The AdcACB/AdcAll system is essential for zinc homeostasis and an important contributor of Enterococcus faecalis virulence

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ABSTRACT

Bacterial pathogens require a variety of micronutrients for growth, including trace metals such as iron, manganese, and zinc (Zn). Despite their relative abundance in host environments, access to these metals is severely restricted during infection due to host-mediated defense mechanisms collectively known as nutritional immunity. Despite a growing appreciation of the importance of Zn in host-pathogen interactions, the mechanisms of Zn homeostasis and the significance of Zn to the pathophysiology of E. faecalis, a major pathogen of nosocomial and community-associated infections, have not been thoroughly investigated. Here, we show that E. faecalis encoded ABC-type transporter AdcACB and an orphan substrate-binding lipoprotein AdcAll that work cooperatively to maintain Zn homeostasis. Simultaneous inactivation of adcA and adcAll or the entire adcACB operon led to a significant reduction in intracellular Zn under Zn-restricted conditions and heightened sensitivity to Zn-chelating agents including human calprotectin, aberrant cell morphology, and impaired fitness in serum ex vivo. Additionally, inactivation of adcACB and adcAll significantly reduced bacterial tolerance toward cell envelope-targeting antibiotics. Finally, we showed that the AdcACB/AdcAll system contributes to E. faecalis virulence in a Galleria mellonella invertebrate infection model and in two catherin-associated mouse infection models that recapitulate many of the host conditions associated with enterococcal human infections. Collectively, this report reveals that high-affinity Zn import is important for the pathogenesis of E. faecalis establishing the surface-associated AdcA and AdcAll lipoproteins as potential therapeutic targets.

Introduction

Iron (Fe), manganese (Mn), and zinc (Zn) are essential trace metals to all forms of life. They serve structural, catalytic, and regulatory functions to metalloproteins involved in a variety of biological processes [1–4]. As a result of this essentiality, hosts deploy a variety of strategies to deprive access of invading pathogens to trace metals, an active process termed nutritional immunity [2,5–8]. To date, the best characterized nutritional immunity strategy is based on mobilization of metal-chelating proteins to the infection site by host immune cells [2]. Among them, calprotectin, a member of the S100 protein family produced by neutrophils and other types of immune cells that is secreted in large quantities during infection and inflammatory processes, is the main host protein responsible for Mn$^{2+}$ and Zn$^{2+}$ sequestration [9–11]. To overcome trace metal limitation, microbial pathogens evolved effective metal-scavenging systems that include expression of surface-associated high-affinity metal uptake systems and, in some bacterial species, synthesis and trafficking of organic extracellular molecules known as metallophones [8].

Although the importance of Fe in host-pathogen interactions has been extensively examined [6], the role of Mn and Zn in host-pathogen interactions and the mechanisms utilized by bacteria to maintain their cellular levels and ratios properly balanced are less understood [7,12–17]. The second most abundant trace metal in vertebrates, Zn, is estimated to be incorporated into approximately 5% of the bacterial proteome and plays structural and catalytic roles in multiple biological processes [18,19]. In bacteria, Zn acquisition under severe Zn-restricted conditions such as those that can be encountered in host environments depends on the activity of surface-associated Zn uptake systems from the ATP-binding cassette (ABC) transporter family (reviewed in Refs. 20–22). Moreover,
major human pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* produce Zn-binding metallophores, also known as zincophores [23].

To date, the contributions of Zn uptake systems to virulence have been demonstrated in a number of bacterial species, including several Gram-positive pathogens that are phylogenetically related to *Enterococcus faecalis*, the subject organism of the present study. In *S. aureus*, inactivation of either the ABC-type transporter AdcABC, the staphylophone (Stp) zincophore, or its cognate multi-metal transporter CntABCDF was sufficient to impair bacterial growth under Zn-restricted conditions in vitro [24]. Loss of both AdcABC and Stp/CntABCDF systems resulted in further growth impairment under Zn-restricted conditions and attenuated virulence in a mouse retro-orbital infection model [24]. In streptococci, which to date reportedly do not synthesize zincophores, Zn acquisition is mediated by the ABC-type transporter AdcABC. In addition to AdcABC, streptococcal species encode an additional *adcA* homologue, known as AdcAII (reviewed in [25]), coding for a second Zn-binding lipoprotein. In *Streptococcus pyogenes*, strains lacking *adcC*, *adcA*, or *adcAII* grew poorly in the presence of purified human calprotectin and displayed attenuated virulence in a necrotizing fasciitis mouse model [26] and in a humanized-plasminogen skin infection mouse model [27]. Moreover, *S. pyogenes* Δ*adc* strains retained wild-type strain levels of virulence in calprotectin-negative (*S100a9<sup>−/−</sup>*) mice [26], which validates the central role of calprotectin in host-mediated Zn sequestration and protection against bacterial infection. Similarly, virulence of *Streptococcus pneumoniae* Δ*adcA*Δ*adcAII* and *Streptococcus agalactiae* Δ*adcA*Δ*adcAII*Δ*imb* (Δ*imb* encodes for a 3<sup>rd</sup> Zn-binding lipoprotein) strains was significantly attenuated in mouse models of systemic and nasopharyngeal colonization [16,28]. Finally, in the oral pathogen *S. mutans*, one of the few streptococci that do not encode the orphan *adcAII* gene, inactivation of the *adcABC* system significantly impaired bacterial growth under Zn-restricted conditions and reduced bacterial colonization of the dental biofilm in a rat model [12,29].

A commensal of the gastrointestinal (GI) tract, *E. faecalis*, is also a prevalent opportunistic pathogen of localized and systemic infections, including but not limited to infective endocarditis, catheter-associated urinary tract infections (CAUTI), and wound infections [30–33]. A major virulence trait of *E. faecalis* is its remarkable capacity to adapt to adverse conditions in the GI tract (their natural host environment) and several other host tissues, and to survive exposure to hospital-grade disinfectants and antibiotic treatments [34,35]. Because very little is known about the mechanisms of Zn homeostasis in enterococci, we sought to characterize the Zn acquisition systems of *E. faecalis* in this study. Similar to streptococci, the core genome of *E. faecalis* encodes for a conserved AdcACB system (originally annotated as znuABC) and an orphan substrate-binding lipoprotein AdcA-II that is annotated as *adcA*. In this report, we isolated a panel of *E. faecalis* Δ*adc* strains, including strains lacking every *adc* gene (Δ*adcABCΔadcAII*) or both genes coding for the substrate-binding lipoproteins (Δ*adcAΔadcAII*), and then used these mutants to define the role of AdcACB and AdcAII in *E. faecalis* pathophysiology. Our results revealed that simultaneous inactivation of *adcA* and *adcAII* or of the entire *adcABC* operon yielded the most impactful phenotypes, which included severe growth/survival defects in the presence of calprotectin or in human serum, and attenuated virulence in both invertebrate and vertebrate infection models. We also discovered that the inability to maintain Zn homeostasis diminished the recognized high tolerance of *E. faecalis* to antibiotics that target the cell envelope. Collectively, this study reveals that AdcACB and AdcAII work cooperatively to maintain *E. faecalis* Zn homeostasis during infection such that the surface-associated AdcA and AdcAII lipoproteins can be considered potential targets for the development of antimicrobial interventions.

**Results**

*AdcACB and AdcAII work in concert to promote growth under Zn-restricted conditions*

Using the NCBI BLASTn tool, we identified the genes coding for the highly conserved ABC-type transporter AdcACB (*OG1RF_RS00260-RS00270*), the orphan substrate-binding AdcAII lipoprotein (*OG1RF_RS12625*) and the transcriptional repressor Zur (*OG1RF_RS09465*) in the *Enterococcus faecalis* OG1RF genome (GenBank: CP002621.1) (Figure 1a). The translated gene products of *OG1RF_RS00260* (AdcA; Accession ID: AEA92738.1, protein ID: WP_002367576.1) and *OG1RF_RS12625* (AdcAII; Accession ID: AEA95159.1, protein ID: WP_002392710.1) display respectively, 57% and 64% amino acid similarity to the *S. pneumoniae* AdcA and 42% and 39% similarity to *S. pneumoniae* AdcAII [16] (Figure S1). Pairwise alignment between *E. faecalis* AdcA and AdcAII also revealed 53% similarity, indicative of functional redundancy (Figure S1). AdcAII, the larger of the two Zn-binding lipoproteins of *E. faecalis*, contains a ZnT-like domain at the C-terminus that is also observed in the *S. pneumoniae* AdcA and was shown to mediate Zn binding via the so-called trap door
Figure 1. Growth characteristics of *E. faecalis* and its Zn-deficient mutants under Zn-restricted conditions. (a) Schematic of the gene locus of the Zn transport system in *E. faecalis* OG1RF core genome. Growth curves of *E. faecalis* wild type (WT) and its isogenic mutants in BHI (b), BHI supplemented with 100 μM ZnSO₄ (c), 10 μM TPEN (d), combination of TPEN and ZnSO₄ (e), TPEN and MnSO₄ (f) or TPEN and FeSO₄ (g). In (b) and (d-f), data points represent the average of nine biological replicates. Finally, the growth curve of *E. faecalis* wild type (WT) and genetically complemented Δadc mutants in BHI supplemented with 10 μM TPEN (h). In (c) and (g), data points represent the average of six biological replicates. Error bar represents the standard error of margin (SEM). Statistical analysis was performed using simple linear regression of the exponential growth phase, and slope of each mutant’s growth kinetics was compared with that of the parent strain.
mechanism [17,36–38]. By contrast, the S. pyogenes AdcA utilizes two domains for Zn binding although it is structurally more distinct from E. faecalis OG1RF AdcA/AdcAII with 36% and 34% similarity, respectively (Figure S1) [39]. Using AlphaFold and Chimera to predict protein structures, we found that S. pneumoniae R6 AdcA (NP_359566.1) and AdcAII (NP_358500.1) structurally overlap with E. faecalis AdcAII (WP_002392710.1) and AdcA (WP_002367576.1), respectively (Figure S2). Moreover, the hinge region identified for Zn binding in S. pneumoniae AdcA [38] and S. pyogenes AdcA [39] was present in both E. faecalis AdcA and AdcAII (Figure S2). In previous transcriptome-based studies conducted with E. faecalis strain V583, the adcABC (originally annotated as znuABC) and adcAII genes were shown to be repressed after exposure to high Zn levels and strongly induced after treatment with the Zn-chelating agent TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)-1,2-ethanediamine) [40,41]. Based on the presence of conserved domains and amino acid similarities with homologous systems of closely related streptococci, we renamed the OG1RF_RS00260-OG1RF_RS00270 gene cluster adcABC keeping the adcAII designation for the lone OG1RF_RS12625. Of note, none of the enterococcal genomes surveyed, including E. faecalis OG1RF, encode biosynthetic gene clusters and cognate transporters of opine-like zincophore systems that are found in a small number of bacterial pathogens [23].

To probe the role of AdcABC and AdcAII in Zn acquisition and, most importantly, determine the significance of these systems in E. faecalis pathophysiology, we used a markerless in-frame deletion strategy [42] to generate strains lacking one or both substrate-binding lipoproteins (ΔadcA, ΔadcAII, and ΔadcAΔadcAII) and the entire adcABC operon alone or in combination with adcAII (ΔadcACB and ΔadcACBΔadcAII). In addition, we isolated a strain lacking the transcriptional repressor Zur (Δzur). Next, we compared the ability of E. faecalis OG1RF (wild-type strain) and mutant derivatives to grow in BHI, a complex media that contains ~10 µM Zn [43], or in BHI supplemented with TPEN [44]. In BHI, inactivation of either adcA or adcAII alone did not impact growth kinetics or growth rates, whereas simultaneous inactivation of adcA and adcAII (ΔadcAΔadcAII) or the entire adcABC operon alone (ΔadcACB) or in combination with adcAII (ΔadcACBΔadcAII) resulted in slower growth rates without affecting final growth yields (Figure 1b). Inactivation of the zur regulator did not affect growth rates but led to a slight increase in the final growth yield in BHI (Figure 1b). BHI supplementation with 100 µM ZnSO₄ (10-fold in excess of labile Zn pools in BHI media) restored the growth defects of ΔadcAΔadcAII, ΔadcACB, and ΔadcACBΔadcAII strains (Figure 1c). Addition of 10 µM TPEN to BHI (BHI+TPEN) minimally impacted the growth of the parent OG1RF strain (OG1RF growth is severely impaired at TPEN concentrations ≥20 µM, Figure S3). On the other hand, the ΔadcAII single mutant grew poorly in BHI+TPEN, while growth of the ΔadcACB and ΔadcACBΔadcAII strains was completely inhibited by 10 µM TPEN (Figure 1d). Finally, the addition of 10 µM ZnSO₄ to the BHI + TPEN media restored growth of the ΔadcAII, ΔadcACB, and ΔadcACBΔadcAII strains (Figure 1e). To verify if the inhibitory effect of TPEN on growth of Δadc strains was indeed Zn-specific, we tested if addition of Mn (10 µM MnSO₄) or Fe (10 µM FeSO₄) could also restore cell growth. With the exception of ΔadcAII that was able to grow in BHI+TPEN after Mn supplementation, Fe or Mn supplementation did not restore growth of the other mutants in BHI+TPEN (Figure 1f-g, compared to Figure 1d). In trans complementation of ΔadcACB and of ΔadcAII fully or partially rescued their growth defects in BHI+TPEN (Figure 1h). The reasons for Mn rescuing growth of ΔadcAII in BHI + TPEN and the partial complementation of ΔadcAII are at present unknown.

Next, we sought to determine the ability of our panel of Δadc strains to grow in the presence of human calprotectin, a potent Mn and Zn chelator. We carried out growth kinetic assays to compare the ability of wild-type (WT) and Δadc mutants to grow in BHI supplemented with purified human calprotectin (hCP) or recombinant calprotectin (hCPΔMn-tail) defective in Mn sequestration [10]. In the presence of hCP, growth of ΔadcA was not significantly different when compared to WT, whereas ΔadcAII, ΔadcACBΔadcAII, ΔadcACB, and ΔadcACBΔadcAII mutants displayed reduced growth or were fully inhibited by the native version of calprotectin (Figure 2a-b). While inactivation of the Mn-binding residue in hCPΔMn-tail improved growth of WT, ΔadcA and ΔadcAII strains, the other mutants remained highly sensitive to hCPΔMn-tail (Figure 2c). In trans complementation fully rescued the growth defects of ΔadcACB mutant in the presence of both versions of calprotectin, whereas growth of the complemented ΔadcAII was partially rescued (Figure S4). The reason for the partial complementation of ΔadcAII in the presence of hCP is at present unknown. Taken together, these findings reveal that AdcABC and AdcAII work independently but cooperatively to mediate E. faecalis growth under Zn-restricted conditions. Based on the identical phenotypes of ΔadcACB and ΔadcACBΔadcAII strains, these results also indicate that both AdcA and AdcAII associate with AdcB (inner membrane permease) and AdcC (cytoplasmic ATPase) to form tripartite Zn
transporters. Finally, growth kinetics in the presence of the Zn-chelating agents TPEN and calprotectin hint that AdcAII might be a more effective Zn scavenger than AdcA, at least under the more severe Zn-restricted conditions.

Next, we used inductively coupled optical emission spectrometry (ICP-OES) to determine intracellular Zn pools in mid-log grown cultures of WT and derivative Δadc strains grown in BHI or in BHI supplemented with 7.5 µM TPEN (Figure 3a). In BHI, the ΔadcACB accumulated less Zn when compared to the WT strain albeit this difference was rather small (~12%) and not supported by a similar or greater decrease in Zn pools in the ΔadcAΔadcAII and ΔadcACBΔadcAII strains.
(Figure 3a). In agreement with the predicted role of Zur as a transcriptional repressor of adcABC and adcAII, the Δzur strain accumulated two times more Zn than the WT strain when grown in BHI. The addition of TPEN to the growth media led to an unexpected increase in intracellular Zn pools in the WT strain when compared to cells grown in BHI (~50% increase). Nonetheless, all mutants accumulated less Zn when compared to the WT strain when grown in BHI +TPEN. We suspected that the higher intracellular levels of Zn in the WT strain grown in BHI+TPEN compared to BHI only correlated with increased transcription of the adcACB and adcAII genes and that this response was controlled by Zur. To verify this possibility, we used quantitative RT-PCR to determine mRNA levels of adcA and adcAII in the WT and Δzur strains grown to mid-log phase in BHI and then treated with either 30 μM TPEN or 4 mM ZnSO₄ for 1 h. In line with previous transcriptional studies [40,41], TPEN treatment significantly induced adcA (~1-log) and adcAII (~2-log) transcription, whereas Zn supplementation reduced adcA levels by ~2-log and adcAII by ~1-log when compared to the BHI control (Figure 3b). As expected, inactivation of zur resulted in increased transcription of adcA and adcAII grown in BHI (~1-log adcA, ~2-log adcAII) or BHI+Zn (~2-log both genes) when compared to the WT strain grown under the same conditions. Taken together, these results confirm that Zn deprivation (TPEN-treated cells) alleviates Zur repression triggering a strong induction of adcACB and adcAII expression that allows E. faecalis overcome Zn starvation.

**AdcACB and AdcAII contribute to growth in serum but not in urine ex vivo**

To determine the contribution of Adc-mediated Zn uptake to E. faecalis virulence, we first monitored the ability of the Δadc strains to grow and survive in pooled human serum or human urine ex vivo. When incubated in serum, growth of ΔadcA and ΔadcAII strains did not significantly differ from WT, whereas ΔadcAΔadcAII, ΔadcACB and ΔadcACBΔadcAII grew poorly and, most relevantly, displayed sharp decreases in survival after 8 hours and onward, ultimately showing a ~3-log reduction in colony-forming unit (CFU) recovered after 48 hours of incubation in serum (Figure 4a). These growth and survival defects were fully reversed by the addition of 500 μM ZnSO₄ (Figure 4b) or in trans complementation (Figure 4c). On the other hand, the ability of all Δadc strains to grow/survive in urine was not found to differ from WT (Figure 4d), suggesting that Zn is not a growth-limiting factor in urine (at least ex vivo). Finally, the inactivation of zur did not impact growth nor survival in serum or urine (Figure 4a-b,d).

While Zn levels and bioavailability in serum or in urine were not determined, the strong phenotype of the ΔadcAΔadcAII, ΔadcACB and ΔadcACBΔadcA strains in serum was expected as Zn levels in blood circulation are low with most Zn sources bound or sequestered by host cells and proteins [45,46]. On the other hand, Zn is abundant in the bladder environment as any excess Zn, typically from dietary sources, is excreted through urine via the gastrointestinal route [47–50].

**Disruption of AdcABC-AdcAll lowers tolerance toward cell envelope-targeting antibiotics**

Because the ΔadcAΔadcAII, ΔadcACB and ΔadcACBΔadcAII strains displayed a reduced growth rate under Zn-restricted conditions, like S. pneumoniae ΔadcAΔadcAII [16], we wondered if this was due to altered cell division. To investigate this, we observed bacterial morphology using a light microscope. Indeed, these mutants formed longer chains when compared to the WT, ΔadcA and ΔadcAII strains that primarily formed only short chains or diplococcus (Figure 5). This observation and the fact that S. pneumoniae ΔadcAΔadcAII mutant displayed aberrant cell septation [16] led us to wonder if expression of virulence traits that occur at the cell surface interface were similarly affected in the mutant strains. First, we compared the capacity of WT and mutants to form biofilms after 24 hours of incubation in BHI supplemented with 10 mM glucose. The total biofilm biomass of ΔadcA and ΔadcAII single mutants was significantly reduced when compared to WT, but the very small differences observed (5 to 10% reduction) are unlikely to have major biological implications (Figure 6a). On the other hand, the ΔadcAΔadcAII, ΔadcACB and ΔadcACBΔadcAII strains formed more robust biofilms with an ~30 to 50% increase in biofilm biomass (Figure 6a).

Next, we tested the capacity of WT and mutants to grow in BHI supplemented with antibiotics that target different steps of cell wall biosynthesis (ampicillin, bacitracin, and vancomycin) or disrupt membrane integrity (daptomycin) by determining the minimal inhibitory concentration (MIC) for each antibiotic. Once again, relevant phenotypes were restricted to the ΔadcAΔadcAII, ΔadcACB, and ΔadcACBΔadcAII strains. Specifically, ΔadcAΔadcAII, ΔadcACB, and ΔadcACBΔadcAII showed lower MICs for ampicillin, bacitracin, and daptomycin (Figure 6b-d). However,
WT and all mutants showed the same MIC for vancomycin (Figure 6e).

**Zn is critical for *E. faecalis* virulence during infection**

In the last series of experiments, we used three *in vivo* models to probe the contributions of AdcABC and AdcAII to virulence. In conformity with *in vitro* and *ex vivo* phenotypes, virulence of ΔadcAΔadcAII, ΔadcACB and ΔadcACBΔadcAII but not the single mutants (ΔadcA, ΔadcAII and Δzur) was highly attenuated in the *Galleria mellonella* model (Figure 7a). Next, we used two catheter-associated mouse infection models that recapitulate some of the environmental and immunological conditions that promote enterococcal infections in human. From this...
point, we did not include the $\DeltaadcA\DeltaadcAII$ and $\DeltaadcACB$ strains as these mutants consistently phenocopied the $\DeltaadcACB\DeltaadcAII$ mutant. In the catheter-associated peritonitis model, the $\DeltaadcA$ and $\DeltaadcAII$ single mutants colonized the peritoneal cavity, catheter and spleen (infection becomes systemic after 12 to 24 h) in significantly fewer numbers than the WT strain (Figure 7b). Not surprisingly, colonization defects of single mutants were significantly more pronounced in the $\DeltaadcACB\DeltaadcAII$ strain. Finally, in a catheter-associated urinary tract infection (CAUTI) model, virulence of $\DeltaadcA$ and $\DeltaadcACB\DeltaadcAII$ strains was attenuated, but bacterial burden recovered from bladders of retrieved catheter of animals infected with WT or $\DeltaadcAII$ strains were nearly identical (Figure 7c). Moreover, $\DeltaadcAII$ was recovered from kidneys and spleen in higher bacterial titers, albeit the trend of the latter was not considered significant (Figure 7c).

**Discussion**

While there has been increasing appreciation of the multiple contributions of trace metals other than Fe to bacterial fitness and virulence [2,5,20], the mechanisms utilized by *E. faecalis* to maintain Zn homeostasis and their specific contributions to pathogenesis were, until now, poorly understood. Previously, our group showed that *E. faecalis* encodes three high-affinity Mn transporters and that while it was necessary to inactivate all three Mn transport systems (efaCBA, mntH1 and mntH2) to severely impair Mn uptake *in vitro*, the inactivation of only two of them (efaCBA and mntH2) was sufficient to abolish *E. faecalis* virulence [51]. In this report, we showed that both AdcACB and the orphan AdcAII predicted to mediate Zn import in a Zur-regulated manner and are critical for the ability of *E. faecalis* to grow and survive Zn-restricted conditions. Although the differences were not as striking as those seen with the Mn transport mutants [51], virulence of strains lacking the AdcACB/AdcAII system was significantly attenuated in both invertebrate and vertebrate infection models.

During characterization of the *adc* mutants, we noted that simultaneous deletion of *adcACB* and *adcAII* led to readily discernible morphological and biophysical alterations, that is increased cell chaining and cell-cell aggregation, which led us to wonder if the inability to maintain Zn homeostasis affected cell envelope homeostasis. Indeed, the $\DeltaadcA\DeltaadcAII$, $\DeltaadcACB$, and $\DeltaadcACB\DeltaadcAII$ strains showed heightened sensitivity to ampicillin, bacitracin, and daptomycin, with the latter showing a striking 16-fold lower MIC than the WT strain MIC ($\leq$16 µg ml$^{-1}$ml, compared to 256 µg ml$^{-1}$). Previous studies have shown that exposure to bacitracin resulted in downregulation of *adcA* (0.2-fold) in *E. faecalis* V583, whereas *adcAII* transcription increased (8-fold) after vancomycin treatment [52]. In
addition, exposure to chlorhexidine, a cationic antimicrobial agent that targets the cell membrane, resulted in upregulation of adcCB (~4-fold) [53]. While the roles played by Zn in E. faecalis envelope homeostasis are unknown, previous studies have associated loss of Zn transporters or Zn-dependent enzymes to surface-associated defective phenotypes. For example, in S. pneumoniae, deletion of adcACB and adcAII resulted in asymmetrical septa formation, abnormal cell division patterns, and emergence of small, aborted cells when S. pneumoniae was forced to grow under Zn-restricted conditions [16]. In the distantly related Gram-negative pathogen Acinetobacter baumannii, inactivation of a Zn-dependent peptidase, ZrLA, increased cell permeability and susceptibility to the β-lactam antibiotic carbencillin [54].

Similar to streptococci, enterococcal genomes do not encode the machinery to synthesize opine-like zinco phosphes and their cognate transporters. In Gram-positive cocci, Zn acquisition is primarily mediated by adcABC (also known as znuABC) and adcAII, both of which are under AdcR (ZuR) negative control [16,22,26,29,55]. Similar to our findings showing the additive contribution of E. faecalis adcA and adcAII genes to Zn homeostasis, simultaneous inactivation of both adcA and adcAII is necessary to (nearly) abolish Zn import and, as a result, drastically impairs virulence of major human pathogens such as S. pyogenes and S. pneumoniae [16,26,27].

Although E. faecalis AdcA and AdcAII share 52% identity, they do not have a similar domain organization. Biochemical and biophysical characterizations of S. pneumoniae AdcA and AdcAII proteins indicated that these functionally redundant proteins employ distinct Zn acquisition mechanisms [17]. AdcAII_Spu has two Zn-binding domains, an amino terminal cluster A-I domain typical of solute-binding proteins and a C-terminal domain that is structurally related to ZinT, a periplasmic Zn chaperone of Gram-negative bacteria, whereas AdcA_Spu has only the terminal cluster A-I domain [17]. Moreover, AdcAII_Spu-mediated Zn uptake in vivo has been shown to depend on proteins
Figure 7. Virulence of *E. faecalis* in different animal models. (a) Percentage survival of *G. mellonella* larvae 96 hours post-infection with *E. faecalis* WT or indicated mutants. Each curve represents a group of 15 larvae injected with \( \sim 1 \times 10^5 \) CFU of selected *E. faecalis* strain. Data points represent the average of 6 biological replicates. Statistical analysis was performed using the log-rank (Mantel-Cox) test.

(b) Total CFU recovered after 48 hours from spleen, peritoneal wash, and catheter of mice infected with \( 2 \times 10^8 \) CFU of bacteria. (c) Total CFU recovered after 24 hours from bladder, kidney, spleen, and catheter of mice infected with \( 1 \times 10^7 \) CFU of WT or indicated mutants. In (b and c), ten mice were infected with two biological replicates and data points shown were a result of using the ROUT outlier test. The black line represents the median. Statistical analysis was performed using the Mann-Whitney test. * \( p \leq 0.05 \), ** \( p \leq 0.01 \), *** \( p \leq 0.001 \), and **** \( p \leq 0.0001 \). The dashed line represents the limit of detection (LOD = 50 CFUs).
Table 1. Strains and plasmids used in this study.

| Strain name | Relevant characteristics | Plasmid | References |
|-------------|--------------------------|---------|------------|
| E. faecalis parent strain | OG1RF wild type Laboratory strain, RifR, Fusα | - | [57] |
| E. faecalis deletion mutants | OG1RF ΔadcA OG1RF ΔadcA-II OG1RF ΔadcAΔadcA- II OG1RF ΔadcACB OG1RF ΔadcAΔadcA-II OG1RF Δzur | OG1RF_RS00260 deletion OG1RF_RS12625 deletion OG1RF_RS00260 and OG1RF_RS12625 deletion OG1RF_RS00260-70 deletion OG1RF_RS12625 deletion OG1RF_RS00260 deletion | pCJ47 [42] |
| E. faecalis conjugative strains and plasmid | CK11 | OG15p upp4::P23repA4, SpecR | pCJ47: This study |
| E. faecalis complementation strains | OG1RF gcp123 Empty vector, KanR | gcp123 | This study |
| E. coli strains for cloning | EC1000 Host and carrier for RepA-dependent cloning using pCJ47 vector, carries phe57, ErmA | pCJ47: This study |

with poly-histidine (Pht) triad (HxxHxH) motifs that scavenge extracellular Zn and then transfer it to AdcAIISpn for internalization [56]. While streptococcal species have been shown to encode as many as four Pht protein homologues (e.g., S. pneumoniae PhtA, PhtB, PhtD and PhtE), E. faecalis genomes do not contain genes with HxxHxH motifs. Of interest, a small open-reading frame (OG1RF_RS12620) (40 amino acids) coding for a putative uncharacterized protein is located upstream and separated by only 21-bp from the adcAII_Ef start codon. While the predicted amino acid sequence of the OG1RF_RS12620 contains only a single histidine residue and is not enriched for other amino acids (such as cysteine and methionine) that typically coordinate Zn, it will be interesting to explore the possible role of OG1RF_RS12620 in Zn acquisition in future studies.

Because an E. faecalis strain lacking the entire adcACB operon is phenotypically similar to the ΔadcAΔadcAII double mutant, an important observation from this study is that, most likely, AdcA and AdcAII can only form functional complexes with AdcB (inner membrane permease) and AdcC (cytoplasmic ATPase). Also of interest was the distinct importance of AdcA and AdcAII to colonization and systemic dissemination in the two mouse models. While attenuated virulence of ΔadcA and ΔadcAII single mutants was comparable in the peritonitis model, adcAII was dispensable for bladder and cather colonization in the CAUTI model. In addition, the recovery of viable bacteria from kidney and spleen of mice infected with ΔadcAII indicates, at first glance, that the loss of AdcAII promotes bacterial dissemination to kidneys and spleen. While speculative at this point, we believe that these phenotypes are due to differences in the expression levels or activity of AdcA and AdcAII in the bladder environment. One possibility is that adcA responds more strongly than adcAII to environmental cues encountered in the bladder, such that adcAII becomes dispensable for E. faecalis proliferation in urine. To test this possibility, studies to compare the adc transcriptional profiles of ΔadcA and ΔadcAII in the bladder, peritoneal cavity, and bloodstream environments will soon be underway. Alternatively, the large and constant fluctuations in the bladder environment in solute concentrations and of other important biophysical and biochemical parameters such as pH caused by intermittent cycles of urination can somehow compromise Zn-binding capacity of AdcAII or ability to interact with the AdcBC partner proteins.

In summary, our findings reveal that the AdcACB/AdcAII system is a bona fide Zn acquisition system of
Table 2. Primers used in this study.

| Primer name | Primer sequence (5’ → 3’) | Restriction sites* |
|-------------|---------------------------|-------------------|
| **Primers for qRT-PCR** | | |
| adcA_F’ | TCGGGAAGAGGCCAGTCAA | - |
| adcA_R’ | TTTGCGTAAACGGGCTAT | - |
| adcA-II_F’ | TTTCGTCAGGCAGACCA | - |
| adcA-II_R’ | GTTGGGCAGGTGGTCTG | - |
| adcA_Arm1 F’ | TCCGTTATGTTAGGGCCGCATT | Notl |
| adcA_Arm1 R’ | TGTTCCAGACATGCTCTCTTCTTAT | - |
| adcA_Arm2 F’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcA_Arm2 R’ | CTATGGCAGGCTATT | SmaI |
| adc_CB_Arm1 F’ | TCGCTATGTTAGGGCCGCATT | Notl |
| adc_CB_Arm1 R’ | GTTGGGCAGGTGGTCTG | - |
| adc_CB_Arm2 F’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adc_CB_Arm2 R’ | CTATGGCAGGCTATT | SmaI |
| **Primers for cloning in pCJK47 vector** | | |
| adcA_F’ | GCCAATTGCGCAAGCCTGTATTTTAAATG | - |
| adcA_R’ | TCGACGTCGCTGATGATAATTCC | - |
| adcA-II_F’ | AATTCGATCGCGCATTTTATC | - |
| adcA-II_R’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcCB_Arm1 F’ | TCGCTATGTTAGGGCCGCATT | Notl |
| adcCB_Arm1 R’ | GTTGGGCAGGTGGTCTG | - |
| adcCB_Arm2 F’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcCB_Arm2 R’ | CTATGGCAGGCTATT | SmaI |
| **Primers for screening deletion mutants** | | |
| adcA_F’ | TTGACGGTCGAGTTAGCTAT | - |
| adcA_R’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcA-II_F’ | AATTCGATCGCGCATTTTATC | - |
| adcA-II_R’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcCB_Arm1 F’ | TCGCTATGTTAGGGCCGCATT | Notl |
| adcCB_Arm1 R’ | GTTGGGCAGGTGGTCTG | - |
| adcCB_Arm2 F’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcCB_Arm2 R’ | CTATGGCAGGCTATT | SmaI |
| **Primers for cloning in gpc123 vector** | | |
| gcpC123 F’ | GTAAAACGAGCGCCAGCAGG | - |
| gcpC123 R’ | CCAAGAAGAACCAGTACGATAG | - |
| adcA_Arm1 F’ | GTGAAGCTGTATGAGTTAGGCTA | - |
| adcA_Arm1 R’ | ATTTTTCGTTCTGCTATT | SmaI |
| adcA_Arm2 F’ | AGGAGAACCAGTGCAACAGAAGG | - |
| adcA_Arm2 R’ | CTATGGCAGGCTATT | SmaI |
| adcA_Arm2 F’ | CGTCTAGATAGAATTAGTACTCC | - |
| adcA_Arm2 R’ | GATCAAGCTGATGAGTTAGGCTA | - |
| adcA_Arm2 F’ | CGTCTAGATAGAATTAGTACTCC | - |
| adcA_Arm2 R’ | GATCAAGCTGATGAGTTAGGCTA | - |
| **Primers for cloning in pGCP123 vector** | | |
| adcA_Arm1 F’ | CGGAAAGACCAGCAGCTGAT | - |
| adcA_Arm1 R’ | CTATGGCAGGCTATT | SmaI |

*Restriction sites are underlined and in bold in the primer sequence.

E. faecalis contributing additively to the maintenance of Zn homeostasis. More importantly, this report demonstrates that the AdcACB/AdcAII system mediates E. faecalis virulence. Therefore, both the substrate-binding and surface-associated AdcA and AdcAII proteins can be viewed as suitable targets for the development of antimicrobial therapies to treat or prevent enterococcal infections.

Material and methods

**Bacterial strains and growth conditions**

The bacterial strains and vectors used in this study are listed in Table 1. Bacteria were routinely grown in brain heart infusion (BHI broth (BD Difco™, for E. faecalis) and Luria-bertani (BD Difco™, for E. coli) at 37°C under static conditions. Strains possessing the pGCP123 plasmid [59] were grown in the presence of kanamycin (300 µg ml⁻¹ for E. coli and 500 µg ml⁻¹ for E. faecalis). For growth kinetics assays, overnight cultures were normalized by cell density to an OD₆₀₀ of 0.25 and inoculated into BHI media at a 1:50 ratio, with the OD₆₀₀ monitored in an automated growth reader (Bioscreen c, Oy Growth Curves AB). Native calprotectin (HCP) and Mn-deficient (Cp ΔMn-tail) calprotectin were gifts from Dr. Walter Chazin (Vanderbilt University, USA) [10]. Experiments using purified calprotectin were performed in BHI supplemented with 20% (v/v) CP buffer (40 mM NaCl, 0.5 mM β-mercaptoethanol, 1.2 mM CaCl₂, 8 mM Tris-HCl, pH 7.5). Normalization of starter cultures for most experiments included centrifugation or overnight cultures at 4000 rpm for 10 mins to remove spent media, two cell
pellet washes in PBS, and adjustment of cell density to an OD$_{600}$ of 0.25 ($\sim$1 x 10$^6$ CFU ml$^{-1}$) also in PBS. For E. faecalis CFU determination from ex vivo and in vivo studies, serially diluted aliquots were plated on BHI agar supplemented with 200 µg ml$^{-1}$ rifampicin and 10 µg ml$^{-1}$ fusidic acid. TPEN (N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine), FeSO$_4$, MnSO$_4$, ZnSO$_4$, lysozyme, ampicillin, bacitracin, daptomycin, fusidic acid, kanamycin, rifampicin, and vancomycin were purchased from Sigma Aldrich.

Homology searches and structural modeling of proteins using BlastP, AlphaFold, and Chimera

Amino acid sequences from AdcA and AdcAII proteins of E. faecalis OG1RF (AdcA; WP_002367576.1, AdcAII; WP_002392710.1), S. pneumoniae R6 (AdcA; WP_000724074.1, AdcAII; WP_001844050.1), and S. pyogenes MGAS5005 (AdcA; WP_011285462.1, AdcAII; WP_002987954.1) were queried for conservation of sequences using NCBI BlastP multiple sequence alignment to generate an alignment highlighting consensuses. For structural modeling, the amino acid sequences of E. faecalis OG1RF AdcA and AdcAII and S. pneumoniae R6 AdcA and AdcAII were retrieved from PubMed NCBI database. Tertiary structures were obtained using AlphaFold Colab notebook [60,61], and image files (PDB) were constructed using ChimeraX1.3 [62].

General cloning techniques

Bacterial genomic DNA (gDNA) was isolated using a Wizard Genomic DNA purification kit (Promega). Plasmid purification was performed using the Monarch plasmid miniprep kit (New England BioLabs). Isolation of PCR amplified products was performed using the Monarch DNA gel extraction kit (New England BioLabs). Typical cloning, either directional or non-directional depending on plasmid used, was performed using the In-Fusion HD cloning kit (TaKaRa Bio). Colony PCR was performed using PCR 2x Master Mix (Promega) with primers listed in Table 2.

Construction of deletion and genetically complemented strains

Deletion of zur, adcA, and AdcAII, or the entire adcACB operon was carried out using a markerless genetic exchange system based on the pCJK47 vector [42]. Briefly, nucleotide sequences flanking zur, adcA, adcCB, and AdcAII were amplified using the primers listed in Table 2. These PCR-amplified products were directly cloned into pJK47, electroporated into E. faecalis CK111 (donor strain), followed by conjugation into E. faecalis OG1RF. The deletion mutants were isolated by following the steps for markerless counterselection detailed elsewhere [42]. The ∆adcA∆adcAII and ∆adcAB&∆adcAII double mutants were obtained by conjugating the pCJK-adcAII plasmid with the ∆adcA and ∆adcACB mutants, respectively. Because the adcACB operon is ~3kb, we first isolated ∆adcA and then performed conjugation using the pCJK-adcBC vector to generate the ∆adcACB strain. PCR sequencing was performed to confirm the absence of zur, adcA, and AdcAII, or the entire adcACB operon in these deletion mutant strains. For genetic complementation, adcACB and AdcAII genes were amplified by PCR and cloned into the pGCP123 plasmid [59] using the primers listed in Table 2. The full-length adcACB and AdcAII nucleotide sequences were incorporated into linearized pGCP123 plasmid using the In-Fusion HD cloning kit. Plasmids were propagated in E. coli and transformed into E. faecalis as described previously [59].

Inductively coupled plasma-optical emission spectrometry (ICP-OES)

The metal (Zn) concentration in BHI media and in bacteria was determined as previously described [51]. For quantification of metals in BHI, 9 ml of broth was digested with 1 ml of trace-metal grade 35% nitric acid (HNO$_3$) prior to analysis. For quantification of intracellular metal, overnight E. faecalis cultures were washed with PBS twice and inoculated at a ratio of 1:40 in BHI or BHI supplemented with 7.5 µM TPEN. Mid-log grown cultures (OD$_{600}$ of 0.5) were harvested by centrifugation and washed twice with PBS containing 0.5 mM EDTA. After washing, cell pellets were collected in a polyethylene scintillation vial (Fisher Scientific) and digested in 1 ml of trace-metal grade 35% nitric acid (HNO$_3$) at 90°C for 1 hour. Digested bacterial cells were diluted at a ratio of 1:10 in reagent-grade water prior to analysis. Intracellular Zn pools were then quantified using a 5300DV ICP Atomic Emission Spectrometer (Perkin Elmer) at the University of Florida Institute of Food and Agricultural Sciences (UF-IFAS) Analytical Services Laboratories. The bicinechonic acid (BCA) assay kit (Pierce™) was used to calculate the total protein content for normalization of the metal concentration obtained.
**MIC determinations**

Overnight *E. faecalis* cultures were normalized to an OD<sub>600</sub> of 0.25 and diluted at a ratio of 1:1000. The diluted cultures were inoculated at a ratio of 1:20 into BHI containing antibiotics (ampicillin, bacitracin, daptomycin, and vancomycin). The absorbance at OD<sub>600</sub> after incubation at 37°C for 24 hours was measured using a Synergy H1 microplate reader (Molecular Devices).

**Growth and survival in serum and urine**

Inoculum was prepared from overnight *E. faecalis* cultures adjusted to OD<sub>600</sub> of 0.5 and inoculated into pooled human serum or human urine (Lee Biosolutions) at a ratio of 1:1000. Aliquots were obtained at several time points and then plated on BHI agar containing fusidic acid and rifampicin to determine CFU.

**Biofilm assay**

Inoculum was prepared from overnight *E. faecalis* cultures adjusted to OD<sub>600</sub> of 0.5 that were diluted 1:25 in BHI supplemented with 10 mM glucose. Biofilm assay was performed using 96-well polystyrene plates (Grenier) that were incubated at 37°C for 24 hours. Post-incubation, spent media was discarded, biofilms washed twice with PBS, and the biomass stained with 0.1% crystal violet for 25 mins. A 33% acetic acid solution was used to dissolve the precipitated crystal violet-stained biomass, and absorbance was determined at OD<sub>595</sub>.

**Quantitative real-time PCR**

Overnight *E. faecalis* cultures were normalized to an OD<sub>600</sub> of 0.5, inoculated at a ratio of 1:20 into fresh BHI, and incubated for 1 hour at 37°C. After incubation, cells were collected by centrifugation at 4000 rpm for 10 mins, washed with PBS, and incubated in the presence of lysozyme (20 mg ml<sup>-1</sup>) at 37°C for 30 mins. After treatment, cells were harvested by centrifugation and the total RNA was extracted using the PureLink RNA minikit (Invitrogen). Next, a Turbo DNA-free kit (Thermo Fisher) was used for purification of RNA and removal of contaminating gDNA. A High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for the synthesis of cDNA. Quantitative real-time PCR (qRT-PCR) was performed using iTaq Universal SYBR supermix (BioRad) with the primers listed in Table 2. For quantification of transcript numbers, *E. faecalis* OG1RF gDNA was used as a template to generate standard curves.

**Galleria mellonella infection**

To assess *E. faecalis* virulence, the larvae of *G. mellonella* were used as described previously [51,63]. Briefly, larvae (groups of 15) were injected with exponentially grown cells (~5 × 10<sup>5</sup> CFU), heat-killed *E. faecalis* (30 mins at 100°C negative control), or PBS (vehicle control). Post-injection, larvae were kept at 37°C and their survival were recorded over time.

**Catheter-Associated peritonitis mouse model**

The methods for the catheter-associated peritonitis model have been described previously [64], and hence, only a brief overview describing minor modifications are described here. Female C57BL/6J 8-weeks old mice were purchased from Jackson Laboratories and allowed to acclimatize for at least 2 days prior to initiation of the study. Three hours post-catheter implantation, mice were injected intra-peritoneally with ~5 × 10<sup>8</sup> CFU of bacteria from an overnight culture. Forty eight hours post-injection, mice were euthanized and the bacterial burden was determined by plating serially diluted aliquots on selective BHI plates supplemented with rifampicin and fusidic acid. This procedure was approved and performed in compliance with the University of Florida Institutional Animal Care and Use Committee (protocol# 201910705).

**Mouse catheter-associated urinary tract infection (CAUTI) model**

The full description of the method for the CAUTI infection model has also been described in detail previously [65], and hence, a brief overview and modifications are described here. Female C57BL/6Ncr 6-weeks old mice were purchased from Charles River Laboratories and subjected to transurethral catheter implantation. Post-catheter implantation, mice were infected with ~1 × 10<sup>7</sup> CFU of bacteria from an overnight culture. 24 hours post-infection, mice were euthanized, and bacterial burden were determined by plating on selective BHI plates supplemented with rifampicin and fusidic acid. This procedure follows the University of Notre Dame Institutional Animal Care and Use Committee (protocol #18 August 4792MD).
**Bright field microscopy**

Overnight cultures grown in BHI were normalized to OD_{600} 0.5 (~5x10^8 CFU/ml) and washed once with 1 ml of PBS. Aliquot of 5 µl of bacterial inoculum was placed on microscope glass sides that were washed once in filtered 70% ethanol followed by milliQ water and left to dry at room temperature. Samples were covered with 5 µl of mounting media (Vectashield) and covered with a glass coverslip. Bright field microscopy was performed using a Leica DM2500 LED optical microscope fitted with a 100X/1.3 oil objective lens. Images acquired were further processed using FIJI software [66].

**Statistical analysis**

Data obtained from this study were analyzed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). Data from multiple experiments conducted on nonconsecutive days were collated, and applicable statistical tests were used.

**Data availability statement**

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials. Vectors and strains created from this study will be available from the corresponding author upon reasonable request.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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