing in bacteria. Proceedings of the National Academy of Sciences of the United States of America, 108, 20172–20177. https://doi.org/10.1073/pnas.1113521108

target gene transcription and polymyxin B susceptibility. Journal of Bacteriology, 200, e00050-18. https://doi.org/10.1128/JB.00050-18
Gertz, S., Engelmann, S., Schmid, R., Ohlsen, K., Hacker, J., & Hecker, M. (1999). Regulation of the α-specificity transcription of sigB and asp23 in two different Staphylococcus aureus strains. Molecular and General Genetics, 261, 558–566. https://doi.org/10.1007/s004380051001
CATALAN-MORENO ET AL.

Lee, J., Jeong, K.-W., Jin, B., Ryu, K.-S., Kim, E.-H., Ahn, J.-H., & Kim, Y. (2013). Structural and dynamic features of cold-shock proteins of Listeria monocytogenes, a psychrophilic bacterium. Biochemistry, 52, 2492–2504. https://doi.org/10.1021/bi301641b

Liu, C.-I., Liu, G. Y., Song, Y., Yin, F., Hensler, M. E., Jeng, W.-Y., ... Oldfield, E. (2008). A cholesterol biosynthesis inhibitor blocks Staphylococcus aureus virulence. Science, 319, 1391–1394. https://doi.org/10.1126/science.1153018

Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., ... Nizet, V. (2005). Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. Journal of Experimental Medicine, 202, 209–215. https://doi.org/10.1084/jem.20050846

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., ... Heinemann, U. (2007). The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Research, 47, W636–W641. https://doi.org/10.1093/nar/gkz268

Max, K. E. A., Zeeb, M., Bienert, R., Balbach, J., & Heinemann, U. (2006). T-rich DNA single strands bind to a preformed site on the bacterial cold shock protein Bs-CspB. Journal of Molecular Biology, 360, 702–714. https://doi.org/10.1016/j.jmb.2006.05.044

Max, K. E. A., Zeeb, M., Bienert, R., Balbach, J., & Heinemann, U. (2007). Common mode of DNA binding to cold shock domains. Crystal structure of hexathymidine bound to the domain-swapped form of a major cold shock protein from Bacillus caldotryicus. FEBS Letters, 571, 1265–1279. https://doi.org/10.1111/j.1742-4658.2007.05672.x

Michaux, C., Holmovist, E., Vasicek, E., Sharan, M., Barquist, L., Westermann, A. J., ... Vogel, J. (2017). RNA target profiles direct the discovery of virulence functions for the cold-shock proteins CspC and CspE. Proceedings of the National Academy of Sciences of the United States of America, 65, 201620772–201620848. https://doi.org/10.1073/pnas.1620772114

Mihalovich, M., Militti, C., Gabaldón, T., & Gebauer, F. (2010). Eukaryotic cold shock domain proteins: Highly versatile regulators of gene expression. BioEssays, 32, 109–118. https://doi.org/10.1002/bies.200901122

Moellerling, R. C. (2011). Discovering new antimicrobial agents. International Journal of Antimicrobial Agents, 37, 2–9. https://doi.org/10.1016/j.ijantimicag.2010.08.018

Neuhaus, K., Rapposch, S., Francis, K. P., & Scherer, S. (2000). Restart of exponential growth of cold-shocked Yersinia enterocolitica occurs after down-regulation of cspA1/A2 mRNA. Journal of Bacteriology, 182, 3285–3288. https://doi.org/10.1128/JB.182.11.3285-3288.2000

Newkirk, K., Feng, W., Jiang, W., Tejero, R., Emerson, S. D., Inouye, M., & Montelione, G. T. (1994). Solution NMR structure of the major cold shock protein (CspA) from Escherichia coli: Identification of a binding epitope for DNA. Proceedings of the National Academy of Sciences of the United States of America, 91, 5114–5118. https://doi.org/10.1073/pnas.91.11.5114

Phadtare, S., & Inouye, M. (1999). Sequence-selective interactions with RNA by CspB, CspC and CspE, members of the CspA family of Escherichia coli. Molecular Microbiology, 33, 1004–1014. https://doi.org/10.1111/j.1365-2958.1999.01541.x

Phadtare, S., Tyagi, S., Inouye, M., & Severinov, K. (2002). Three amino acids in Escherichia coli CspE surface-exposed aromatic patch are critical for nucleic acid melting activity leading to transcription antitermination and cold acclimation of cells. Journal of Biological Chemistry, 277, 46706–46711. https://doi.org/10.1074/jbc.M208118200

Rennella, E., Sára, T., Juen, M., Wunderlich, C., Imbert, L., Solomy, Z., ... Konrat, R. (2017). RNA binding and chaperone activity of the E. coli cold-shock protein CspA. Nucleic Acids Research, 45, 4255–4268. https://doi.org/10.1093/nar/gkx044

Sachs, R., Max, K. E. A., Heinemann, U., & Balbach, J. (2012). RNA single strands bind to a conserved surface of the major cold shock protein in crystals and solution. RNA, 18, 65–76. https://doi.org/10.1261/rna.02809212

Schindelin, H., Jiang, W., Inouye, M., & Heinemann, U. (1994). Crystal structure of CspA, the major cold shock protein of Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America, 91, 5119–5123. https://doi.org/10.1073/pnas.91.11.5119

Schröder, K., Graumann, P., Schnuchel, A., Holak, T. A., & Marahiel, M. A. (1995). Mutational analysis of the putative nucleic acid-binding surface of the cold-shock domain, CspB, revealed an essential role of aromatic and basic residues in binding of single-stranded DNA containing the Y-box motif. Molecular Microbiology, 16, 699–708. https://doi.org/10.1111/j.1365-2958.1995.tb02431.x

Schrodinger, LLC. (2015). The PyMOL molecular graphics system, Version 1.8. Retrieved from https://pymol.org/

Shi, Y., Mowery, R. A., Ashley, J., Hentz, M., Ramirez, A. J., Bilgicer, B., ... Shaw, B. F. (2012). Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. Protein Science, 21, 1197–1209. https://doi.org/10.1002/pro.2107

Toledo-Aranas, A., Merino, N., Vergara-Irigaray, M., Débarbouillé, M., Penadés, J. R., & Lasa, I. (2005). Staphylococcus aureus develops an alternative, ica-independent biofilm in the absence of the arlRS two-component system. Journal of Bacteriology, 187, 5318–5329. https://doi.org/10.1128/JB.187.15.5318-5329.2005

Treisman, J., Gönçzy, P., Vashishtia, M., Harris, E., & Desplan, C. (1989). A single amino acid can determine the DNA binding specificity of homeodomain proteins. Cell, 59, 553–562. https://doi.org/10.1016/0092-8674(89)90038-X

Ünal, C. M., & Steinert, M. (2014). Microbial Peptidyl-Prolyl cis/trans Isomerases (PPIases): Virulence factors and potential alternative drug targets. Microbiology and Molecular Biology Reviews, 78, 544–571. https://doi.org/10.1128/MMBR.00015-14

Valle, J., Echeverz, M., and Lasa, I. (2019) α6 inhibits poly-N-acetylglucosamine exopolysaccharide synthesis and biofilm formation in Staphylococcus aureus. Journal of Bacteriology, 201(11), e00098-19. https://doi.org/10.1128/JB.00098-19

Valle, J., Toledo-Aranas, A., Berasain, C., Ghio, J.-M., Amorena, B., Penadés, J. R., & Lasa, I. (2003). SarA and not α6 is essential for biofilm development by Staphylococcus aureus. Molecular Microbiology, 48, 1075–1087. https://doi.org/10.1111/j.1365-2958.2003.03493.x

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., ... Stevens, R. L. (2017). Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. Nucleic Acids Research, 45, D535–D542. https://doi.org/10.1093/nar/gkw1017

Wiomial, R. E., Cech, S. M., Meyer, N. M., Burke, C. A., Weiss, A., ... Nizet, V. (2005). The EMBL-EBI search and sequence analysis tools API in 2019. Nucleic Acids Research, 47, W636–W641. https://doi.org/10.1093/nar/gkz268
Zeeb, M., Max, K. E. A., Weininger, U., Löw, C., Sticht, H., & Balbach, J. (2006). Recognition of T-rich single-stranded DNA by the cold shock protein Bs-CspB in solution. *Nucleic Acids Research*, 34, 4561–4571. https://doi.org/10.1093/nar/gkl376

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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