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Adaptation of *Listeria monocytogenes* to perturbation of c-di-AMP metabolism underpins its role in osmoadaptation and identifies a fosfomycin uptake system

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Summary

The human pathogen *Listeria monocytogenes* synthesizes and degrades c-di-AMP using the diadenylate cyclase CdaA and the phosphodiesterases PdeA and PgpH respectively. c-di-AMP is essential because it prevents the uncontrolled uptake of osmolytes. Here, we studied the phenotypes of *cdaA*, *pdeA*, *pgpH* and *pdeA pgpH* mutants with defects in c-di-AMP metabolism and characterized suppressor mutants restoring their growth defects. The characterization of the *pdeA pgpH* mutant revealed that the bacteria show growth defects in defined medium, a phenotype that is invariably suppressed by mutations in *cdaA*. The previously reported growth defect of the *cdaA* mutant in rich medium is suppressed by mutations that osmotically stabilize the c-di-AMP-free strain. We also found that the *cdaA* mutant has an increased sensitivity against isoleucine. The isoleucine-dependent growth inhibition of the *cdaA* mutant is suppressed by *codY* mutations that likely reduce the DNA-binding activity of encoded CodY variants. Moreover, the characterization of the *cdaA* suppressor mutants revealed that the Opp oligopeptide transport system is involved in the uptake of the antibiotic fosfomycin. In conclusion, the suppressor analysis corroborates a key function of c-di-AMP in controlling osmolyte homeostasis in *L. monocytogenes*.

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Introduction

The second messenger cyclic di-AMP (c-di-AMP) has been studied since many years in a variety of bacteria (Corrigan and Gründling, 2013; Stülke and Krüger, 2020). c-di-AMP was identified during structural and functional studies with the DNA integrity scanning protein A (DisA), which is involved in DNA metabolism in the Gram-positive model organism *Bacillus subtilis* (Bejerano-Sagie *et al*., 2006; Witte *et al*., 2008; Oppenheimer-Shaanan *et al*., 2011; Gándara and Alonso, 2015; Torres *et al*., 2019a, 2019b). DisA contains a DNA-binding motif and a diadenylate cyclase domain converting two molecules of ATP to c-di-AMP and pyrophosphate (P_iP_i) (Witte *et al*., 2008). In the meantime, other diadenylate cyclases belonging to the CdaA-, CdaM-, CdaS- and CdaZ-type have been identified (Commichau *et al*., 2019; Stülke and Krüger, 2020). While some bacteria like *B. subtilis* produce three different diadenylate cyclases, most other bacteria known to synthesize c-di-AMP possess only one diadenylate cyclase (Luo and Helmann, 2012; Stülke and Krüger, 2020). CdaA is the most-abundant diadenylate cyclase, which has been intensively studied (Rosenberg *et al*., 2015; Heidemann *et al*., 2019).

c-di-AMP is essential for viability of many bacteria and archaea under standard growth conditions, suggesting that the second messenger fulfils important functions in the cell (Woodward *et al*., 2010; Luo and Helmann, 2012; Barker *et al*., 2013; Blötz *et al*., 2017; Braun *et al*., 2019). Indeed, c-di-AMP controls potassium homeostasis and transport of other osmolytes (Nelson *et al*., 2013; Bai *et al*., 2014; Chin *et al*., 2015; Moscoso *et al*., 2015; Huynh *et al*., 2016; Schuster *et al*., 2016; Devaux *et al*., 2018a; Devaux *et al*., 2018b; Rubin *et al*., 2018; Quintana *et al*., 2019; Wang *et al*., 2019; Sikkema *et al*., 2020; Zeden *et al*., 2018, 2020; Cereija *et al*., 2021). Therefore, c-di-AMP is a key component in the regulation of the cellular turgor, a physical variable that needs to be tightly regulated (Commichau *et al*., 2018). Besides its role in osmoadaptation, c-di-AMP is implicated in the regulation of central metabolism (Sureka *et al*., 2014; Choi *et al*., 2017; Whiteley *et al*., 2017), DNA damage repair caused by H_2O_2 (Gándara and Alonso, 2015), cell wall metabolism (Witte *et al*., 2013; St-Onge and Elliot, 2017; Massa *et al*., 2020), biofilm formation (Du *et al*., 2014; Gundlach *et al*., 2016; Peng *et al*., 2016; Townsley *et al*., 2018; Fahmi *et al*., 2019; The *et al*., 2019; Faozia *et al*., 2021) and of genetic competence (Zarrella *et al*., 2020). Pathogenic bacteria producing c-di-AMP secrete the nucleotide, which is recognized by infected host cells and triggers an innate immune response (Woodward *et al*., 2010). The released c-di-AMP exacerbates infections as it probably avoids immune recognition by the host cell (Devaux *et al*., 2018a; Devaux *et al*., 2018b).

In addition to c-di-AMP secretion, the cellular concentration of the nucleotide can be reduced by c-di-AMP-specific phosphodiesterases. Five different types of phosphodiesterases have been identified. While the phosphodiesterases belonging to the GdpP- and PgpH-type are membrane-bound enzymes, the DhhP- and AtaC-type phosphodiesterases are soluble enzymes (Commichau *et al*., 2019; Latoscha *et al*., 2020). All bacteria and archaea that were shown to synthesize c-di-AMP possess at least one phosphodiesterase-degrading c-di-AMP. The presence of a phosphodiesterase is crucial for the viability of a cell because under hyperosmotic conditions c-di-AMP needs to be degraded to allow the influx of osmolytes into the cytoplasm for maintaining the cellular turgor (Smith *et al*., 2012; Zhu *et al*., 2016; Commichau *et al*., 2018; Pham *et al*., 2018). The extracellular cell wall-anchored phosphodiesterase CdnP has been identified in the human pathogen *Streptococcus agalactiae* (Andrade *et al*., 2016). CdnP degrades secreted c-di-AMP to dampen the activation of the innate immune system and to promote virulence of *S. agalactiae* (Andrade *et al*., 2016).

Recently, two studies that were aimed at identifying the function of the c-di-AMP-binding proteins DarB and CbpB in *Bacillus subtilis* and *Listeria monocytogenes* respectively uncovered a link between c-di-AMP signalling and (p)ppGpp, a second messenger orchestrating stringent response in bacteria (Ronneau and Hallez, 2019; Peterson *et al*., 2020; Krüger *et al*., 2021a; Krüger *et al*., 2021b). A cross-talk between the c-di-AMP and (p)ppGpp signalling pathways also exists in *Staphylococcus aureus* (Corrigan *et al*., 2015). In *B. subtilis* it has been demonstrated that apo-DarB directly interacts and activates the pyruvate carboxylase under conditions of potassium limitation (Krüger *et al*., 2022). Thus, DarB links potassium availability to control central carbon and amino acid metabolism. There are also still c-di-AMP targets of unknown function, among them CbpA from *L. monocytogenes* and the P_{II}-like signal transduction protein DarA from *B. subtilis* (PstA in *L. monocytogenes* and *S. aureus*) (Campeotto *et al*., 2015; Choi *et al*., 2015; Müller *et al*., 2015; Gundlach *et al*., 2015a; Gundlach *et al*., 2015b; Mains *et al*., 2021).

We are interested in c-di-AMP metabolism in *L. monocytogenes*, which possesses a CdaA-type diadenylate cyclase and the GdpP- and PgpH-type phosphodiesterases of which the former is designated as PdeA in this organism (Fig. 1). The activity of CdaA is controlled by the extracytoplasmic protein CdaR and the phosphoglucosamine mutase GlmM (Rismondo *et al*., 2015; Gibhardt *et al*., 2020) (Fig. 1). *Listeria monocytogenes* also secretes c-di-AMP via multidrug efflux pumps (MDRs) (Fig. 1). In fact, the characterization of *L. monocytogenes* mutants overexpressing MDRs led to the identification of c-di-AMP and CdaA (Woodward *et al*., 2010). A number of c-di-AMP targets have been identified in *L. monocytogenes*, among them the pyruvate carboxylase PycA as well as the CbpA and PstA proteins of unknown function (Fig. 1). Recently, we could show that c-di-AMP inhibits the potassium uptake systems KtrCD and KimA of *L. monocytogenes* (Gibhardt *et al*., 2019). Moreover, c-di-AMP directly binds to and inhibits the ATPase subunit of the carnitine importer OpuC (Huynh *et al*., 2016). Surprisingly, in contrast to *B. subtilis* and *S. aureus*, a c-di-AMP-free mutant of *L. monocytogenes* is not intoxicated by potassium ions (Gibhardt *et al*., 2019). Thus, the physiological importance of the c-di-AMP-dependent control of potassium transport is less pronounced in *L. monocytogenes*. Indeed, c-di-AMP is essential for growth of *L. monocytogenes* in rich medium because it controls the uptake of osmotically active oligopeptides via the Opp oligopeptide transporter (Whiteley *et al*., 2015) (Fig. 1). During growth in rich medium, c-di-AMP-bound CbpB does not activate the (p)ppGpp-synthetase/hydrolase

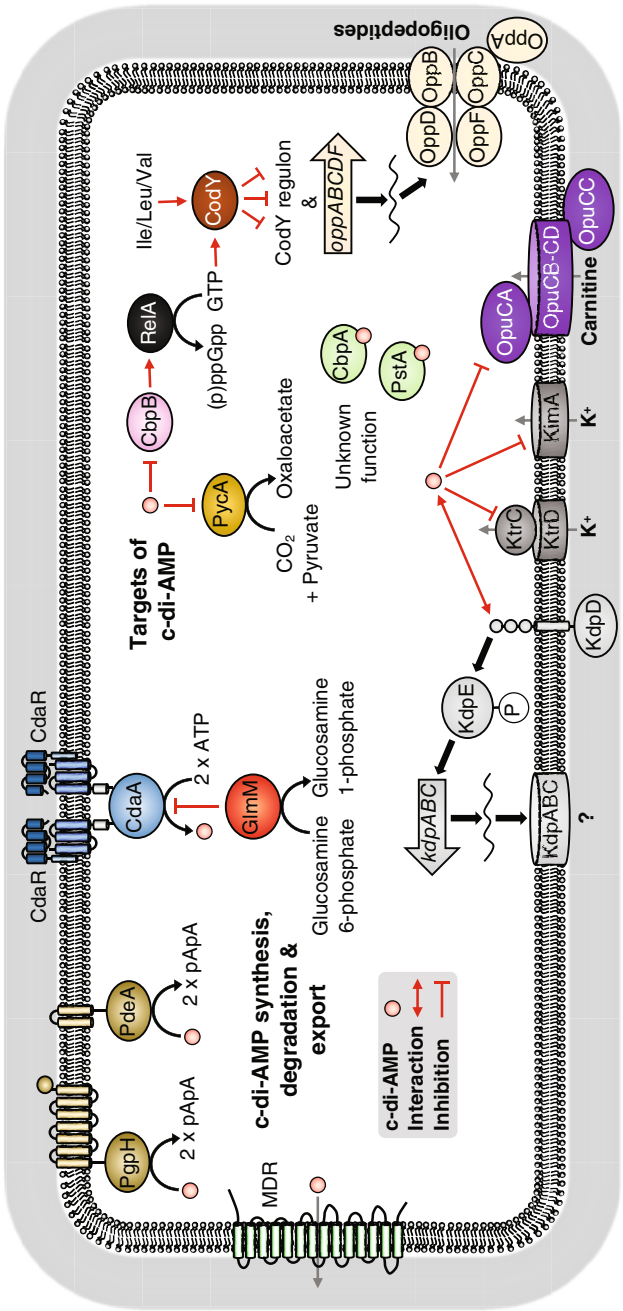

Fig. 1. Schematic illustration of c-di-AMP signalling in *L. monocytogenes*. The activity of the diadenylate cyclase CdaA, the only c-di-AMP synthesizing enzyme in *L. monocytogenes*, is modulated by CdaR and GlmM. c-di-AMP is degraded by the phosphodiesterases PgpH and PdeA and secreted via multidrug efflux pumps (MDRs). Binding of c-di-AMP to KtrC and OpuCA inhibits uptake of potassium ions and carnitine respectively. The interaction between c-di-AMP and the sensor kinase KdpD of the KdpDE two-component system probably affects the expression of the putative *kdpABC* potassium transporter genes. The cellular concentration of c-di-AMP indirectly affects the expression of the *opp* oligopeptide transporter genes belonging to the CodY regulon via CbpB-dependent regulation of RelA activity. c-di-AMP allosterically regulates the activity of the PycA pyruvate carboxylase. The c-di-AMP targets CbpA and PstA are proteins of unknown function.

RelA and a high cellular GTP pool is maintained (Peterson *et al*., 2020). The branched-chain amino acid (BCAA)- and GTP-responsive pleiotropic transcription factor CodY inhibits the transcription of the *opp* oligopeptide transporter genes (Whiteley *et al*., 2015; Biswas *et al*., 2020) (Fig. 1). In a c-di-AMP-free strain, CbpB directly binds to activates RelA and CodY does not inhibit the expression of the *opp* genes, which leads to oligopeptide uptake by the Opp transporter to toxic levels (Whiteley *et al*., 2015). Thus, phylogenetically related bacteria have evolved species-specific mechanisms to regulate the cellular turgor using different osmolytes, but they all use c-di-AMP in this process (Commichau *et al*., 2018).

Here, we have characterized *L. monocytogenes* mutant strains lacking the phosphodiesterase PgpH and PdeA and the diadenylate cyclase CdaA. We found that perturbation of c-di-AMP metabolism affects biofilm formation, osmosensitivity and integrity of the cell envelope. A suppressor analysis of the Δ*pgpH* Δ*pdeA* double mutant revealed that the growth defect of the strain in defined medium is relieved by the acquisition of mutations in *cdaA*. Moreover, the previously reported growth defect of the diadenylate cyclase mutant in rich medium is suppressed by mutations that osmotically stabilize the c-di-AMP-free strain. We also show that an isoleucine-dependent growth inhibition of the *cdaA* mutant is suppressed by mutations in *codY* that likely reduce the DNA-binding activity of encoded CodY variants. Finally, the characterization of the *cdaA* suppressor mutants uncovered that the Opp oligopeptide transport system is involved in fosfomycin uptake.

Experimental procedures

Chemicals, media, bacterial strains and growth conditions

Chemicals and media were purchased from Sigma-Aldrich (Munich, Germany), Carl Roth (Karlsruhe, Germany) and Becton Dickinson (Heidelberg, Germany). Primers were purchased from Sigma-Aldrich and are listed in Table S1. *Escherichia coli* strains were grown in lysogeny broth (LB). Agar plates were prepared with 15 g agar L^{-1} (Roth, Germany). *Escherichia coli* transformants were selected on LB plates containing ampicillin (100 μg ml^{-1}). *Listeria monocytogenes* was grown in brain–heart infusion (BHI) medium (Sigma-Aldrich) or in LSM (Whiteley *et al*., 2017), as previously described (Gibhardt *et al*., 2019). All bacteria used in this study are listed in Table S2. Growth in liquid medium was monitored using 96-well plates (Microtest Plate 96-Well, F, Sarstedt) that were incubated at 37°C in a SpectraMax iD5 multi-mode microplate reader. The plates were horizontally shaken (230 rpm) and the OD_{600} was measured in 15 min intervals.

DNA manipulation, construction of plasmids and mutant strains

The plasmids that were used and constructed in this study are listed in Table S2. Transformation of *E. coli* was performed using standard procedures. Plasmids were isolated from *E. coli* using the Nucleospin Extract Kit (Macherey and Nagel, Germany). PCR products were purified using the PCR Purification Kit (Thermo Scientific, Germany). DNA polymerases, restriction enzymes, DNA ligases were purchased from Thermo Scientific (Germany) and used according to the manufacturer's instructions. Other DNA sequencing was performed at the SeqLab Sequence Laboratories (Göttingen, Germany). *Listeria monocytogenes* chromosomal DNA was isolated using the NucleoSpin Microbial DNA Kit (Macherey and Nagel). *Listeria monocytogenes* mutant strains were constructed using the pMAD plasmid system (Arnaud *et al*., 2004), as previously described (Gibhardt *et al*., 2019). Deletion of genes in *L. monocytogenes* was confirmed by colony PCR (Dussurget *et al*., 2002) and DNA sequencing. The insertion of pIMK3-derived plasmids (Monk *et al*., 2008) into the *attB* site of the $tRNA^{Arg}$ locus in the *L. monocytogenes* genome was confirmed by PCR (Rismondo *et al*., 2016). The plasmids pBP355, pBP356, pBP569 and pBP570 were constructed for the deletion of the *pgpH*, *pdeA*, *oppF* and *hpt* genes respectively, in *L. monocytogenes*. The DNA fragments surrounding the *pgpH*, *pdeA*, *oppF* and *hpt* genes were amplified by PCR with the primer pairs JH17/JH18 and JH19/JH20 for *pgpH*, JH13/JH14 and JH15/JH16 for *pdeA*, MI56/MI57 and MI58/MI59 for *oppF*, and MI69/MI70 and MI71/MI72 for *hpt*, and fused in a second PCR. The fusion product was digested with the enzymes EcoRI or NcoI and BamHI, and ligated with the plasmid pMAD (Arnaud *et al*., 2004). The plasmid pBP568 contains a translational P_{oppA} promoter-*lacZ* fusion and was constructed for monitoring the activity CodY. The 597 bp long P_{oppA} promoter fragment was amplified by PCR using the primer pair MI38/MI44. The PCR product was digested with PstI and BamHI and ligated to the plasmid pBP117 (Hauf *et al*., 2019).

Electron microscopy

Strains BPL23 (Δ*pgpH*), BPL24 (Δ*pdeA*) and BPL89 (Δ*pgpH* Δ*pdeA*) were pre-grown overnight in BHI broth at 37°C and subsequently transferred to LSM. Cells were cultured to mid-logarithmic phase, centrifuged (5000*g*, 2 min) and fixed (1% *para*-formaldehyde, 2.5%

glutardialdehyde in 0.05 M HEPES buffer, pH 7.2) for 2 h at room temperature. Strains EGD-e (wild type) and BPL77 (Δ*cdaA*) were grown in LSM at 37°C to an OD_{600} of 0.8 and were then back-diluted to an optical density of 0.5 in BHI. Cells were afterwards cultured for 1 h at 37°C and then harvested for fixation. Scanning electron microscopy was performed essentially as described earlier (Rismondo *et al*., 2015) with the following modifications. A Leica EM CPD300 device was used and the thickness of the sputter coating was increased to 5 nm.

Biofilm assay

The *L. monocytogenes* strains were cultivated overnight in LSM at 30°C. The OD_{600} was adjusted to 0.1 and 100 µl of the diluted cultures were transferred to the wells of a 96-well microplate. Uninoculated medium served as a control. The plate was covered and incubated overnight at 37°C. The wells were rinsed by submerging the plate in clear water in the rinsing tray. The plate faced was turned down and the remaining water was removed by firmly patting the plate on a paper towel. 125 µl 0.1% (wt./vol.) CV solution was added to the wells to cover the biofilm with the stain, the plate was incubated for 10 min and the liquid was removed by inverting the plate over a waste tray. The plate was submerged in a second tray for allowing water to enter the wells. The plate faced was again turned down and the remaining water was removed by firmly patting the plate on a paper towel. The plate was dried for several hours prior to proceeding with quantitation. Biofilm formation was quantified by the addition of 2 × 200 ml of 95% ethanol to each CV-stained microplate. The ethanol was transferred to a 1.5 ml reaction tube, the volume brought to 1 ml with deionized water and the absorbance determined at a wavelength of 540 nm in a spectrophotometer. Since the Δ*cdaA*, Δ*pdeA*, Δ*pgpH* and Δ*pdeA* Δ*pgpH* mutants have a growth defect, biofilm formation was normalized to planktonic growth.

Genome sequencing

To identify the mutations in the *L. monocytogenes* suppressor mutants, genomic DNAs were subjected to sequencing at Azenta Life Sciences (Griesheim, Germany). The reads were mapped on the *L. monocytogenes* reference genome NC_003210 from GenBank (Glaser *et al*., 2001) as previously described (Widderich *et al*., 2016) using the Geneious software package (Biomatters) (Kearse *et al*., 2012).

β-Galactosidase assay

Quantitative studies of *lacZ* expression in *L. monocytogenes* were performed as described previously (Hauf *et al*., 2019).

Disc diffusion assay

The strains were grown overnight in LSM and next day, the OD_{600} was adjusted to 1.0. 100 µl of the diluted cells suspension were propagated on the plates. 10 µl of the antibiotic solutions (penicillin G, 1 mg ml^{-1}; vancomycin, 20 mg ml^{-1}; bacitracin, 100 mg ml^{-1}; fosfomycin, 50 mg ml^{-1}; cycloserine, 30 mg ml^{-1}) were added to a paper disc that was placed in the centre of the plate. The plates were incubated for 48 h at 37°C.

Analysis of c-di-AMP pools

Listeria monocytogenes was cultivated overnight in 10 ml LSM. The pre-cultures were used to inoculate 25 ml LSM to an OD_{600} of 0.1. Bacteria were incubated at 37°C with agitation (220 rpm) until they reached an OD_{600} of 0.5–0.6. At this time point (time 0 min) two times 10 ml samples for the determination of the c-di-AMP concentration and two times 1 ml samples for the determination of the protein concentration were taken (see below). The 1 ml samples for protein concentration determination were harvested by centrifugation at 20 000*g* for 1 min at 4°C and further processed as described earlier (Rismondo *et al*., 2016). The 10 ml samples for determination of the c-di-AMP concentration were rapidly cooled by swirling in liquid nitrogen, centrifuged for 5 min at 3300*g* and 4°C and the pellets were frozen in liquid nitrogen. Samples were further processed and analysed via HPLC/MS–MS as described previously (Rismondo *et al*., 2016).

Results

Phenotypes of L. monocytogenes *mutants defective in c-di-AMP synthesis and degradation*

Listeria monocytogenes cdaA mutants derived from the wild type strains 10403S and EGD-e do not grow in rich medium and 10403S-derived mutants with altered cellular c-di-AMP levels are sensitive to cell wall-targeting antibiotics (Witte *et al*., 2013; Whiteley *et al*., 2015; Rismondo *et al*., 2016; Whiteley *et al*., 2017; Massa *et al*., 2020). To gain further insights into the role of c-di-AMP for viability of the *L. monocytogenes* EGD-e strain, we inspected the phenotypes of mutants with altered cellular c-di-AMP levels. For this purpose, we constructed Δ*pgpH*, Δ*pdeA* and Δ*pgpH* Δ*pdeA* mutants lacking PgpH, PdeA and both phosphodiesterases respectively. The Δ*cdaA* mutant was previously constructed (Gibhardt

et al., 2019). The determination of the relative cellular c-di-AMP levels in cells cultivated in LS liquid medium revealed that the amounts of c-di-AMP were slightly increased in the Δ*pgpH* and Δ*pdeA* mutants, and about 1.7-fold higher in the Δ*pgpH* Δ*pdeA* mutant compared to the wild type (Fig. S1A). No c-di-AMP could be detected in the Δ*cdaA* mutant (Fig. S1A). Next, we analysed the growth of the wild type and of the Δ*cdaA*, Δ*pgpH*, Δ*pdeA* and Δ*pgpH* Δ*pdeA* mutants on BHI rich and synthetic medium (LSM) plates as well as in BHI and LS liquid medium. As previously shown for the *L. monocytogenes* 10403S strain (Whiteley *et al*., 2015), also the EGD-e-derived Δ*cdaA* mutant did not grow on BHI plates and in BHI liquid medium and the Δ*pgpH* Δ*pdeA* mutant showed a slight growth defect in BHI liquid medium (Fig. S2A and B). On listeria synthetic medium (LSM) plates and in LS liquid media, both, the Δ*cdaA* and the Δ*pgpH* Δ*pdeA* mutant had a significant growth defect (Fig. S2A and B), which was more pronounced for the Δ*pgpH* Δ*pdeA* mutant in LS liquid medium. As previously shown for the *L. monocytogenes* 10403S strain (Whiteley *et al*., 2017), the lack and accumulation of c-di-AMP also adversely affects growth of *L. monocytogenes* EGD-e under defined conditions. Previous reports describing that perturbation of c-di-AMP metabolism affects biofilm formation in a variety of bacteria (Du *et al*., 2014; Gundlach *et al*., 2016; Peng *et al*., 2016; Townsley *et al*., 2018; Fahmi *et al*., 2019; The *et al*., 2019; Faozia *et al*., 2021) stimulated us to carry out a microtiter dish-based biofilm assay, in which the extent of biofilm formation is measured using the dye crystal violet (CV). As shown in Fig. 2A, biofilm formation by the Δ*pdeA* and the Δ*pgpH* Δ*pdeA* mutants was slightly increased compared to the wild type. By contrast, the ability of the Δ*cdaA* mutant to form biofilms was severely affected (Fig. 2A). Thus, perturbation of c-di-AMP metabolism also affects biofilm formation in *L. monocytogenes* and related Gram-positive bacteria.

Next, we assessed the salt sensitivity of mutants with altered cellular c-di-AMP levels. For this purpose, we cultivated the wild type as well as the Δ*cdaA*, Δ*pgpH*, Δ*pdeA* and Δ*pgpH* Δ*pdeA* mutants on BHI and LSM plates in the absence and in the presence of 1 M NaCl. The Δ*cdaA* mutant was not propagated on BHI medium because it does not grow under these conditions (Fig. S2A and B). As shown in Fig. S2C, the Δ*pgpH* Δ*pdeA* mutant showed a growth defect on LSM and BHI plates supplemented with NaCl. On LSM plates, the Δ*cdaA* mutant also showed a growth defect, which was more pronounced in the presence of NaCl (Fig. S2C). Thus, perturbation of the cellular c-di-AMP concentration increases salt sensitivity of the *L. monocytogenes* EGD-e strain as well as of the 10403S strain (Huynh *et al*., 2016; Whiteley *et al*., 2017).

To assess the cell morphology of *L. monocytogenes* strains with altered cellular c-di-AMP concentrations, we performed scanning electron microscopy. As previously shown for the *L. monocytogenes* strain 10403S (Whiteley *et al*., 2015), also the EGD-e-derived Δ*cdaA* mutant showed a growth defect in BHI medium respectively (Fig. S2A and B). Therefore, the Δ*cdaA* mutant was first cultivated in LSM. The cells were transferred to BHI medium, incubated for 1 h at 37°C and analysed by scanning electron microscopy. As shown in Fig. 2B, the cell morphologies of the wild type and of the Δ*pgpH* and Δ*pdeA* mutants were indistinguishable. By contrast, the cells of the Δ*pgpH* Δ*pdeA* mutant were slightly curved and seemed to be smaller in diameter (Fig. 2B). The Δ*cdaA* mutant showed a strong lytic phenotype, which is likely caused by an enhanced cellular turgor due to the uncontrolled uptake of osmotically active substances via the Opp system (Figs 1 and 2B) (Whiteley *et al*., 2015). To conclude, perturbation of the cellular c-di-AMP levels affects growth, biofilm formation, salt sensitivity and the morphology of *L. monocytogenes*.

Listeria monocytogenes *mutant strains with high cellular c-di-AMP concentrations exclusively accumulate mutations in* cdaA

As shown above, the Δ*pgpH* Δ*pdeA* mutant accumulating c-di-AMP has a growth defect in LSM (Fig. S2A–C). Since the DNA-binding activity of CodY is indirectly controlled via the c-di-AMP-binding protein CbpB, the accumulation of c-di-AMP in the Δ*pgpH* Δ*pdeA* mutant probably enhances the repression of the CodY regulon, among them genes required for *de novo* synthesis and uptake of amino acids (Fig. 1). LSM contains BCAAs among them 0.8 mM isoleucine that activates CodY (Whiteley *et al*., 2015; Biswas *et al*., 2020). The enhanced DNA-binding activity of CodY could therefore cause the amino acid limitation and thus the growth defect in LSM. The supplementation of LSM with casamino acid hydrolysate could indeed slightly enhance the growth of the Δ*pgpH* Δ*pdeA* mutant (Fig. S3). To find the cause for the growth defect of the Δ*pgpH* Δ*pdeA* mutant on LSM, we performed a suppressor screen. For this purpose, the Δ*pgpH* Δ*pdeA* mutant was propagated on LSM plates that were incubated for 96 h until individual colonies emerged. In this initial screen, we isolated 16 suppressor mutants that could be assigned to two classes: seven mutants only grew on LSM plates and nine mutants grew on both, LSM and BHI plates (Table 1). Genome sequencing analyses of one mutant of each class revealed that always the *cdaA* gene was mutated. In the remaining mutants, the sequence of the *cdaA* gene was determined by Sanger sequencing. With the exception of one mutant (suppressor S15), the

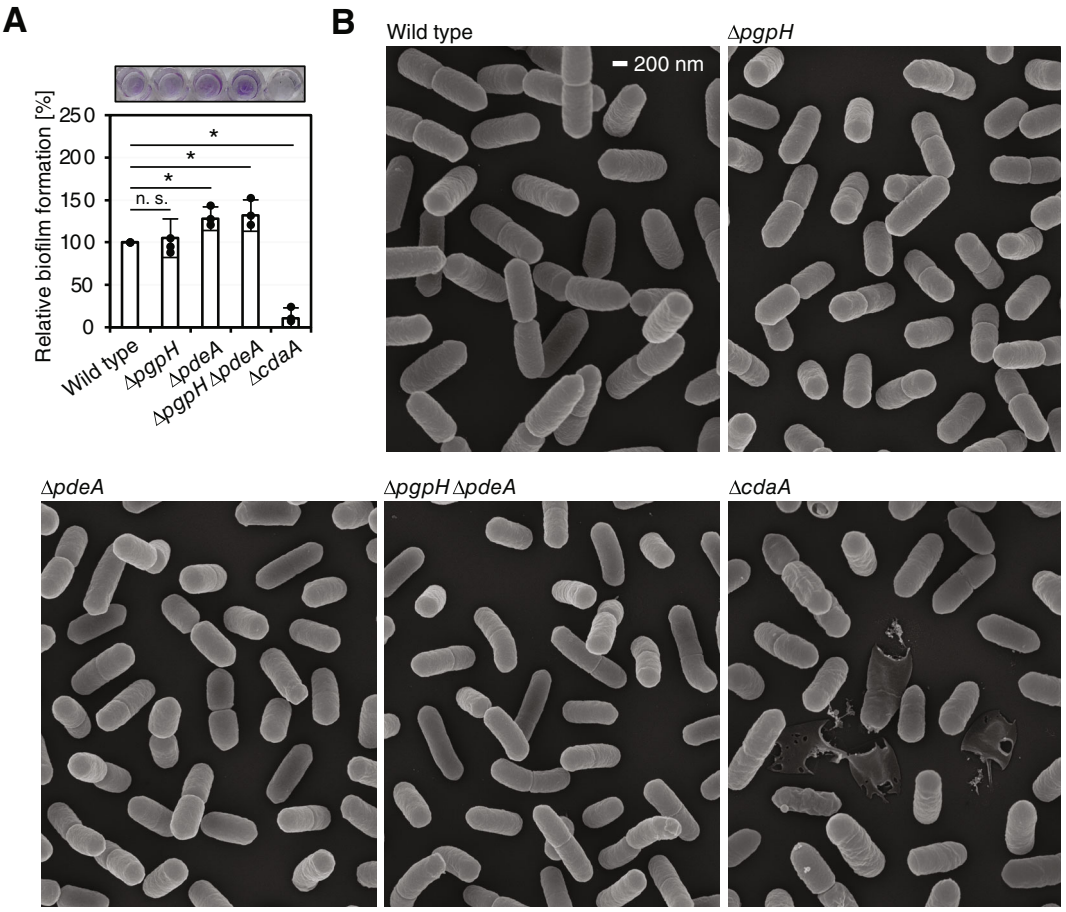

Fig. 2. Biofilm formation and morphological characterization of *L. monocytogenes* mutants with defects in c-di-AMP metabolism.
A. Relative biofilm formation of the wild type and the strains BPL23 (Δ*pgpH*), BPL24 (Δ*pdeA*), BPL89 (Δ*pgpH* Δ*pdeA*) and BPL77(Δ*cdaA*) using a microtiter dish- and CV-based assay. Since the Δ*cdaA*, Δ*pdeA*, Δ*pgpH* and Δ*pdeA* Δ*pgpH* mutants have a growth defect, biofilm formation was normalized to planktonic growth. For statistical analysis, we performed multiple *t*-tests and *p*-values less than 0.05 were considered as statistically significant (*): wild type versus Δ*pgpH*, $p = 0.7224$ (no significant difference, n.s.); wild type versus Δ*pdeA*, $p = 0.0251$; wild type versus Δ*pgpH* Δ*pdeA*, $p = 0.0411$; wild type versus Δ*cdaA*, $p = 0.0002$.
B. Scanning electron microscopy images of the *L. monocytogenes* wild type EGD-e and the strains BPL23 (Δ*pgpH*), BPL24 (Δ*pdeA*), BPL89 (Δ*pgpH* Δ*pdeA*) and BPL77 (Δ*cdaA*).

mutants that only grew on LSM had acquired single-nucleotide polymorphisms (SNPs) that likewise would truncate CdaA (Table 1). These mutants probably no longer synthesize c-di-AMP, which is essential for growth on rich medium such as BHI (Whiteley *et al*., 2015). The mutants that grew on LSM and BHI plates had acquired SNPs in the *cdaA* gene and in the promoter region of *cdaA* (Fig. 3A and B; Table 1). These mutants likely still synthesize c-di-AMP, albeit to a lesser extent. To conclude, the Δ*pgpH* Δ*pdeA* mutant suppresses the growth defect on LSM plates either by inactivating the *cdaA* gene or by acquiring mutations to reduce c-di-AMP biosynthesis.

To decrease the probability that Δ*pgpH* Δ*pdeA* suppressors would emerge on agar plates that did inactivate the *cdaA* gene, we constructed the strain BPL91 (Δ*pgpH* Δ*pdeA* I*cdaA*) carrying the native *cdaA* gene and an IPTG-inducible *cdaA* copy in the genome. Compared to the strain BPL89 carrying only one *cdaA* copy, growth of the strain BPL91 was strongly inhibited in BHI and LSM containing 1 mM IPTG (Fig. 3C). Next, we propagated the strain BPL91 on LSM agar plates supplemented with 1 mM IPTG. This time, the suppressors emerged only after 72 h of incubation, indicating that the selective pressure acting on the bacteria was stronger. We isolated four suppressor mutants designated as Sa, Sb, Sc and Sd and determined the sequences of both *cdaA* alleles by Sanger sequencing. As in the previous suppressor analysis, the mutants had acquired SNPs in one of the two *cdaA* copies that likely reduce the activity of the encoded enzyme (Table 1). To conclude, the Δ*pgpH* Δ*pdeA* mutant preferentially mutates the *cdaA* gene to reduce c-di-AMP accumulation.

Table 1. Suppressor mutants derived from the strains BPL89 (Δ*pgpH* Δ*pdeA*), BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*) and BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*).

CdaA loss-of-function mutants

Suppressor mutant	Strain	Parental strain (genotype)	Phenotype	Affected genes, mutations	Effect on the protein
S2	BPL106	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, ΔC359	C-terminally truncated by 143 amino acids
S13	BPL107	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, C469T	C-terminally truncated by 117 amino acids
S14	BPL108	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, G257T	R86L
S15	BPL109	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaAR* deletion	No synthesis of CdaA and CdaR
S16	BPL110	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, ΔA412	C-terminally truncated by 124 amino acids
S17	BPL111	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, ΔA539	C-terminally truncated by 61 amino acids
S18[a]	BPL112	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM plates	*cdaA*, G216A *rsbU* Δ286-389	C-terminally truncated by 202 amino acids
LSM2	BPL130	BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*)	Growth on LSM plates +200 mM NaCl, blue colonies	*cdaA*, ΔA13	C-terminally truncated by 262 amino acids

Mutants with decreased CdaA activity or c-di-AMP synthesis

Suppressor mutant	**Strain**	**Parental strain (genotype)**	**Phenotype**	**Affected genes, mutations**	**Effect on the protein**
S3[a]	BPL113	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, C623T	R205W
S4	BPL114	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, G514A	G172R
S7	BPL115	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, G281A	R94Q
S9	BPL116	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, G614A	R205Q
S10	BPL117	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, A251G	E84G
S11	BPL118	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	ΔT-65 nt upstream of ATG of *cdaA*	Reduced *cdaA* expression?
S19	BPL119	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, G574T	D192Y
S20	BPL120	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, G101A	G34D
S21	BPL121	BPL89 (Δ*pgpH* Δ*pdeA*)	Growth on LSM and BHI plates	*cdaA*, C641T	T214I
Sa	BPL122	BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*)	Growth on LSM plates	*cdaA*, C610T	H204Y
Sb	BPL123	BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*)	Growth on LSM plates	*cdaA*, C274T	L92F
Sc	BPL124	BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*)	Growth on LSM plates	*cdaA*, G128T	G43V
Sd	BPL125	BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*)	Growth on LSM plates	*cdaA*, G595A	E199K
Se	BPL126	BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*)	Growth on LSM plates	*cdaA*, G416A	G139D
BHI1	BPL127	BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*)	Growth on LSM plates +200 mM NaCl and BHI plates, blue colonies	*cdaA*, G420A	M140I
BHI2	BPL128	BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*)	Growth on LSM plates +200 mM NaCl and BHI plates, blue colonies	*cdaA*, C579A	S193R
LSM1	BPL129	BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*)	Growth on LSM plates +200 mM NaCl plates, blue colonies	T → C -65 nt upstream of ATG of *cdaA*	Reduced *cdaA* expression?

[a]Strains were genome sequenced.

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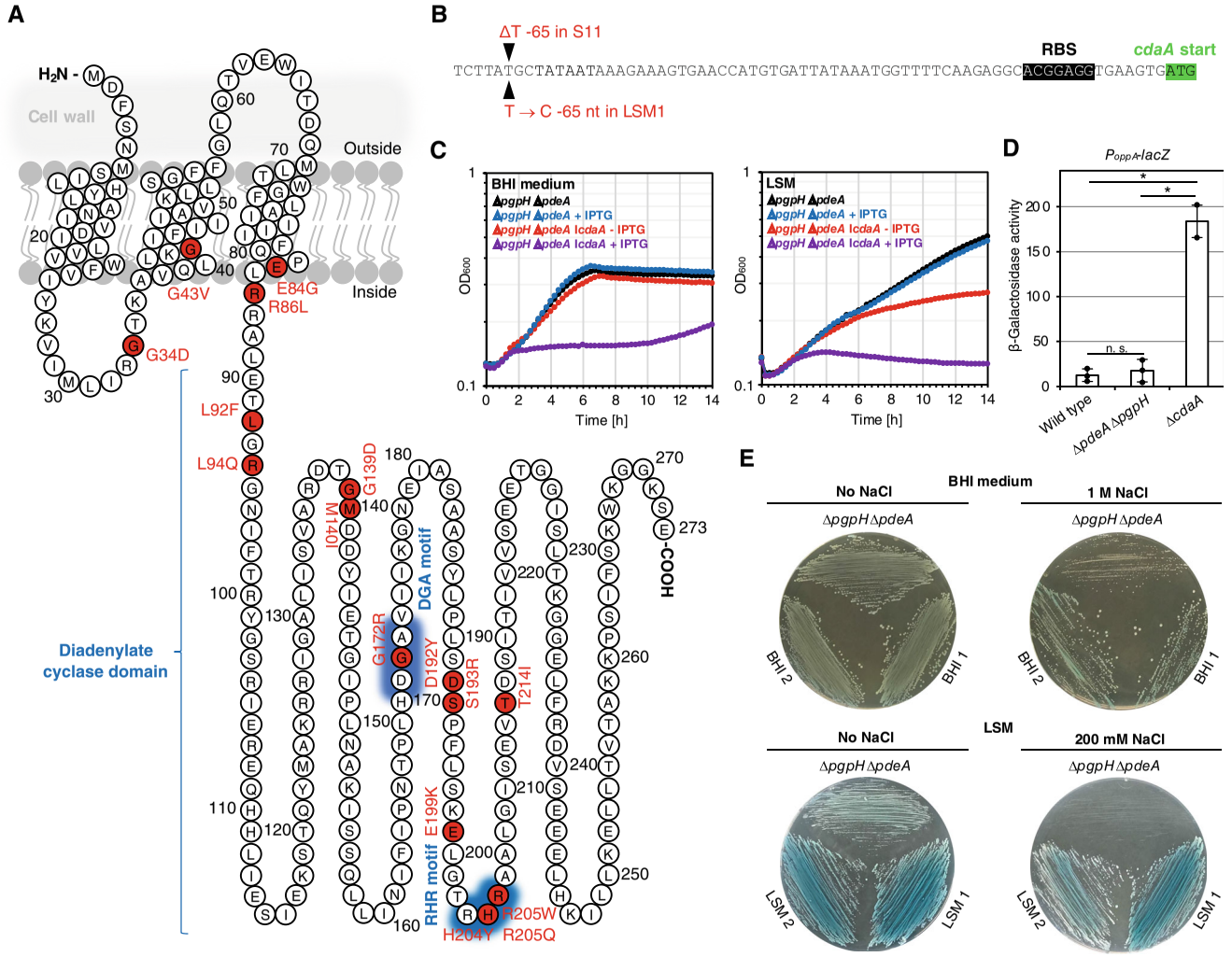

Fig. 3. Characterization of Δ*pgpH* Δ*pdeA* suppressor mutants.
A. Amino acid exchanges in CdaA suppressing the growth defect of the strain BPL89 (Δ*pgpH* Δ*pdeA*). The conserved DGA and RHR motifs highlighted in blue are essential for catalytic activity of CdaA.
B. Mutations identified in the suppressors S11 and LSM1 of the strain BPL89 (Δ*pgpH* Δ*pdeA*) that likely affect the expression of the *cdaA* gene.
C. Growth of the strains BPL89 (Δ*pgpH* Δ*pdeA*) and BPL91 (Δ*pgpH* Δ*pdeA* lc*daA*) in BHI and LSM in the absence and in the presence of 1 mM IPTG at 37°C.
D. β-Galactosidase activity assays to monitor the effect of the *cdaA* deletion on the activity of the P_{oppA} promoter. The assay was performed with the *L. monocytogenes* wild type BPL93 (P_{oppA}-*lacZ*), the *pdeA pgpH* mutant BPL96 (Δ*pdeA* Δ*pgpH* P_{oppA}-*lacZ*) and the *cdaA* mutant BPL99 (Δ*cdaA* P_{oppA}-*lacZ*). The bacteria were cultivated in LSM. Data points represent biologically independent replicates. Bars indicate means of replicates and the standard deviations are shown. β-Galactosidase activities are given as units per milligram of protein. For statistical analysis, we performed multiple *t*-tests and *p*-values less than 0.05 were considered as statistically significant (*): wild type versus Δ*pdeA* Δ*pgpH*, $p = 0.5785$ (no significant difference, n.s.); wild type versus Δ*cdaA*, $p = 0.0001$; Δ*pdeA* Δ*pgpH* versus Δ*cdaA*, $p = 0.0001$.
E. Growth of the strain BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*) and its suppressors BHI1, BHI2, LSM1 and LSM2 on BHI and LSM plates in the absence and in the presence of the indicated amounts of NaCl for 24 h at 37°C.

Development of a screening system indicating the intracellular c-di-AMP levels in L. monocytogenes

The finding that c-di-AMP indirectly controls the activity of CodY inspired us to construct a translational P_{oppA} promoter *lacZ* fusion that could serve as an indicator for the intracellular c-di-AMP levels (Fig. 1). For this purpose, the P_{oppA} promoter (597 bp) was fused in frame to *lacZ* and integrated into the wild type and the Δ*pgpH*, Δ*pdeA*, Δ*pgpH* Δ*pdeA* and Δ*cdaA* strains defective in c-di-AMP degradation and synthesis (Fig. S1A). A subsequent β-galactosidase assay with the strains BPL93 (P_{oppA}-*lacZ*), BPL96 (Δ*pdeA* Δ*pgpH* P_{oppA}-*lacZ*) and BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) grown in LSM showed that the P_{oppA} promoter in the Δ*cdaA* mutant was about 10-fold and 15-fold higher compared to the Δ*pdeA* Δ*pgpH* mutant and the wild type respectively (Fig. 3D). Next, we propagated the Δ*cdaA*, Δ*pgpH*, Δ*pdeA* and Δ*pgpH* Δ*pdeA* mutants on BHI and LSM plates supplemented with X-Gal. As shown in Fig. 4A, the wild type as well as the Δ*pgpH*, Δ*pdeA* and Δ*pgpH* Δ*pdeA* mutants formed white colonies on the plates. Under these conditions, the CbpB-mediated de-repression of the P_{oppA} promoter is not visible due to the higher intracellular c-di-AMP levels (Fig. S1A). By contrast, the cells of the Δ*cdaA* mutant turned blue on LSM plates, indicating that the P_{oppA} promoter-*lacZ* fusion responds to c-di-AMP.

To assess whether the P_{oppA} promoter-*lacZ* fusion could be useful for the initial classification of mutants with altered intracellular c-di-AMP levels, we performed another suppressor screen with the strain BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*) on BHI and LSM plates supplemented with 1 M NaCl and 200 mM NaCl respectively. The salt was added to the media to increase the selective pressure acting on the high-c-di-AMP strain (Fig. S2C). A close inspection of the agar plates containing the

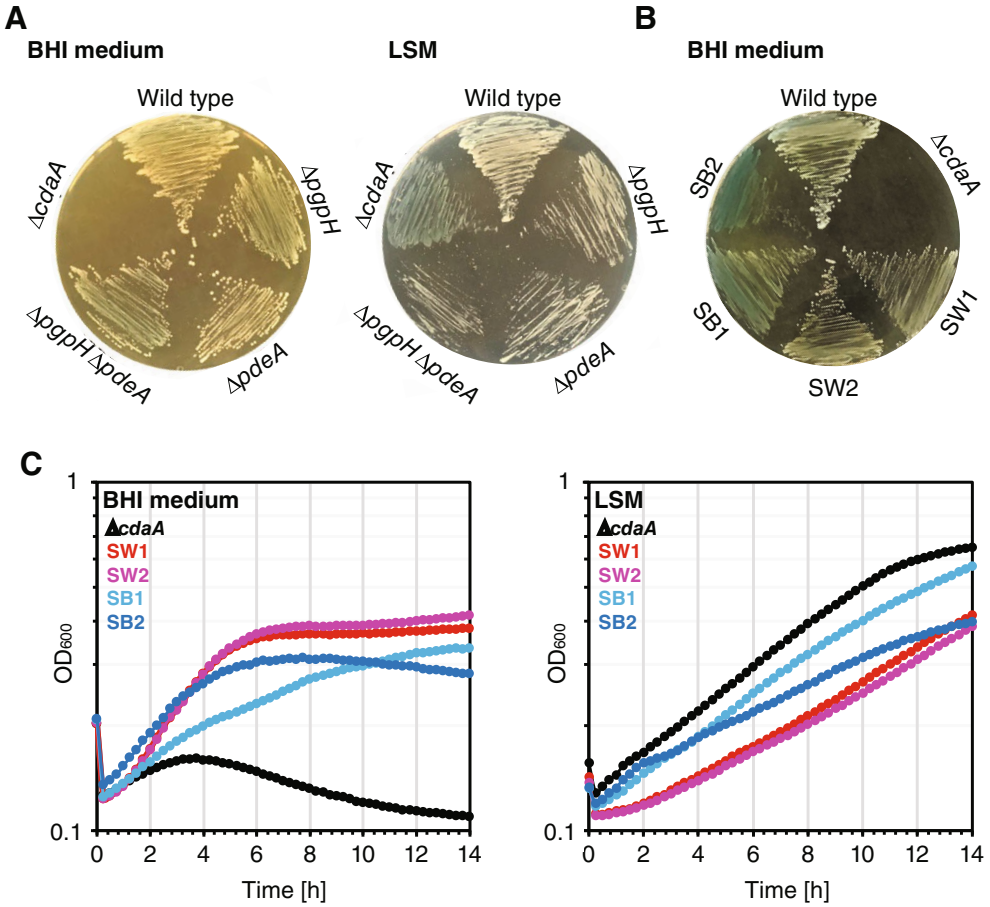

Fig. 4. Characterization of suppressors of a *cdaA* mutant strain.
A. Growth of the *L. monocytogenes* strains BPL93 (P_{oppA}-*lacZ*), BPL102 (Δ*pgpH* P_{oppA}-*lacZ*), BPL103 (Δ*pdeA* P_{oppA}-*lacZ*), BPL96 (Δ*pgpH* Δ*pdeA* P_{oppA}-*lacZ*) and BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) on BHI and LSM plates supplemented with X-Gal.
B. Growth of the wild type BPL93 (P_{oppA}-*lacZ*), the *cdaA* mutant BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) and the *cdaA* suppressors SB1, SB2, SW1 and SW2 on BHI plates. The plates were incubated for 24 h at 37°C.
C. Growth of the strains BPL93 (P_{oppA}-*lacZ*) and BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) and the suppressors SW1, SW2, SB1 and SB2 derived from the strain BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) in BHI and LS liquid medium. X-Gal-containing agar plates were incubated for 24 h at 37°C.

suppressors revealed that without exception all papilla turned blue (Fig. S1B), suggesting that the mutants exclusively accumulated mutations in the *cdaA* gene for reducing c-di-AMP accumulation. Sequencing analyses of two suppressors from BHI and LSM plates (designated as BHI 1, BHI 2 and LSM 1, LSM 2 respectively; Fig. 4B) revealed that the bacteria had indeed acquired mutations in *cdaA*, which would prevent c-di-AMP accumulation (Table 1). Thus, a phosphodiesterase-deficient strain counteracts c-di-AMP accumulation either by *cdaA* inactivation or reduction of CdaA activity. However, the P_{oppA} promoter-*lacZ* fusion is suitable for displaying the intracellular c-di-AMP levels in *L. monocytogenes*.

Isolation and characterization of suppressors of a cdaA *mutant strain*

A c-di-AMP-free *L. monocytogenes* strain is viable in rich medium due to mutations in the *relA* (p)ppGpp synthetase/hydrolase gene. The reduced consumption of GTP by the (p)ppGpp synthetase activity of RelA allows the GTP-responsive transcriptional regulator CodY to prevent the expression of the *opp* genes and thus uptake of oligopeptides (Whiteley *et al*., 2015; Peterson *et al*., 2020) (Fig. 1). A *cdaA* mutant is also viable in rich medium when the *opp* genes and the *gbuABC* glycine betaine uptake genes were inactivated (Whiteley *et al*., 2015; Whiteley *et al*., 2017). Here, we aimed to assess whether the P_{oppA} promoter-*lacZ* fusion is suitable to classify Δ*cdaA* suppressor mutants emerging on rich medium plates. We suspected that the blue/white colouring of the suppressors would be indicative for the type of mutation that could affect the activity of CodY or osmolyte uptake systems (Fig. 1). We also intended to identify novel mutations that could contribute to the dispensability of c-di-AMP for growth of *L. monocytogenes* in rich medium. For this purpose, we propagated the strain BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) on BHI plates supplemented with X-Gal. After 72 h of incubation, suppressors appeared on the plates at a frequency of 2×10^{-6} (24%–29% white and 71%–76% blue suppressors) (Fig. S1C). Two mutants of each class were isolated for further characterization and genome sequencing analysis (Fig. 4B). Next, we performed growth experiments with the parental strain BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) and the suppressor mutants in BHI and LSM. As expected, the parental strain grew only in LSM (Fig. 4C). The four suppressors could grow in both BHI and LSM. While the white mutants grew better than the blue mutants in BHI medium, the opposite was the case in LSM (Fig. 4C). The different phenotypes of the mutants can likely be explained by the acquired mutations (see below).

Genome sequencing analysis revealed that the white mutants SW1 and SW2 had acquired mutations in the *relA* gene. The RelA R295S mutant variant that is synthesized by SW1 was previously shown to have reduced (p)ppGpp synthase activity (Whiteley *et al*., 2015). Therefore, the reduced conversion of GTP to (p)ppGpp in SW1 and probably also in SW2 enables CodY to repress the *opp* genes, which in turn would allow the c-di-AMP-free suppressors to grow in rich medium (Fig. 1) (Whiteley *et al*., 2015). Like the Δ*pgpH* Δ*pdeA* phosphodiesterase mutant also the white mutants showed a growth defect in LSM (see Fig. S2B and Fig. 4C). It is reasonable to assume that both, the reduction of the (p)ppGpp synthase activity of RelA and the accumulation of c-di-AMP, increase the activity of CodY, which could lead to defects in amino acid biosynthesis and uptake during growth of the bacteria in defined medium (Fig. 1). Moreover, the white mutants had acquired mutations preventing the synthesis and the activity of the Gbu glycine betaine transport system (Table 2). Our suppressor analysis is consistent with a previous study showing that the inactivation of the *gbuABC* genes disrupts the intracellular osmotic pressure and enables a Δ*cdaA* mutant to grow in rich medium (Whiteley *et al*., 2017). The mutant SW2 also had acquired an SNP in the *lmo1418* gene (*yqgS* in *B. subtilis*) encoding the polyglycerolphosphate lipoteichoic acid (LTA) synthase (Table 2). Previously, it has been observed that an LTA-deficient *S. aureus* mutant inactivates the *gdpP* phosphodiesterase gene, which enhances cellular c-di-AMP levels and thus decreases the turgor pressure due to reduced uptake of osmolytes (Corrigan *et al*., 2011). Therefore, it is tempting to speculate that altered LTA biosynthesis could help to osmotically stabilize the c-di-AMP-free suppressor SW2. Finally, the mutants SW1 and SW2 had inactivated the *rsbU* and *rsbT* genes (Table 2) respectively, which are part of the stress-inducible sigma factor sigma B (σ^B) operon (Liu *et al*., 2017). Recently, it has been shown that mutations in the *rsbU* and *rsbT* genes attenuating σ^B activity arise during laboratory culture because they confer a growth advantage under mild stress conditions (Guerreiro *et al*., 2020). Therefore, the mutations in the *rsbU* and *rsbT* genes of which one also occurred in the genome-sequenced blue suppressors [see below (Table 2)] probably arose spontaneously during evolution of the Δ*cdaA* mutant and are not linked to c-di-AMP metabolism.

The blue suppressors SB1 and SB2 had acquired SNPs in the *oppF* and *oppC* genes that likewise would truncate the encoded proteins and prevent oligopeptide uptake via the Opp system (Table 2). Moreover, SB1 had inactivated the *gbuA* gene of the Gbu osmolyte transport system. Sequencing analysis of the *opp* genes of three additional blue suppressors (SB3, SB4 and SB5) also uncovered inactivating mutations in *oppF* genes

Table 2. Suppressor mutants derived from the strain BPL77 (Δ*cdaA*).

Suppressor mutant	Strain	Parental strain (genotype)	Phenotype	Affected genes, mutations	Effect on the protein
SW1[a]	BPL132	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, white colonies	*relA*, C883A	R295S
				gbuA, G82T in promoter	Reduced *gbuA* expression?
				rsbU, Δ823-831 GTAACAGAA	ΔVTE 275–277
SW2[a]	BPL133	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, white colonies	*relA*, C844T	L282F
				gbuC, T398A	C-terminally truncated by 168 amino acids
				rsbT, Δ73A	C-terminally truncated by 91 amino acids
				yqgS, C415T	H139Y
				lmo2295, C89A	A30D
SB1[a]	BPL134	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, blue colonies	*oppF*, C397T	C-terminally truncated by 190 amino acids
				gbuB, insertion of C6	C-terminally truncated by 280 amino acids
				rsbU, Δ823-831 GTAACAGAA	ΔVTE 275–277
SB2[a]	BPL135	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI medium, blue colonies	*oppC*, C199T	C-terminally truncated by 278 amino acids
				rsbU, Δ823-831 GTAACAGAA	ΔVTE 275–277
				walK, T812C	V271A
SB3	BPL136	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, blue colonies, increased fosfomycin resistant	*oppF*, insertion of C199	C-terminally truncated by 250 amino acids
SB4	BPL137	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, blue colonies, increased fosfomycin resistant	*oppF*, Δ903-905 AGA	Δ302E Glutamic Acid
SB5	BPL138	BPL99 (Δ*cdaA* P_{oppA}-*lacZ*)	Growth on BHI plates, blue colonies, increased fosfomycin resistant	*oppF*, Δ473-478 GTCAAC	Δ159-160QR Glutamine and Arginine
SL1[a]	BPL139	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*rsbV*, G196T	C-terminally truncated by 49 amino acids
				codY, C533T	S178L
SL2[a]	BPL140	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*rsbU*, G623A	G208E
				codY, G301A	E101K
				metN, G128A	G43D
SL3	BPL141	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*codY*, G128T	R61L
SL4	BPL142	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*codY*, Δ415-417 GAT	ΔD139
SL5	BPL143	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*codY*, Δ494-496 AAG	ΔE165
SL6	BPL144	BPL77 (Δ*cdaA*)	Growth on LSM plates with 15 g L^{-1} isoleucine	*codY*, C181T	R61C

[a]Strains were genome sequenced.

(Table 2). Thus, the blue suppressors having de-repressed the CodY regulon primarily cope with osmosensitivity by acquiring loss-of-function mutations in the *opp* osmolyte transporter genes. The mutant SB2 also had acquired an SNP in the *walK* gene encoding the WalK sensor kinase of essential WalRK two-component system, which controls cell wall metabolism in low GC Gram-positive bacteria (Dubrac *et al*., 2008). The V271A exchange occurred in the PAS signal sensor domain of WalK. To conclude, our suppressor screen confirmed that the essentiality of c-di-AMP for growth of *L. monocytogenes* in rich medium is mainly suppressed in two ways: (i) by mutations increasing CodY activity and (ii), by mutations reducing the import of osmotically active substances. Moreover, alterations in cell wall metabolism may help to osmotically stabilize a c-di-AMP-free *L. monocytogenes* mutant.

The L. monocytogenes Δ*cdaA mutant is sensitive to isoleucine*

A Δ*cdaA* mutant is unable to grow in BHI and in LSM supplemented with casamino acids due to the presence of oligopeptides (Rismondo *et al*., 2015; Whiteley *et al*., 2015, 2017) (Figs S2A, B and S3). Here, we assessed whether also individual amino acids are toxic for the Δ*cdaA* mutant. For this purpose, we cultivated the c-di-AMP-free strain in LSM containing individual amino acids that were added to a final concentration of 5 g l^{-1}. Of the 18 amino acids tested, only isoleucine specifically inhibited growth of the Δ*cdaA* mutant even at lower concentrations (e.g. 2.5 g L^{-1}, 20 mM) (Fig. 5A). Thus, the lack of c-di-AMP increases isoleucine sensitivity of *L. monocytogenes*. To elucidate the reason for isoleucine toxicity, we performed a suppressor screen by propagating the Δ*cdaA* mutant on LSM supplemented with 15 g L^{-1} isoleucine. After 72 h of incubation, several single colonies appeared on the plates. Six suppressor mutants were isolated and cultivation experiments with two suppressors (SL1 and SL2) revealed that they became indeed resistant to 2.5 g L^{-1} isoleucine (compare Fig. 5A and B). Genome sequencing analyses uncovered that both mutants had acquired a mutation in the *codY* gene resulting in the amino acid exchanges S178L and E101K in CodY (Fig. 5C; Table 2). The mutant SL2 contains an additional SNP in the *lmo2419* gene, causing the G43D amino acid exchange in the ATP-binding subunit of a putative MetN methionine transporter (Hullo *et al*., 2004). Sanger sequencing revealed that the remaining Δ*cdaA* isoleucine suppressors produce the CodY R61L, R61C, ΔE139 and ΔE165 variants (Fig. 5C; Table 2). Previously, it has been observed that the CodY R61A variant synthesized in *L. monocytogenes* and *B. subtilis* shows reduced isoleucine responsiveness and DNA-binding activity (Villapakkam *et al*., 2009). Moreover, the CodY E101K and R61K variants were isolated in a suppressor screen with a *B. subtilis* strain whose growth depends on CodY with reduced DNA-binding activity (Brinsmade and Sonenshein, 2011). In fact, CodY E101K and R61K are loss-of-function variants. Since the S178 residue is located in the winged helix–turn–helix motif of CodY, the S178L exchange probably negatively affects the DNA-binding activity of the protein (Fig. 5C). The ability of the truncated CodY ΔE139 and ΔE165 variants to bind to DNA is likely also impaired. To conclude, the lack of c-di-AMP seems to facilitate the uptake of isoleucine via unknown transport systems which in turn leads to CodY hyperactivity and thus reduced expression of genes for uptake and/or *de novo* synthesis of amino acids (see Discussion). The isoleucine-dependent growth defect of the *cdaA* mutant is likely relieved by mutations in the *codY* gene encoding CodY variants with reduced DNA-binding activity.

Inactivation of the opp *genes confer resistance to fosfomycin*

As shown above, the suppressors of the Δ*cdaA* mutant had acquired mutations in LTA and cell wall biosynthesis genes (see above). Therefore, we hypothesized that alterations in cell envelope integrity could alter the sensitivity of the suppressors towards cell wall-targeting antibiotics. To test this idea, we performed disc diffusion assays with the parental strain BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) and the suppressors SW1, SW2, SB1 and SB2 (see Table 2) using the cell wall-targeting antibiotics bacitracin, cycloserine fosfomycin, penicillin G and vancomycin. All strains showed a comparable sensitivity towards vancomycin and the other antibiotics (Fig. S4; data not shown). By contrast, fosfomycin sensitivity of the strains SB1 and SB2 was strongly reduced (Fig. 6A). Since SB1 and SB2 both had acquired loss-of-function mutations in the *opp* genes, the Opp oligopeptide system is probably involved in the uptake of fosfomycin, which inhibits the essential UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase MurA catalysing the first committed step in peptidoglycan (PG) biosynthesis (Kahan *et al*., 1974; Raz, 2012; Chekan *et al*., 2016). To assess whether the inactivation of the *opp* system alone reduces fosfomycin sensitivity in the *L. monocytogenes* wild type background, we deleted the *oppF* gene in the wild type strain and performed growth assays. The *oppF* mutant showed a strong growth defect in BHI medium (Fig. 6B), confirming a major role of the Opp system in oligopeptide uptake in *L. monocytogenes* (Borezee *et al*., 2000). Despite the importance of the Opp system for growth in rich medium, only the *oppF* mutant could grow in the

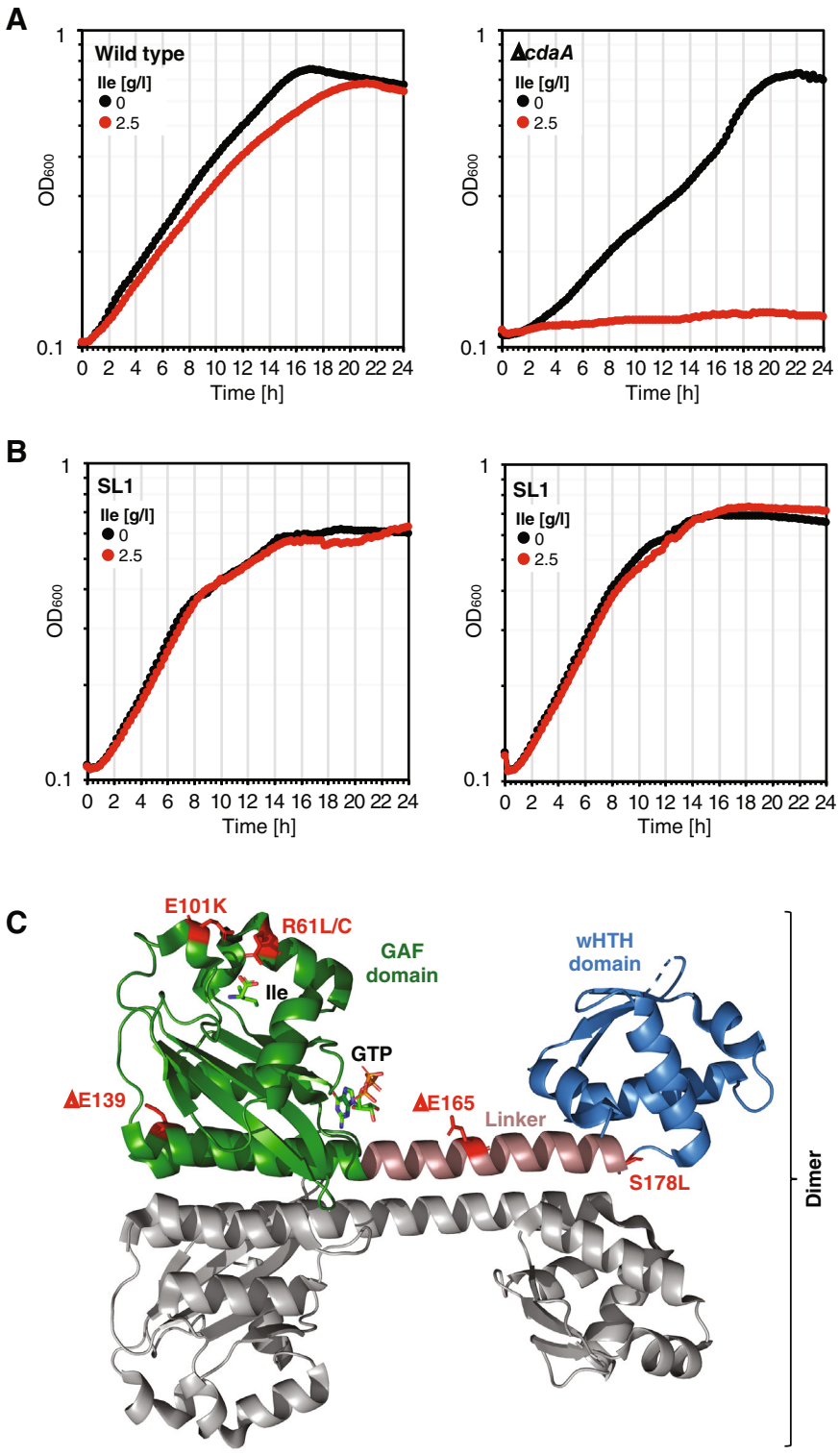

Fig. 5. Characterization *cdaA* isoleucine suppressors.
A. Growth of the wild type and of the *cdaA* mutant BPL77 (Δ*cdaA*) in LSM in the absence and presence of isoleucine at 37°C.
B. Growth of the *cdaA* isoleucine suppressors SL1 and SL2 in LSM in the absence and presence of isoleucine at 37°C.
C. Localization of the amino acid exchanges in CodY that affect the DNA-binding activity. Amino acid residues that are exchanged or missing in the *cdaA* isoleucine suppressors are indicated in red in a CodY structure model (PDBid 5EY0; Han *et al*., 2016).

presence of 100 μg ml^{-1} fosfomycin (Fig. 6B). Next, we compared the fosfomycin resistance of the *oppF* mutant with that of a newly constructed *hpt* mutant in a disc diffusion assay. The Hpt hexose phosphate transporter was previously shown to be involved in fosfomycin uptake in *L. monocytogenes* (Scortti *et al*., 2006, 2018). The *oppF* mutant and to a lesser extent also the *hpt* mutant showed increased fosfomycin resistance on BHI medium and LSM plates compared to the wild type strain (Fig. 6C).

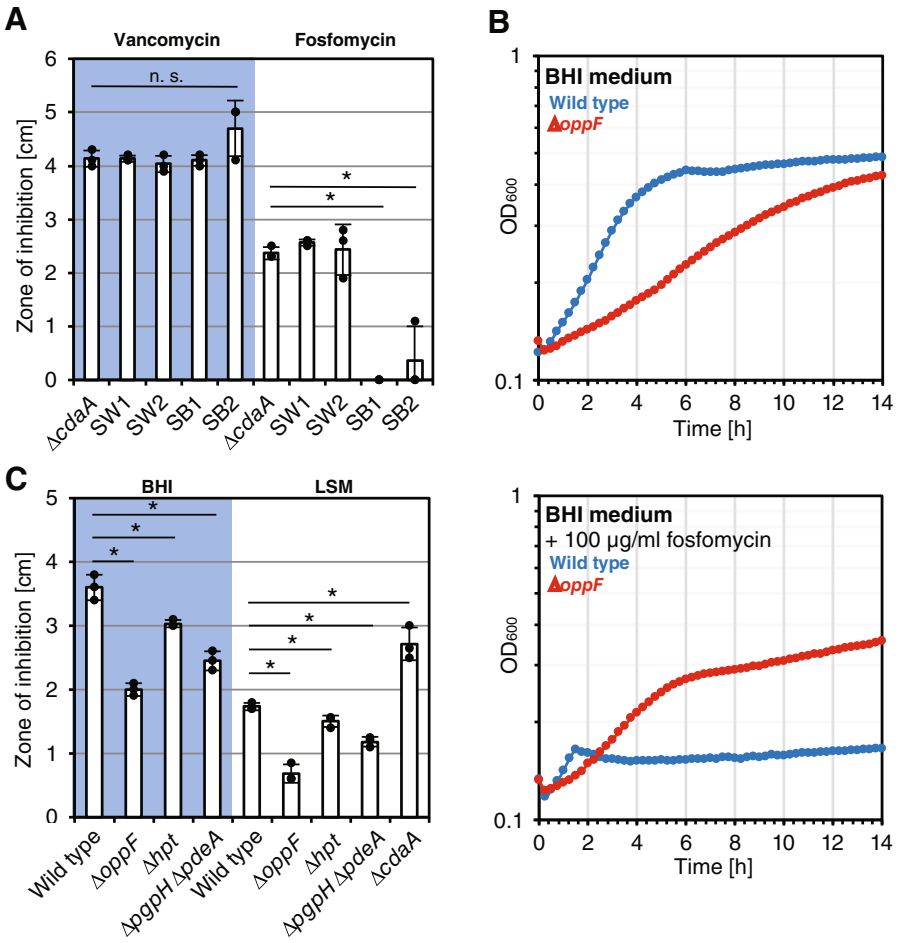

Fig. 6. Inactivation of the *opp* genes confer resistance to fosfomycin.
A. Disc diffusion assay to assess the susceptibilities of the strains BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) and the suppressors SW1, SW2, SB1 and SB2 derived from the strain BPL99 (Δ*cdaA* P_{oppA}-*lacZ*) towards vancomycin and fosfomycin on LSM plates. Each data point represents an independent replicate and bars indicated means of replicates. For statistical analysis, we performed multiple *t*-tests and *p*-values less than 0.05 were considered as statistically significant (*). Vancomycin assay: Δ*cdaA* versus SW1, $p > 0.9999$ (no significant difference, n.s.); Δ*cdaA* versus SW2, $p = 0.4676$ (n.s.); Δ*cdaA* versus SB1, $p = 0.7676$ (n.s.); Δ*cdaA* versus SB2, $p = 0.1441$ (n.s.). Fosfomycin assay: Δ*cdaA* versus SW1, $p = 0.0551$ (n.s.); Δ*cdaA* versus SW2, $p = 0.8240$ (n.s.); Δ*cdaA* versus SB1, $p = 0.0000$; Δ*cdaA* versus SB2, $p = 0.0058$.
B. Effect of fosfomycin on growth of the *L. monocytogenes* EGD-e wild type strain and the strain BPL105 (Δ*oppF*) in BHI medium at 37°C.
C. Disc diffusion assay to assess the susceptibilities of the *L. monocytogenes* EGD-e wild type strain and of the strains BPL105 (Δ*oppF*), BPL106 (Δ*hpt*), BPL89 (Δ*pgpH* Δ*pdeA*) and BPL77 (Δ*cdaA*) towards fosfomycin on LSM and BHI plates. Each data point represents an independent replicate and bars indicated means of replicates. Each data point represents an independent replicate and bars indicated means of replicates. For statistical analysis, we performed multiple *t*-tests and *p*-values less than 0.05 were considered as statistically significant (*). BHI plates: Wild type versus Δ*oppF*, $p = 0.0002$; Wild type versus Δ*hpt*, $p = 0.0092$; Wild type versus Δ*pgpH* Δ*pdeA*, $p = 0.0013$. LSM plates: Wild type versus Δ*oppF*, $p = 0.0003$; Wild type versus Δ*hpt*, $p = 0.0202$; Wild type versus Δ*pgpH* Δ*pdeA*, $p = 0.0006$; Wild type versus Δ*cdaA*, $p = 0.0029$.

As shown above, the expression of the CodY-dependent *opp* genes was strongly increased in the c-di-AMP-free *cdaA* mutant (Fig. 3D). Therefore, alterations of the cellular c-di-AMP concentrations could result in altered fosfomycin uptake via the Opp system and thus in a sensitivity change of *L. monocytogenes* to fosfomycin. To test this idea, we performed disc diffusion assays with the wild type and the Δ*pgpH* Δ*pdeA* and Δ*cdaA* mutants defective in c-di-AMP degradation and synthesis respectively. As shown in Fig. 6C, the Δ*pgpH* Δ*pdeA* mutant with elevated cellular c-di-AMP levels was less sensitive to fosfomycin than the wild type and the Δ*cdaA* mutant. By contrast, the sensitivity of the c-di-AMP-free Δ*cdaA* mutant was increased compared to the wild type and the Δ*pgpH* Δ*pdeA* mutant on LSM plates. Thus, the high-level expression of the *opp* genes in the *cdaA* mutant indeed increase the sensitivity of the bacteria towards fosfomycin. It is unclear why the expression of the *opp* genes does not differ in the Δ*pgpH* Δ*pdeA* mutant and in the wild type, although the two strains differ in terms of

fosfomycin resistance (Figs 3D and 6C). It is tempting to speculate that the accumulation of c-di-AMP in the Δ*pgpH* Δ*pdeA* mutant inhibits the uptake of fosfomycin via other transport systems. However, along with other transporters such as Hpt, the Opp system might contribute to the fosfomycin uptake in *L. monocytogenes*.

Effect of enhanced PG precursor biosynthesis on growth of a cdaA *mutant*

For several bacteria it has been shown that a reduction of the cellular c-di-AMP levels and the lack of c-di-AMP result in uncontrolled uptake of osmolytes and thus cell lysis (Commichau *et al*., 2018). Previously, it has been shown that ReoM serves as an effector protein for ClpCP-dependent proteolytic degradation of the enzyme MurA (Wamp *et al*., 2020). Thus, increased cell wall strength, e.g. by enhanced cell wall biosynthesis via stabilization of MurA should counteract lysis of the c-di-AMP-free Δ*cdaA* mutant overproducing the Opp system involved in fosfomycin uptake. To test the idea, we compared the growth of the wild type and the Δ*cdaA*, Δ*reoM* and Δ*cdaA* Δ*reoM* mutants in BHI and LSM. As shown in Fig. 7, while the lack of ReoM did only slightly enhance the growth of the bacteria in LSM, there was a clear effect of enhanced PG biosynthesis on the growth of the c-di-AMP-free strain in BHI medium. Thus, enhanced cell wall biosynthesis allows growth of the mutant lacking CdaA in rich medium. Next, we tested the effect of *reoM* deletion on fosfomycin sensitivity of the Δ*cdaA* mutant during growth in BHI medium and LSM. For this purpose, the Δ*cdaA* and Δ*cdaA* Δ*reoM* mutants were cultivated in the presence of increasing amounts of fosfomycin. As shown in Fig. 7, the Δ*cdaA* Δ*reoM* mutant was much less sensitive towards fosfomycin than the Δ*cdaA* mutant in LSM. To conclude, enhanced cell wall biosynthesis via reduced MurA degradation improves growth of the c-di-AMP-free Δ*cdaA* mutant in BHI medium and enhances resistance to fosfomycin targeting MurA in LSM (Fig. 7).

Discussion

In the present study, we have phenotypically characterized *L. monocytogenes* Δ*pgpH* Δ*pdeA* and Δ*cdaA* mutant strains defective in c-di-AMP degradation and biosynthesis respectively. We have also examined how Δ*pgpH* Δ*pdeA* and Δ*cdaA* mutant strains respond to perturbation of c-di-AMP metabolism at the genome level.

Compared to the wild type, the cellular c-di-AMP concentration in the Δ*pgpH* Δ*pdeA* mutant strain is about 1.7-fold higher (Fig. S1A). While the Δ*pgpH* Δ*pdeA* mutant with the elevated cellular c-di-AMP levels did not exhibit a growth defect in rich medium, growth of the strain was significantly impaired in LSM (Fig. S2). Previously, it has been demonstrated that the BCAA- and GTP-responsive global regulator CodY controls large regulons in response to BCAA limitation

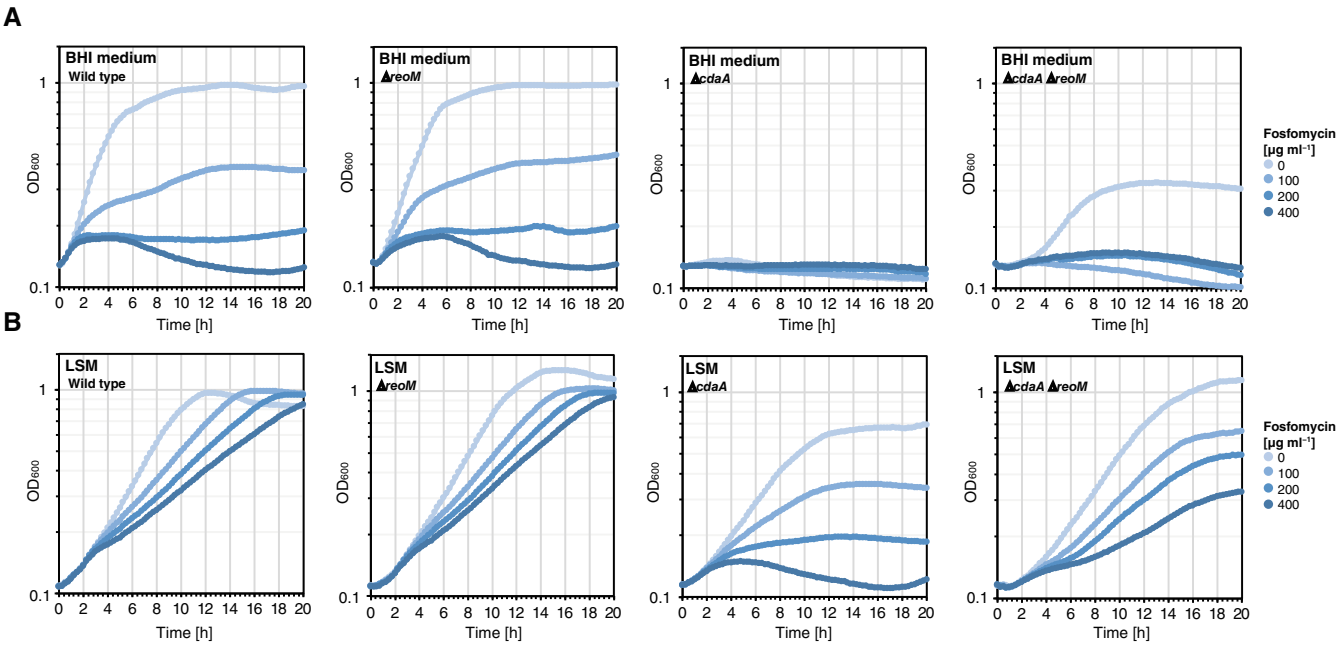

Fig. 7. Effect of MurA stabilization growth and fosfomycin resistance of the *cdaA* mutant.
A and B. Growth of the wild type and the strains BPL77 (Δ*cdaA*) LMSW32 (Δ*reoM*) and BPL104 (Δ*cdaA* Δ*reoM*) in BHI medium and LSM respectively, supplemented with the indicated amounts of fosfomycin at 37°C.

in *L. monocytogenes* and *B. subtilis* (Belitsky and Sonenshein, 2013; Biswas *et al*., 2020). Moreover, in *L. monocytogenes* and *B. subtilis*, the c-di-AMP receptor protein CbpB (DarB in *B. subtilis*) indirectly controls the DNA-binding activity of CodY by modulating the activity of the (p)ppGpp synthetase/hydrolase RelA (Fig. 1) (Peterson *et al*., 2020; Krüger *et al*., 2021a; Krüger *et al*., 2021b). Therefore, the growth defect of the *L. monocytogenes* Δ*pgpH* Δ*pdeA* mutant in LSM could be caused by elevated CodY activity, which in turn would result in reduced amino acid biosynthesis (e.g. BCAAs) and oligopeptide uptake via the Opp system as well as other CodY-regulated transporters (Fig. 1). This hypothesis is supported by the observation that the addition of casamino acids to LSM slightly improved growth of the Δ*pgpH* Δ*pdeA* mutant (Fig. S3). A previous study with *B. subtilis* revealed that the accumulation of GTP causes phenotypic amino acid auxotrophy (Kriel *et al*., 2014). The Δ*pgpH* Δ*pdeA* mutant strain also showed a salt-sensitive phenotype (Fig. S2C). Since oligopeptides act as osmolytes in *L. monocytogenes* (Maria-Rosario *et al*., 1995; Whiteley *et al*., 2017), the accumulation of c-di-AMP might cause the salt-sensitive phenotype of the Δ*pgpH* Δ*pdeA* mutant (Fig. S2C) (Huynh *et al*., 2016; Whiteley *et al*., 2017). The growth defect and salt-sensitive phenotype of the Δ*pgpH* Δ*pdeA* mutant are rapidly suppressed by loss-of-function mutations in the *cdaA* gene and by the acquisition of mutations in *cdaA* that reduce c-di-AMP production (Fig. 3A and B). Like *L. monocytogenes*, also *B. subtilis* and *L. lactis* phosphodiesterase mutants with elevated c-di-AMP levels preferentially acquire mutations in the *cdaA* gene (Gundlach *et al*., 2015a; Gundlach *et al*., 2015b; Zhu *et al*., 2016). In conclusion, our phenotypic and genetic analyses validated that the intracellular concentration of c-di-AMP must be precisely adjusted to allow rapid growth of the bacteria (Fig. 8).

Several studies revealed that perturbation of c-di-AMP metabolism affects the formation of biofilms by *B. subtilis* and *Streptococci* (Gundlach *et al*., 2016; Fahmi *et al*., 2019; The *et al*., 2019; Faozia *et al*., 2021; Rorvik *et al*., 2021). Here, we show that the cellular levels of c-di-AMP influence biofilm formation by *L. monocytogenes* (Fig. 2A). However, since the potassium ion channels enable electrical communication

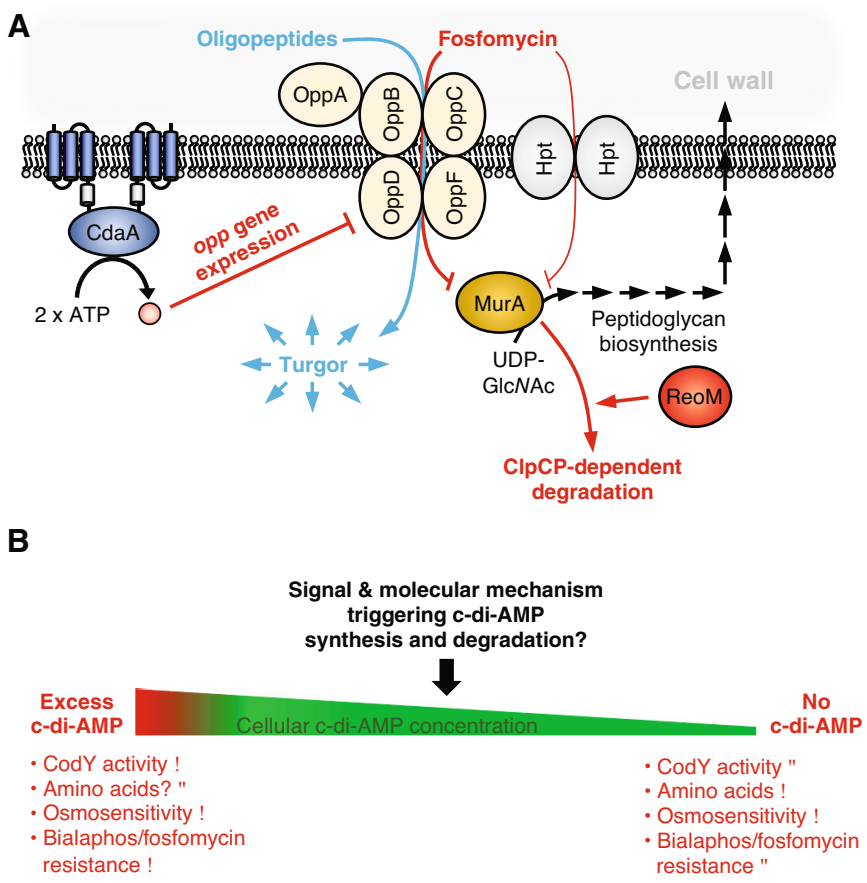

Fig. 8. Link between c-di-AMP metabolism and Opp-dependent growth phenotypes.
A. Fosfomycin uptake is mediated by the Opp oligopeptide transport system and by the Hpt transporter. The c-di-AMP-dependent repression of the *opp* genes is essential to prevent uptake of oligopeptides to toxic levels. Fosfomycin targets the MurA catalysing the committed step in peptidoglycan synthesis. ReoM controls the ClpCP-dependent degradation of MurA (Wamp *et al*., 2020).
B. Perturbation of c-di-AMP metabolism results in pleiotropic phenotypes. The wild type strain can control synthesis and degradation depending on the environmental condition. The signal and underlying molecular mechanism regulating the cellular c-di-AMP concentration remains to be elucidated.

within bacterial communities (Prindle *et al*., 2015) and c-di-AMP controls the potassium ion uptake systems in *B. subtilis* as well as in *L. monocytogenes* (Bai *et al*., 2013, 2014; Gundlach *et al*., 2017; Gibhardt *et al*., 2019; Krüger *et al*., 2020; Cereija *et al*., 2021; Krüger *et al*., 2021a; Krüger *et al*., 2021b), it is not surprising that the signalling nucleotide regulates biofilm formation. It has indeed been reported that the biofilm phenotype of a *Streptococcus pyogenes cdaA* mutant can be rescued by a mutation in the *ktrB* gene encoding the subunit of the KtrAB potassium ion transport system (Faozia *et al*., 2021). It will be interesting to uncover the biological relevance of the c-di-AMP-dependent control of biofilm formation in *B. subtilis*, *L. monocytogenes* and *Streptococci*.

Previously, it has been reported that c-di-AMP is essential for growth of *L. monocytogenes* in rich medium to prevent the uptake of oligopeptides to toxic levels (Whiteley *et al*., 2015, 2017). Here, we confirmed that the *L. monocytogenes* Δ*cdaA* mutant lacking c-di-AMP shows a very strong lytic phenotype (Fig. 2B). Moreover, our suppressor screen confirmed that the reduced CodY activity and thus the overexpression of the *opp* oligopeptide transporter genes in the Δ*cdaA* mutant cause the growth defect of the c-di-AMP-free bacteria in rich medium. Thus, like in other bacteria, c-di-AMP is required to adjust the cellular turgor in *L. monocytogenes*, depending on the osmolarity of the environment (Fig. 8) (Gundlach *et al*., 2017; Whiteley *et al*., 2017; Pham *et al*., 2018, 2021; Devaux *et al*., 2018a; Devaux *et al*., 2018b). We also found that a high extracellular concentration of isoleucine is toxic for the *L. monocytogenes* Δ*cdaA* mutant (Fig. 5A). In this mutant, amino acid transporters that transport isoleucine either specifically or non-specifically are probably overexpressed. The characterization of *cdaA* isoleucine suppressors revealed that all mutants had acquired mutations in the *codY* gene that certainly reduce the DNA-binding activity of CodY (Villapakkam *et al*., 2009). The strong activation of CodY by isoleucine probably results in amino acid auxotrophy of *L. monocytogenes* as it has been observed with a *B. subtilis* mutant accumulating GTP, which is the second activating effector of CodY (Fig. 1) (Kriel *et al*., 2014). We also observed that high amounts of isoleucine partially restore the growth defect of the *cdaA* mutant in rich medium, which is likely due to the CodY-dependent repression of the *opp* genes (data not shown). The characterization of the isoleucine-resistant Δ*cdaA* suppressors confirmed that isoleucine is an important nutritional signal for *L. monocytogenes*. Previously, it has been shown that CodY serves as a global regulator of amino acid metabolism and as an isoleucine sensor controlling virulence gene expression in *L. monocytogenes* (Lobel *et al*., 2012, 2015; Lobel and Herskovits, 2016; Brenner *et al*., 2018; Biswas *et al*., 2020). BCAAs also serve as a signal for virulence gene expression in *Bacillus anthracis* and other Gram-positive bacteria (Stenz *et al*., 2011; Dutta *et al*., 2022). It is interesting to note that we did not identify Δ*cdaA* suppressor mutants that had inactivated isoleucine uptake systems. This observation could indicate that, like in *B. subtilis*, there are also multiple uptake systems for isoleucine present in *L. monocytogenes* (Belitsky, 2015). It is indeed rather unlikely to isolate mutants that have adapted to a toxic substance by inactivating multiple uptake systems simultaneously (Zaprasis *et al*., 2014; Wicke *et al*., 2019).

The Δ*cdaA* suppressor screen, which was based on the detection of CodY activity allowed us to discriminate between mutants having either inactivated the *opp* oligopeptide or the *relA* (p)ppGpp synthetase/hydrolase genes. We found that some of the mutants also had acquired mutations in genes involved in cell wall metabolism. Thus, as described in other studies, alterations in cell wall metabolism may help to osmotically stabilize mutant strains with altered c-di-AMP levels (Commichau *et al*., 2018). The characterization of the Δ*cdaA* suppressors coincidentally led to the identification of a novel system involved in the uptake of the antibiotic fosfomycin in *L. monocytogenes* (Fig. 8). In fact, we could show that the Opp transporter is the major fosfomycin uptake system in *L. monocytogenes*, at least during controlled cultivation (Fig. 8). Previously, it has been shown that fosfomycin enters the *L. monocytogenes* cell via the hexose phosphate transporter Hpt (Scortti *et al*., 2006, 2018). Since Hpt is an *in vivo*-induced virulence factor, the susceptibility of *L. monocytogenes* to fosfomycin is only enhanced during infection (Scortti *et al*., 2006, 2018). Therefore, Hpt can be considered as the minor fosfomycin transporter during controlled cultivation. Like the Hpt transporter, also the Opp oligopeptide transport system is promiscuous because both uptake systems show a relaxed substrate specificity. In addition to fosfomycin, the *L. monocytogenes* Opp system transports the oligopeptides KLLLLK, KAAAAK and AQ as well as the tripeptide antibiotic bialaphos (Borezee *et al*., 2000; Whiteley *et al*., 2015). The transport of bialaphos was also reported for the oligopeptide transport systems from *B. subtilis*, GAS *Streptococcus* and *Sinorhizobium meliloti* (Perego *et al*., 1991; Podbielski *et al*., 1996; Nogales *et al*., 2009). Thus, substrate promiscuity may be a general property of oligopeptide transport systems in bacteria.

To conclude, the present study confirmed a central role of c-di-AMP in osmoadaptation of *L. monocytogenes*. Even though *L. monocytogenes* possesses c-di-AMP-regulated potassium transporters (Gibhardt *et al*., 2019), oligopeptides that are imported via the Opp system might be the dominant osmolytes that regulate the cellular

turgor in this organism. In the future, it is crucial to answer the question of how the bacteria sense the environmental osmolarity to adjust the cellular turgor. Certainly, the c-di-AMP-producing and -degrading enzymes are involved in the sensing process because they are attached to the membrane (Fig. 1). In *B. subtilis*, *L. lactis*, *L. monocytogenes* and *S. aureus*, CdaA is inhibited by the phosphoglucosamine mutase GlmM (Fig. 1) (Zhu *et al*., 2016; Tosi *et al*., 2019; Gibhardt *et al*., 2020; Pathania *et al*., 2021). Moreover, for *L. monocytogenes* it has been shown that the GlmM-dependent inhibition of CdaA occurs when the bacteria encounter hyperosmotic stress (Gibhardt *et al*., 2020). However, the precise underlying molecular mechanism of how the environmental signal is transmitted to the players involved in c-di-AMP metabolism remains to be elucidated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relative amounts of c-di-AMP and emergence of suppressor mutants.

Fig. **S2**. Phenotypes of *L*. *monocytogenes* mutant strains with defects in c-di-AMP synthesis and degradation.

Fig. **S3**. Effect of casaminoacids on the growth of the *L*. *monocytogenes* wild type and the Δ*pgpH* Δ*pdeA* and Δ*cdaA* mutants in LSM.

Fig. **S4**. Inactivation of the *opp* genes confers resistance to fosfomycin.

Table S1. Primers.

Table S2. Bacterial strains and plasmids.