Cold-shock RNA-binding protein CspR is also exposed to the surface of Enterococcus faecalis

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CspR has been characterized recently as a cold-shock RNA-binding protein in Enterococcus faecalis, a natural member of the gastro-intestinal tract capable of switching from a commensal relationship with the host to an important nosocomial pathogen. In addition to its involvement in the cold-shock response, CspR also plays a role in the long-term survival and virulence of E. faecalis. In the present study, we demonstrated that anti-CspR immune rabbit serum protected larvae of Galleria mellonella against a lethal challenge of the WT strain. These results suggested that CspR might have a surface location. This hypothesis was verified by Western blot that showed detection of CspR in the total as well as in the surface protein fraction. In addition, identification of surface polypeptides by proteolytic shaving of intact bacterial cells followed by liquid chromatography-MS-MS revealed that cold-shock proteins (EF1367, EF2939 and CspR) were present on the cell surface. Lastly, anti-CspR immune rabbit serum was used for immunolabelling and detected with colloidal gold-labelled goat anti-rabbit IgG in order to determine the immunolocalization of CspR on E. faecalis WT strain. Electron microscopy images confirmed that the cold-shock protein RNA-binding protein CspR was present in both cytoplasmic and surface parts of the cell. These data strongly suggest that CspR, in addition to being located intracellularly, is also present in the extracellular protein fraction of the cells and has important functions in the infection process of Galleria larvae.

INTRODUCTION

Enterococcus faecalis, a natural member of the intestinal tract, is versatile and can switch from a commensal relationship with the host to a leading cause of nosocomial infections (Murray, 1990; Sreeja et al., 2012; Wisplinghoff et al., 2004). This bacterium, well known to be able to cope with hostile environments (Ogier & Serror, 2008) and used as an indicator of faecal contamination, is responsible for serious infections such as endocarditis or surgical wound infection, especially in immunocompromised patients (Gilmore et al., 2002; Shepard & Gilmore, 2002). Therefore, the virulence of E. faecalis has been intensively studied over the past 20 years and ~12 putative virulence genes have been reported, including several transcriptional and post-transcriptional regulators. The recently identified cold-shock RNA-binding protein CspR is part of this last category of virulence-associated factors (Fox et al., 2009; Hancock & Gilmore, 2002; Lebreton et al., 2009; Michaux et al., 2011, 2012; Qin et al., 2001; Shankar et al., 2001). In some Gram-positive bacteria such as Staphylococcus aureus and Bacillus subtilis, cold-shock polypeptides have been shown to be involved in several aspects of bacterial life, i.e. survival under starvation and low-temperature conditions or resistance to anti-microbial agents (Duval et al., 2010; Graumann & Marahiel, 1999, 1996; Graumann et al., 1997). In addition, the cold-shock polypeptides usually present the conserved RNA-binding motifs RNP-1 and RNP-2 that enable them to act as RNA chaperones (Phadtare & Inouye, 1999; Schröder et al., 1995). Thus, these proteins are key actors in the post-transcriptional...
regulation of bacteria (Ermolenko & Makhatadze, 2002; Graumann & Marahiel, 1998; Gualerzi et al., 2003; Horn et al., 2007). Among the six putative cold-shock proteins found in the genome of *E. faecalis* V583 strain, one, CspR, has been identified by coprecipitation with RNA (Michaux et al., 2012). Analysis performed with the ΔcspR mutant showed that it is impaired in its growth under cold conditions and in its long-term survival under nutrient starvation. Moreover, the ΔcspR mutant demonstrated attenuated virulence in an insect (*Galleria mellonella*) infection model as well as reduced persistence in mouse kidney and in peritoneal macrophages (Michaux et al., 2012). This study by Michaux et al. (2012) was the first report on the implication of a cold-shock protein in the virulence of Gram-positive bacteria. However, it has been shown that other RNA-binding proteins, e.g. the well-documented Hfq, have a role in the regulation of the expression of genes involved in virulence (Brennan & Link, 2007; Chao & Vogel, 2010; Christiansen et al., 2004; Vogel & Luisi, 2011). Of note, enterococci and streptococci do not harbour *hfq*-like genes (Sun et al., 2002).

The aim of the present study was to further investigate the role of CspR in the virulence of *E. faecalis*. Interestingly, we observed that the addition of anti-CspR immune rabbit serum protected larvae of *G. mellonella* against killing by *E. faecalis*. Using different approaches, we also demonstrated a dual localization of this cold-shock RNA-binding protein, both intracellularly and on the surface of the bacteria. Taken together, these data revealed possible new functions of this protein.

**METHODS**

**Bacterial strains, growth conditions and survival experiments.** Bacterial strains used in this study are listed in Table 1. The ΔcspR mutant was constructed from the parental *E. faecalis* strain EryS, an erythromycin-sensitive-cured derivative of the vancomycin-resistant clinical isolate V583 (Rigottier-Gois et al., 2011). *E. faecalis* EryS and its derivatives were grown without shaking at 37 °C in M17 medium supplemented with 0.5% glucose (GM17).

**G. mellonella infection experiments.** Infection of *G. mellonella* larvae with *E. faecalis* was accomplished as previously described by Lebreton et al. (2009). Briefly, using a syringe pump (KD Scientific), larvae (about 0.3 g and 3 cm long) were infected subcutaneously with washed *E. faecalis* WT, ΔcspR and corresponding complemented strains from an overnight culture in GM17, with $6 \times 10^6 \pm 0.6 \times 10^6$ c.f.u. per larvae administered in 10 µl of sterile saline buffer. In each test, 15 insects were infected and the experiments were repeated at least five times. Larval killing was then monitored at 18 h post-infection. For determination of putative anti-CspR polyclonal antibody and CspR protein effects, equivalences of 0.0025, 0.0125 and 0.025 µl anti-CspR serum with or without the purified CspR protein at a concentration of 10 µg ml⁻¹ per larvae were mixed within the different bacterial cells preparations, and then injected in the caterpillars. As a control, identical experiments were carried out with a rabbit non-immune serum and with the anti-lipopolysaccharide EF0685 polyclonal antibody obtained under the same conditions as the polyclonal antibody anti-CspR and kindly provided by Fany Reffuveille (Reffuveille et al., 2012). According to the protocol published by Joyce & Gahan (2010), we also performed the same experiments using the Gram-positive food-borne human pathogen *Listeria monocytogenes*. The variability of the results is expressed by the mean values for all biological replicates ($n=5$ ± SE and the different effects were subjected to statistical analysis using Microsoft® Excel 2010/unpaired Student's *t*-tests provided by the Jussieu University software (http://www.physics.csbsju.edu/stats/t-test_NROW_.html).

**Protein extractions.** Cells from *E. faecalis* strain were harvested from exponential growth phase in GM17 medium (OD₅₆₀=0.5) for the extraction of surface-exposed, secreted and total cytoplasmic proteins.

Surface-exposed proteins were extracted by exposure of cells to high pH using a protocol described by Hempel et al. (2011). Briefly, a cell pellet from a 50 ml culture was washed with a PBS sucrose solution [NaCl 100 mM, sucrose 60 mM, sodium phosphate 55 mM (pH 7.2)] and then shaken gently for 1 h at room temperature in 2 ml NaOH glycine sucrose [glycine 50 mM, sucrose 60 mM (pH 12.4)]. After centrifugation (30 min, 10,000 g), 108 µl 1 M HCl and 100 µl 1 M Tris/HCl (pH 7.0) were added to 1 ml supernatant. Proteins were precipitated at 4 °C by addition of 8 ml cold acetone. The protein pellet obtained after centrifugation (10 min, 10,000 g) was resuspended in 20 µl Tris/HCl (pH 7.5).

For extraction of the secreted proteins, as described by Becher et al. (2009), the supernatant of 50 ml culture volume was separated from cells by centrifugation (10 min, 7000 g, 4 °C) and transferred into a new tube. Then, 5 ml fresh pure TCA (final concentration 10% (v/v)) was added to the supernatant and the proteins were precipitated overnight at 4 °C. The precipitate was centrifuged for 1 h, and the supernatant was decanted very carefully and washed with 20 ml

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant properties</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>Strain V583 (clinical isolate), without erythromycin resistance</td>
<td>Rigottier-Gois et al. (2011)</td>
</tr>
<tr>
<td>EryS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔcspR</td>
<td>EryS isogenic derivative CspR mutant</td>
<td>Michaux et al. (2012)</td>
</tr>
<tr>
<td>ΔcspRC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Strain ΔcspR mutant derivative complemented</td>
<td>Michaux et al. (2012)</td>
</tr>
<tr>
<td>CLIP 21369</td>
<td>Clinical isolate with fluoroquinolone resistance</td>
<td>Godreuil et al. (2003)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M15pRep4</td>
<td>M15 (<em>recA</em>⁺ <em>uvrA</em>+ <em>F</em> <em>mtl</em> <em>gal</em> <em>ara</em> <em>lac</em>) harbouring pRep4 plasmid</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M15/pQE30cspR</td>
<td>Strain harbouring pQE30cspR</td>
<td>Michaux et al. (2012)</td>
</tr>
</tbody>
</table>
100 % (v/v) ice-cold ethanol, centrifuged for 10 min (8000 × g, 4 °C) and decanted. The washing procedure was repeated twice. Afterwards the pellet was removed from the tube, mixed with 10 ml 100 % ice-cold ethanol, transferred to a 15 ml Falcon tube and centrifuged for 10 min (8000 g, 4 °C). The supernatant was decanted very carefully. This washing procedure was repeated at least six times and the last step was done with 70 % (v/v) ice-cold ethanol. Then, the supernatant containing the proteins was dried at room temperature. The protein pellet was dissolved in 8 M urea/2 M thiourea and mixed by shaking for 20 min at room temperature. The solution was finally centrifuged for 15 min at 21 000 g and the supernatant was transferred into a new microtube.

To obtain the total cytoplasmic proteins extracts as described by Becher et al. (2009), 50 ml of culture was centrifuged (7000 g) for 10 min at 4 °C. Cell pellets were washed twice with 1 ml ice-cold TE buffer after resuspension in 1 ml TE buffer and transferred into screw-top tubes containing 500 µl glass beads. Cells were disrupted using a Fast Prep instrument (MP Biomedical) for 30 s at 6.5 m s⁻¹. The lysate was centrifuged for 25 min at 21 000 g at 4 °C to remove the cell debris, and the supernatant was transferred into a new tube and centrifuged for 45 min at 21 000 g at 4 °C in order to remove insoluble and aggregated proteins.

**Western blotting.** After electrophoresis, the proteins were transferred into a PVDF membrane that was then stained with Coomassie blue in order to verify that equal amounts of protein were present in all lanes. Blocking, incubation with the polyclonal antibody against CspR (Michaux et al., 2012) and enhanced chemiluminescence detection (GE Healthcare) were carried out as described previously by Riboulet-Bisson et al. (2008). As a control we also used two polyclonal antibodies: the first reacting with the transcriptional regulator Ers and the second reacting against the lipoprotein EF0685, kindly provided by Eliette Riboulet-Bisson (Riboulet-Bisson et al., 2008) and Fany Reffuveille (Reffuveille et al., 2012), respectively.

**Identification of surface proteins by ‘surface shaving’ experiments.** Extractions were performed as described by Tjalsma et al. (2008). Briefly, two aliquots of 2 ml of each bacterial cultures of the EryS and ΔcspR mutant strains were harvested at OD₅₆₀=0.3 by centrifugation (11 000 r.p.m., 2 min) and washed twice with 400 µl Bicam (tryptihylammonium bicarbonate buffer) 100 mM (Sigma-Aldrich) (pH 8). The first aliquots containing EryS or ΔcspR mutant cells were then mixed with 75 µl of trypsin-agarose (10 U; Thermo Scientific) prewashed with 500 µl Bicam. The other aliquots were resuspended in Bicam without any trypsin. All the samples were incubated for 1 h at 37 °C under gentle shaking. After centrifugation (7500 r.p.m., 5 min), the cell pellets were removed and the supernatants were treated with 1 mM DTT for 30 min, followed by 1 mM iodoacetamide, also for 30 min at room temperature. Fresh trypsin (0.5 µg; Thermo Scientific) was added and trypsin digestion was continued for 18 h at 37 °C. Two sets of samples were then obtained: protein extractions from EryS and ΔcspR mutant cells treated with trypsin-bead resin and trypsin overnight. Protein extractions from EryS and ΔcspR mutant cells treated only with Bicam and digested with trypsin overnight were considered as the ‘controls’ of the procedure.

**MS analyses.** MS analyses were performed after the overnight trypsin digestion of protein samples obtained by shaving extraction. Trypsin-cleaved samples were desalted and concentrated on a tip microC18 Omix (Varian) before nano-liquid chromatography (LC)-MS-MS analysis. The chromatography step was performed using a Prominence nano-LC system (Shimadzu).

**Immunolabelling and electron microscopy protocol.** The 100 nm ultrathin sections from blocks of EryS and ΔcspR mutant strains from exponential growth (OD₅₆₀=0.5) were prepared according to Spühner & Calvairial (2008). Briefly, the cells were fixed with 2.5 % paraformaldehyde and 0.5 % glutaraldehyde in Sorensen’s phosphate buffer 0.1 M (pH 7.4) at 4 °C for 30 min and rinsed three times in Sorensen’s buffer. The samples were then embedded in 12 % gelatin. The obtained pellets were cryosubstituted in progressive baths of ethanol (30–100 %) and in resin K4M, and finally polymerized 48 h at −30 °C under UV light (FS8500; RMC). Ultrathin sections were prepared using a RMC powertome and picked up on nickel-embedded grids (Spühner & Calvairial, 2008). For the whole-cell observations, 10 µl samples of bacterial suspensions were applied to carbon-coated Formvar copper grids and negatively stained with 1 % (w/v) phosphotungstic acid (pH 7.2) for 10 s. For the immunolabelling experiments as described by Webster et al. (2008) and according to the manufacturer’s instructions (Aurion), the grids were washed three times in PBS buffer (pH 7.2). The non-specific binding sites were masked by floating the grids section-side down on a first drop of PBS 0.5 % BSA-G for 30 min and on a second drop of PBS 0.5 % cold water fish skin gelatin (Aurion) for 30 min. The primary polyclonal antibody (anti-CspR antibody) was diluted under the same conditions as used in the G. mellonella experiments (0.025 µl per 10 µl of PBS). Grids containing EryS strain or ΔcspR mutant strain were transferred on the diluted anti-CspR antibody drop for 1 h of incubation at 4 °C. A similar experiment was also performed with diluted non-immune rabbit serum as a control. The grids were then washed six times for 5 min each with PBS drops and incubated at room temperature for 1 h with 15 nm colloidal gold-labelled goat anti-rabbit IgG (Aurion) diluted 1/40 in blocking buffer and rinsed six times for 5 min with PBS drops. A 1% glutaraldehyde fixation was performed on each grid for 5 min followed by six washes of 5 min with distilled water and the sections were contrasted with uranyl acetate for 5 min.

The cryosections and whole cells were observed with a JEOL 1011 transmission electron microscope at the Electron Microscopy Center of the University of Caen Basse-Normandie.

**RESULTS**

**Anti-CspR antibody protects against E. faecalis infection**

As previously observed by Michaux et al. (2012), larvae of G. mellonella infected with the ΔcspR mutant strain survived much better than those infected with the E. faecalis WT strain EryS (Fig. 1). After 18 h of infection, <9% of the EryS-infected larvae were still alive, whereas >76% of the animals infected with the ΔcspR strain survived (P<0.001). Thus, we used different concentrations of polyclonal antibody against CspR (anti-CspR) to test whether it could protect larvae against E. faecalis infection. Interestingly, a graduated titration of the antibody was observed. At 18 h post-infection, 37.3% and 49 % of the infected larvae survived when 0.0025 and 0.0125 µl anti-CspR per larvae were added, respectively. As shown in Fig. 1, WT bacteria appeared less virulent in the larvae model when 0.025 µl anti-CspR antibody was added (P<0.001). This addition of CspR antibody to WT cells led to a phenotype similar to that of the ΔcspR mutant: 70 % of the infected larvae survived (Fig. 1). In control experiments, the WT strain was treated with a polyclonal antibody raised against the EF0685 lipoprotein, a peptidyl-prolyl isomerase anchored to the...
membrane and predisposed in the folding of secreted proteins, also involved in the virulence of *E. faecalis* in the *G. mellonella* model (Reffuveille *et al.*, 2012). However, addition of anti-EF0685 did not significantly modify the virulence of the WT strain (Fig. 1). To prove that the protective effect of anti-CspR antibody was due to its specific interaction with CspR, we mixed anti-CspR and purified CspR in the WT culture. Virulence of *E. faecalis* was conserved under these conditions, likely due to the precipitation of the antibody by the recombinant CspR protein (Fig. 1).

As a control, we also performed injections of *G. mellonella* with the *E. faecalis* WT or ΔcspR strains in the presence of non-immune rabbit serum. No significant difference was observed compared with the previous data obtained for the larvae infected with the strains only (data not shown).

Assays conducted with the complemented ΔcspR mutant strain showed that the virulence was restored to the WT level (Michaux *et al.*, 2012) (Fig. 1), confirming that the observed phenotype for the ΔcspR mutant was due to the lack of CspR. To answer the question whether CspR may have toxic activity, larvae were infected with suspensions of the WT or ΔcspR mutant strains containing purified CspR protein and with recombinant purified CsrR protein only. No difference in terms of virulence in our animal model was observed, arguing for the absence of any intrinsic toxicity of the CspR protein (data not shown). Lastly, using *L. monocytogenes*, survival of the larvae infected only with the WT and those infected with *L. monocytogenes* mixed with the anti-CspR serum was similar (Fig. 1).

**Western blot analysis**

As anti-CspR was able to protect larvae of *G. mellonella* against infection by *E. faecalis*, we speculated that, despite a lack of leader peptide or putative transmembrane domain, CspR might also have a surface location. This hypothesis was also supported by the presence of CspR (EF2925) among the surface proteins identified by Bøhle *et al.* (2011). Thus, detection of CspR on the total cytoplasmic, secreted and surface protein fractions by Western immunoblotting was undertaken. Using a polyclonal antibody against CspR, we tested the presence of the protein on the three protein extractions (Fig. 2a). A cross-reactive band of 7.3 kDa corresponding to the molecular mass of CspR was found.
detected in the total (Fig. 2a, lane 2) and the surface (Fig. 2a, lane 4) protein fractions. As a control and to exclude possible contamination with cytoplasmic proteins, we tested for the presence of the transcriptional regulator Ers (expected to be an intracellular protein) (Riboulet-Bisson et al., 2008) and the lipoprotein EF0685 (expected to be mainly present in the surface protein fraction) (Reffuveille et al., 2012) on the three same samples as used for the localization of CspR. As expected, with the anti-Ers serum, a cross-reactive band (~25 kDa) was observed only with the total cytoplasmic protein extraction (Fig. 2b, lane 2). This suggests a negligible contamination of the surface fraction with intracellular proteins (Fig. 2b). Using anti-EF0685 serum, a signal of ~37 kDa was observed with the total and cytoplasmic protein extract (Fig. 2c, lane 2) and surface (Fig. 2c, lane 4) protein extractions as well as a faint band in the secreted protein extraction (Fig. 2c, lane 3). Hence, these combined results of Western blot experiments strongly suggest that CspR has both an intracellular and surface location in E. faecalis.

Proteins identified by ‘shaving’ the bacterial surface

The aim of this experiment was to identify surface proteins by LC-MS-MS after proteolytic shaving of intact bacterial cells. To do this, E. faecalis cells were incubated with trypsin beads releasing peptides from surface proteins. As a control, the same experiments were performed with cells incubated without trypsin beads. Proteins identified in the ‘shaved fraction’ by at least two peptides in two independent experiments and not present in the control fraction were considered as present on the surface. This approach led to the identification of 10 proteins: six present only in the WT, three only in the ΔcspR mutant and one in both extractions (Table 2). In addition to CspR (logically not present in the mutant), two other cold-shock proteins were detected: EF1367 identified in the WT and EF2939 observed in the ΔcspR mutant (Table 2). Of note, the only two polypeptides harbouring putative transmembrane domains were EFO394, corresponding to a secreted antigen (common in the shaving fractions of both strains), and EF0443, a LysM domain protein (absent in the mutant strain).

Immunolocalization of CspR

The results obtained with the Western blot experiments and the protein shaving assays argued strongly for the presence of the CspR protein not only in the intracellular compartment, but also on the cell surface. To confirm, we used the polyclonal antibodies raised against the CspR protein to perform immunolocalization by electron microscopy. As can be deduced by analysing the different views of cryosections of the WT strain, the immunogold-labelled polyclonal antibodies were present inside the cells and also on the surface of the bacteria (Fig. 3a). As controls, similar experiments with cryosections of the WT strain incubated with non-immune serum (Fig. 3b) as well as the ΔcspR mutant strain incubated with the gold-labelled anti-CspR antibodies (Fig. 3c) were performed. As expected, in both cases, no labelling was observed (Fig. 3b, c).

Immunolabelling experiments with the anti-CspR antibodies using whole cells of E. faecalis WT strains were also performed. As shown in Fig. 4, CspR proteins were detected on the surface of the cells. In contrast, experiments carried out with the ΔcspR mutant strain and with the non-immune serum did not reveal any significant labelling (data not shown).

DISCUSSION

In order to cope with infection due to enterococci, it is necessary to characterize effectors that are involved in the virulence process and in the ability to survive in harsh environments. Cold-shock proteins are known to act as RNA chaperones and thus play a major role in the post-transcriptional regulation that enables cells to counteract the harmful effects of cold temperature (Phadtare & Inouye, 1999). In the opportunistic pathogen E. faecalis, one of the leading causes of nosocomial infection, we recently characterized CspR as a cold-shock RNA-binding protein involved in the low-temperature stress response, long-term survival as well as virulence in the G. mellonella model (Michaux et al., 2012). We showed in this work that the addition of anti-CspR immune rabbit serum to the WT culture protected the host. Moreover, the purified protein was able to precipitate anti-Csp antibodies, arguing for a specific action of the serum against CspR. However, the absence of any survival difference between the larvae infected with the L. monocytogenes strain and those infected with the same strain and the anti-CspR immune rabbit serum suggested an enterococcal specificity of the antibody. G. mellonella is now a well-developed model host for studying human pathogens because of the good accordance with mammalian virulence models (Junqueira, 2012). Of
note, *G. mellonella* has only an innate immune system composed of different types of haemocytes and the activity of the mammalian antibody used in the experiments should be the blocking reaction by cross-linking of its corresponding antigen. Our previous work and the present study revealed that cells lacking CspR (*ΔcspR* mutant) were more efficiently destroyed by the defence system of the host. It can be therefore suggested that CspR may not correspond to a recognition molecule for phagocytes but rather be involved in the defence against stresses encountered inside cells. This is also supported by the results obtained in the *in vivo*/*in vitro* macrophage infection model, where we showed that the phagocytosis of WT and *ΔcspR* mutant strains was similar and that survival of the mutant was significantly reduced inside the phagocytes (Michaux *et al.*, 2012). Thus, it may be speculated that the extracellular CspR could protect or act as a defence molecule against anti-microbial agents present in the insect’s haemolymph or in the phagocytic cells. Another hypothesis is that this cold-shock protein could play a role as a sensor of deleterious conditions at the cell surface.

Despite the absence of a putative transmembrane motif or leader peptide sequences in CspR, our results strongly suggested that it is located on the surface of the cell. Bôhle *et al.* (2011) identified 69 surface proteins of *E. faecalis*, including CspR (EF2925), which supported our findings.

### Table 2. Proteins identified by ‘trypsin shaving’ in WT and *ΔcspR* mutant strains of *E. faecalis*

<table>
<thead>
<tr>
<th>Name†</th>
<th>Mass (kDa)</th>
<th>Description</th>
<th>WT</th>
<th>ΔcspR mutant</th>
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<tr>
<td>EF0007 (RpsF)</td>
<td>11.6</td>
<td>Ribosomal protein S6</td>
<td>Yes</td>
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</tr>
<tr>
<td>EF0228</td>
<td>24.3</td>
<td>Adenylate kinase</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>EF0394</td>
<td>47.3</td>
<td>Secreted antigen, putative</td>
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<td>Yes</td>
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<tr>
<td>EF0443</td>
<td>21.4</td>
<td>LysM domain protein</td>
<td>Yes</td>
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<tr>
<td>EF1002</td>
<td>26.7</td>
<td>Putative cell division protein divIVA</td>
<td>No</td>
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<tr>
<td>EF1367</td>
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<td>Cold-shock domain family protein</td>
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<td>EF2925 (CspR)</td>
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<td>Cold-shock domain family protein</td>
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<tr>
<td>EF2939</td>
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<tr>
<td>EF3065 (RpsO)</td>
<td>12.2</td>
<td>Ribosomal protein S15</td>
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*Proteins were identified by at least two unique peptides in the trypsin-shaved fraction in two independent experiments.
†ORFs from the V583 sequence (http://cmr.jcvi.org).

### Fig. 3. Immunolocalization of CspR on ultrathin sections of *E. faecalis* WT strain (a, b) and *ΔcspR* mutant strain (c) by electron microscopy. Bacterial cells were successively incubated with the anti-CspR antibody (a, c) or non-immune serum (b) and 15 nm colloidal gold-conjugated goat anti-rabbit IgG. Black and white arrows indicate surface and cytoplasmic localization of CspR, respectively. Bars, 100 nm [a, b (right), c (right)]; 0.2 μm [b (left), c (left)].
As this was unexpected, we verified the extracellular location of CspR by different approaches such as Western blotting, identification of surface polypeptides by proteolytic shaving of intact bacterial cells and immunolocalization. Similar results were obtained for the virulence factor and general stress protein Gls24 of *E. faecalis*. Indeed, Gls24 was supposed, according to the literature, to be a cytoplasmic protein, but was also found to be present on the cell surface (Teng et al., 2005). Moreover, similar to our data, anti-Gls24 immune rabbit serum reduced the virulence of *E. faecalis* in a mouse peritonitis model (Teng et al., 2005). Interestingly, other cold-shock proteins (EF1367 and EF2939) harbouring the two RNP motifs corresponding to the highly conserved nucleic acid-binding motifs have been identified after proteolytic shaving of intact cells. EF1367 and CspR were detected in WT cells, and EF2939 was only observed in the ΔcspR mutant. This indicates that different cold-shock proteins also able to interact with nucleic acid may be present on the surface. Such types of protein have not been found in the reference map of the membrane proteome of *E. faecalis* (Maddalo et al., 2011). Nevertheless, in this study, only 102 unique proteins were identified, which may correspond to only ~10% of the membrane-embedded predicted proteome. Interestingly, among them, 29 were characterized as soluble proteins (Maddalo et al., 2011). Identification of a priori cytoplasmic proteins is not unusual and it is well known that some proteins lacking signal sequences or motifs important for their secretion, such as LuxS of *Salmonella* (Kint et al., 2009), SodA of *Listeria* (Archambaud et al., 2006) or enolase of *E. faecalis* (Boël et al., 2004), may have an extracellular location thanks to ‘non-classical secretion’ (Benachour et al., 2009; Bendtsen et al., 2005; Boëlle et al., 2011). For example, the ribosomal protein L7/L12 identified on the surface of *Helicobacter pylori* or *Brucella abortus* has immunogenic properties (Mallick et al., 2007; Voland et al., 2002). In the same manner, in *L. monocytogenes*, elongation factor EF-Tu, heat-shock protein DnaK and glyceraldehyde 3-phosphate dehydrogenase that are observed at the surface are able to bind human plasminogen, playing a putative adhesive role (Schaumburg et al., 2004). It is possible that CspR may be one of the ‘moonlighting’ protein group, meaning that it can have two unrelated functions according to its intra- or extracellular location. To date, for the prevention of *E. faecalis* infections, in addition to lipoteichoic acid and capsular polysaccharides, three proteins known as virulence factors have been proposed as possible immunotherapy targets: the general stress protein Gls24, the collagen adhesion Ace, and the endocarditis- and biofilm-associated pilus Ebp (Giard et al., 2000; Huebner et al., 2000; Lebreton et al., 2009; Murray, 1990; Singh & Murray, 2012; Singh et al., 2007; Teng et al., 2005). Our results showed that a cold-shock RNA-binding protein can present a double localization, both intercellularly and at the surface of the bacteria, and that anti-CspR antibody protects insect larvae against infection by *E. faecalis*. Alone or in association with the antigens mentioned above, it is conceivable to use CspR for the development of an immune strategy to prevent or fight *E. faecalis* infection.

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