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Aurantimycin resistance genes contribute to survival of *Listeria monocytogenes* during life in the environment

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SUMMARY

Bacteria can cope with toxic compounds such as antibiotics by inducing genes for their detoxification. A common detoxification strategy is compound excretion by ATP-binding cassette (ABC) transporters, which are synthesized upon compound contact. We previously identified the multi drug resistance ABC transporter LieAB in *Listeria monocytogenes*, a Gram-positive bacterium that occurs ubiquitously in the environment, but also causes severe infections in humans upon ingestion. Expression of the *lieAB* genes is strongly induced in cells lacking the PadR-type transcriptional repressor LftR, but compounds leading to relief of this repression in wild type cells were not known. Using RNA-Seq and promoter-*lacZ* fusions we demonstrate highly specific repression of the *lieAB* and *lftRS* promoters through LftR. Screening of a natural compound library yielded the depsipeptide aurantimycin A - synthesized by the soil-dwelling *Streptomyces aurantiacus* - as the first known naturally occurring inducer of *lieAB* expression. Genetic and phenotypic experiments concordantly show that aurantimycin A is a substrate of the LieAB transporter and thus, *lftRS* and *lieAB* represent the first known genetic module conferring and regulating aurantimycin A resistance. Collectively, these genes may support the survival of *L. monocytogenes* when it comes into contact with antibiotic-producing bacteria in the soil.

INTRODUCTION

The Gram-positive bacterium *Listeria monocytogenes* is adapted to two entirely different ecological niches. It is ubiquitously found in nature, where it lives in the soil, on surfaces of plants and on decaying plant material (Freitag *et al.*, 2009), but it also invades epithelial cells of the human gut upon ingestion of contaminated food (Freitag *et al.*, 2009, Seveau *et al.*, 2007). Once inside a host cell, the bacterium multiplies, spreads from cell to cell and is able to breach the intestinal barrier to primarily infect the spleen, the liver or the brain of susceptible persons or the placenta and the fetus in pregnant women (Vazquez-Boland *et al.*, 2001). Such invasive disease manifestations are associated with high mortality (Ramaswamy *et al.*, 2007, Vazquez-Boland *et al.*, 2001).

On one hand, adaptation to life as a pathogen is reflected by the presence of virulence factors mediating attachment, invasion and multiplication inside host cells (Camejo *et al.*, 2011), survival in the gut (Travier *et al.*, 2013, Dussurget *et al.*, 2002) and their precise regulation (Lebreton & Cossart, 2017). On the other hand, the *L. monocytogenes* genome carries many genes for the uptake of different nutrients and for detoxification of various noxious compounds. This illustrates the constant struggle for survival of *L. monocytogenes* within the competitive and nutrient-limited environment outside the human host. For example, 30 complete phosphotransferase systems for the uptake of different sugars are present in the *L. monocytogenes* reference strain EGD-e (Barabote & Saier, 2005). Likewise, over 30 different ATP-binding cassette (ABC) transporters are encoded in the listerial genome (Liu *et al.*, 2013), serving different cellular functions such as nutrient uptake and antibiotic resistance (Fraser *et al.*, 2000, Gopal *et al.*, 2010).

Previously, we have identified the *L. monocytogenes* multidrug resistance (MDR) transporter

LieAB (Kaval *et al.*, 2015), which is annotated as a daunorubicin resistance ABC transporter based on its similarity to LmrCD from *Lactococcus lactis*. Like LmrCD, LieAB is a heterodimeric ABC transporter composed of a cytosolic ATPase (LieA) and a transmembrane protein (LieB). *L. lactis* cells lacking *lmrCD* are hypersensitive to daunorubicin, suggesting that daunorubicin is exported through LmrCD (Lubelski *et al.*, 2006). Expression of the *lmrCD* genes is tightly repressed by the PadR-type transcriptional repressor LmrR and relieved in the presence of daunorubicin (Agustiandari *et al.*, 2008). Daunorubicin binds to a hydrophobic pore in the center of the LmrR dimer and induces a conformational change that prevents LmrR binding to an operator element in the P_{lmrCD} promoter region and thus relieves transcriptional repression of *lmrCD* (Madoori *et al.*, 2009, Agustiandari *et al.*, 2011). Thus, LmrR and LmrCD cooperate in sensing and export of daunorubicin, thereby mediating resistance to the antibiotic.

In contrast, natural substrates of LieAB are not known but ethidium bromide has been described as an artificial substrate (Kaval *et al.*, 2015). Overproduction of LieAB increases ethidium bromide sensitivity of *L. monocyctogenes* in a way that requires the ATPase activity of LieA, indicating that LieAB acts as an ethidium bromide importer consuming ATP for import (Kaval *et al.*, 2015). Why ATP should be invested to import such a harmful compound is not clear. It is also unlikely that *L. monocytogenes* comes in contact with synthetic compounds like this in nature; hence ethidium bromide is considered an unspecific side substrate of LieAB.

The *lieAB* operon is tightly repressed in wild type cells, but strongly induced in cells lacking the LmrR-homologue LftR, suggesting that LftR represses *lieAB* transcription (Kaval *et al.*,

2015). The *lftR* gene is encoded together with *lftS* in the bi-cistronic *lftRS* operon. LftS is supposed to be a soluble cytoplasmic protein containing a DUF1048 domain, the function of which is still not known. Tight *lieAB* repression by LftR seems to be beneficial during infection, since derepression of *lieAB* in a Δ*lftR* mutant impairs invasion of host cells (Kaval *et al.*, 2015). It is currently unclear how LieAB-dependent transport processes affect invasion, but other multidrug resistance transporters such as MdrT are known to interfere with *L. monocytogenes* virulence, when overproduced (Crimmins *et al.*, 2008). MdrT is one of several major facilitator superfamily (MFS) MDR transporters in *L. monocytogenes* that collectively contribute to secretion of cyclic-di-AMP (c-di-AMP), a small molecule important for immune recognition through the cytosolic surveillance pathway in infected host cells (Woodward *et al.*, 2010, Kaplan Zeevi *et al.*, 2013). However, MFS type transporters do not rely on ATP hydrolysis for export (Van Bambeke *et al.*, 2000) and sufficiently explain c-di-AMP secretion. Thus, a similar role for LieAB in c-di-AMP export seems unlikely.

In this manuscript, we present a set of experiments that finally led to the identification of the first naturally occurring LftR effector molecule and greatly improve our understanding of the *lftR* and *lieAB* genes in *L. monocytogenes*.

RESULTS

Identification of LftR-dependent genes by transcriptome sequencing

In order to better understand LftR function, we first sought to identify all genes belonging to the LftR regulon. Knowledge of the complete regulon enables a more targeted approach to gene function determination. LftR-regulated genes were identified by RNA-Seq with samples from wild type and the Δ*lftR* mutant LMKK42, constructed previously (Kaval *et al.*, 2015), grown in BHI broth at 37°C to mid-exponential growth phase (OD_{600}~0.8). This approach confirmed massive de-repression of the *lieAB* operon (~450-fold, Tab. 1) in strain LMKK42.

Transcription of the *lftS* gene was also massively induced in this strain (~190-fold), supporting the idea that the *lftRS* operon is auto-repressed by LftR. Virtually no *lieAB* and *lftS* transcripts were detected in wild type cells, indicating a strong LftR-dependent repression of the P_{lieAB} and P_{lftRS} promoters. The two genes located downstream of *lieAB*, *i. e. lmo0981* encoding a putative efflux transporter and *lmo0982* coding for a putative peptidase, are induced four to seven-fold in the Δ*lftR* mutant. The reason for this effect is unknown, but LftR could also repress expression of these two divergently transcribed genes. Finally, the *kdpDE* genes coding for a two component system that controls expression of the *Escherichia coli* high affinity potassium uptake system *kdpABC* (Polarek *et al.*, 1992, Walderhaug *et al.*, 1992) were two- to three-fold more transcribed in the absence of LftR. 16 genes were downregulated in the Δ*lftR* mutant, among them the *inlB* and *inlH* genes encoding internalins B and H and the *opuCA-D* genes coding for an L-carnitine ABC transporter (Fraser *et al.*, 2000) Remarkably, the 16 downregulated genes identified here are all members of the σ^B regulon (Tab. 1). This effect is explained by a C→T transition changing the 4^{th} codon of *rsbT* into a premature stop codon, which was uncovered by RNA sequencing and confirmed by sequencing of the LMKK42 genome. RsbT is part of the stressosome, a multiprotein complex that regulates σ^B activation due to environmental stimuli (Pane-Farre *et al.*, 2005) and *rsbT* inactivation causes a σ^B null phenotype in *L. monocytogenes* (Chaturongakul & Boor, 2004). It has been shown earlier that mutations in genes controlling σ^B activity can arise randomly during genetic manipulation of *L. monocytogenes* (Quereda *et al.*, 2013).

Identification of LftR-dependent promoters

The repressive effect of LftR on the promoters of the *lieAB* and *lftRS* operons was further

studied using reporter gene experiments. Fragments encompassing putative promoter regions upstream of *lftR* (315 bp) or *lieA* (266 bp, Fig. 1A) as well as their respective start codons were fused in frame to *lacZ* and then integrated into wild type and the Δ*lftR* $rsbT^{I0C\rightarrow T}$ background of strain LMKK42. Both promoter fragments conferred high β-galactosidase activity to *L. monocytogenes* in the mutant background, where 3436±389 Miller units (MU) for P_{lieAB}-*lacZ* and 3313±218 MU for P_{lftRS}-*lacZ* were measured. In good agreement with LftR-dependent repression, activity of both promoter *lacZ* fusions was strongly repressed in wild type cells and in a Δ*sigB* strain that we included as control to rule out the possibility that the $rsbT^{I0C\rightarrow T}$ mutation could affect *lftR* or *lieAB* expression (Fig. 1B). Remarkably, activity of the P_{lieAB} fragment (14±3 MU) was close to the background values of 8±7 MU seen in strain LMSH16 that carries the promoter-less *lacZ* gene, whereas the promoter activity of the P_{lftRS} fragment was higher (32±15 MU, Fig. 1B). Thus, both promoters are tightly repressed by LftR in wildtype cells, but the *lftRS* promoter has a higher background activity. We speculate that this background level guarantees the required level of LftR production needed to maintain full repression of LftR-dependent promoters under non-inducing conditions.

In order to compare P_{lieAB} and P_{lftRS} promoter strengths with that of known housekeeping genes, promoter fragments of the *L. monocytogenes gpsB* (Rismondo *et al.*, 2016) and *divIVA* genes (Halbedel *et al.*, 2012) were fused to *lacZ* and β-galactosidase activity was measured in wild type background. The P_{gpsB}-*lacZ* and P_{divIVA}-*lacZ* fusions conferred LacZ activity levels of 900±107 MU and 982±121 MU, respectively, to the cells. Thus, derepression of the P_{lieAB} and P_{lftRS} promoters in the Δ*lftR* $rsbT^{I0C\rightarrow T}$ mutant results in three- to four-fold higher promoter activities.

Finally, de-repression of *lftRS* transcription in the absence of LftR was analyzed by Northern blotting. A probe specific for *lftS* detected a single ~700 nt transcript in wild type cells (Fig. 1C), consistent with *lftRS* forming a bicistronic operon, which was strongly induced in the

Δ*lftR rsbT*$^{l0C\rightarrow T}$ background of strain LMKK42 (where its length was reduced due to *lftR* deletion). Importantly, a wild type like *lftRS* transcript was observed in the Δ*sigB* mutant (Fig. 1C). These results confirm the assertion that LftR autorepresses *lftRS* transcription.

Rhodamine dyes induce the LftR response

To identify compounds inducing the expression of the LftR regulon, LMSH5 (P_{lieAB}-*lacZ*) cells were poured into BHI agar plates containing X-Gal and compounds to be tested were spotted on top of these plates. No induction of the *lieAB* promoter was observed with several antibiotics (ampicillin, penicillin G, fosfomycin, bacitracin, cycloserin, erythromycin, chloramphenicol, kanamycin, tetracyclin, spectinomycin, nalidixic acid), antimicrobial compounds (benzalkonium, tert-butylhydroquinone, cravacrol, acriflavine) or dyes such as acridine orange. Likewise, no induction was observed with ethanol, DMSO, NaOH, EDTA, KCl, Triton X-100, SDS, deoxycholate or H_2O_2 (data not shown).

PadR-like transcriptional repressors are known to respond to daunorubicin and the fluorescent dye rhodamine 6G (Huillet *et al.*, 2006, Crimmins *et al.*, 2008, Madoori *et al.*, 2009, Takeuchi *et al.*, 2014). While daunorubicin did not induce the P_{lieAB} promoter (Fig. S1A), rhodamine 6G induced the P_{lieAB}-*lacZ* fusion, as a deep blue ring surrounding the zone of growth inhibition was observed (Fig. 2A). Another rhodamine dye, rhodamine B, also induced P_{lieAB}, but induction of P_{lieAB} by rhodamine B required higher concentrations, at least if judged based on the diameter of the induction zone that was violet in this case due to the color mixture of red (rhodamine B) and blue (Fig. 2A). The P_{lftRS} promoter showed the same induction profile, further indicating that the *lftRS* and *lieAB* operons are co-regulated. As expected, the control strain carrying a promoter-less *lacZ* gene (LMSH16) did not reveal any signs of β-galactosidase activity. Also, neither of the two promoters was induced by ethidium bromide (Fig. 2A), the previously identified artificial substrate of LieAB (Kaval *et al.*, 2015).

A gradual induction of β-galactosidase activity in strain LMSH5 (P_{lieAB}-*lacZ*) was observed in the presence of increasing rhodamine 6G concentrations, which reached a 13.5-fold induction in the presence of 0.5 µg/ml (1.04 µM) rhodamine 6G (Fig. 2B), which is below the minimal inhibitory concentration of 2 µg/ml (see below). The same concentration induced the P_{lftRS}-*lacZ* fusion present in strain LMSH7 promoter 6.6-fold (Fig. 2B). Both promoters were only induced to 3.4% (P_{lieAB}) or 6.2% (P_{lftRS}) of their maximal capacity seen in the Δ*lftR* $rsbT^{10C\rightarrow T}$ mutant (Fig. 2B).

Isolation of a Δ*lftR* mutant free of unwanted second site mutations

Based on the experiments with the Δ*sigB* mutant and the clear-cut separation of transcriptionally deregulated genes in LMKK42 into down-regulated σ^B-dependent genes and LftR-dependent genes, which were strongly de-repressed, it seemed reasonable to assume that σ^B does not affect transcription of LftR-dependent genes. Nevertheless, we wondered whether the $rsbT^{10C\rightarrow T}$ mutation in the Δ*lftR* mutant LMKK42 occurred as a result of the Δ*lftR* deletion and massive overproduction of LieAB or whether it just arose by chance. In order to distinguish between these two possibilities, we repeated the mutant construction protocol. New clones lacking *lftR* were readily isolated and whole genome sequencing revealed the absence of unwanted second site mutations in four out of six isolated Δ*lftR* clones, suggesting that the $rsbT^{10C\rightarrow T}$ mutation in LMKK42 must have arisen by chance and is not needed to suppress an otherwise sick or even lethal Δ*lftR* phenotype. One of these new Δ*lftR* clones was designated LMSH26 and *lieAB* was deleted in this background, yielding strain LMSH35 (Δ*lftR* Δ*lieAB*). These two mutants were used in all further experiments.

Induction of the P_{lieAB} promoter requires LftS

The function of *lftS*, the second gene of the *lftRS* operon, is not known. The observation that *lftS* is co-regulated with the *lftR* and *lieAB* genes suggested that LftS could either act in LftR-

dependent gene repression or that it could be a component of the LieAB transporter. The latter possibility seemed unlikely, because a Δ*lftRS* mutant still showed increased susceptibility against ethidium bromide in a way that was dependent on the presence of *lieAB*, indicating that LftS does not contribute to LieAB function (Kaval *et al.*, 2015). The remaining possibility, *i. e.* a possible role of LftS in LftR-dependent repression of the *lieAB* operon, was tested using a strain carrying the P_{lieAB}-*lacZ* reporter but lacking *lftS* (LMSH27). While rhodamine 6G induces β-galactosidase in wild type strains carrying this reporter fusion, the P_{lieAB}-*lacZ* reporter in the Δ*lftS* background was not induced by rhodamine 6G any more (Fig. 3A). This suggests that LftS is dispensable for *lieAB* repression but indispensable for relief of repression in the presence of rhodamine 6G.

Next, the *lftS* gene was reintroduced at its original site in the Δ*lftS* mutant. When the P_{lieAB}-*lacZ* reporter was introduced into this revertant, rhodamine 6G induced the P_{lieAB} promoter again as in wild type bacteria (Fig. 3A), ruling out the possibility that the effect of *lftS* was due to unwanted second site mutations.

Aurantimycin is a naturally occurring inducer of the LftR response

Rhodamines are of synthetic origin (Mudd *et al.*, 2015), questioning their importance as inducers of the LftR response in natural environments. In order to identify naturally occurring compounds inducing *lieAB* expression, a compound collection containing ~700 natural products isolated from myxobacteria, actinobacteria and fungi was tested for β-galactosidase induction on strain LMSH5 (P_{lieAB}-*lacZ*) . This yielded a single hit: Aurantimycin A (for its chemical structure see Fig. S2), a depsipeptide antibiotic isolated from *Streptomyces aurantiacus* (Gräfe *et al.*, 1995). A zone of growth inhibition was observed when a 1 µl droplet of a 1 mM aurantimycin A solution in DMSO was spotted on agar plates containing X-Gal and LMSH5 cells. This inhibition zone was surrounded by a clear blue ring indicating

induction of the P_{lieAB}-*lacZ* reporter (Fig. 3A), an observation not made with DMSO alone (data not shown).

Induction of the P_{lieAB} promoter by aurantimycin A was then tested during growth in BHI broth containing increasing but sub-inhibitory concentrations of aurantimycin A. This approach revealed gradual induction of β-galactosidase activity in strain LMSH5 (P_{lieAB}-*lacZ*) up to 657±117 MU at an aurantimycin A concentration of 100 ng/ml (~0.12 µM) corresponding to a 36.5-fold induction compared to the absence of the inducer (Fig. 3B). Thus, the induction of the P_{lieAB} promoter by a molecule of aurantimycin A is approximately 24 times stronger than by a molecule of rhodamine 6G. Most likely, higher induction levels are prevented by the negative feedback loop that results from autorepression of the P_{liftRS} promoter through LftR.

As observed for rhodamine 6G, induction of P_{lieAB} by aurantimycin was not detected in strain LMSH27 (Δ*lftS* P_{lieAB}-*lacZ*) whether tested on plate (Fig. 3A) or during growth in broth (Fig. 3B). Furthermore, aurantimycin A did induce the P_{lieAB} promoter in the *lftS* revertant LMSH49 as it did in the wild type (Fig. 3A). Thus, LftS is required for P_{lieAB} induction by aurantimycin A in wild type cells. In contrast, P_{lieAB} was maximally de-repressed in cells lacking the entire *lftRS* operon (Fig. 3C), demonstrating that LftS only can exert its effect on de-repression of the P_{lieAB} promoter as long as LftR is present. This suggests that LftS potentially acts on or through LftR. Furthermore, LieAB does not influence P_{lieAB} induction by aurantimycin A (Fig. 3C), demonstrating that aurantimycin A sensing is LieAB-independent.

Evidence for LieAB-dependent aurantimycin A transport

We hypothesized that compounds inducing the LftR response are also transported outside the cell by LieAB. In order to test this hypothesis, minimal inhibitory concentrations (MIC) of

rhodamine 6G and aurantimycin A were determined. Growth of *L. monocytogenes* wild type cells was inhibited in the presence of 2 µg/ml rhodamine 6G and the same MIC was obtained with the Δ*lieAB* mutant (Tab. 2). Even in the absence of LftR, when LieAB is overproduced, no influence of LieAB on rhodamine 6G sensitivity was observed (Tab. 2), clearly indicating that rhodamine 6G is not a substrate of the LieAB transporter. Daunorubicin is also not a substrate of LieAB as resistance against daunorubicin was not affected in the Δ*lftR* (LMSH26) and Δ*lftR* Δ*lieAB* mutants (LMSH35, Fig. S1B).

The MIC of aurantimycin A against wild type was 1.25 µg/ml and two-fold higher (2.5 µg/ml) in the corrected Δ*lftR* mutant LMSH26, in good agreement with LieAB de-repression in this strain. In the absence of *lieAB*, the MIC of aurantimycin dropped to 0.156 µg/ml, whether LftR was present or not (Tab. 2). This shows that LieAB and LftR cooperate to mediate resistance of *L. monocytogenes* against aurantimycin A. LftS did not affect the MIC of aurantimycin A in this assay (Tab. 2), despite its relevance for P_{lieAB} induction (see above). However, Δ*lftS* cells showed longer lag phases to cope with aurantimycin A concentrations the wild type could quickly adapt to (Fig. 4). This late adaptation of the Δ*lftS* mutant to aurantimycin A was LieAB-dependent, as it was not observed in a Δ*lftS* Δ*lieAB* mutant (Fig. 4). To decide whether the late growth of the Δ*lftS* mutant at aurantimycin A concentrations between 156-625 mg/ml (Fig. 4) results from regulatory adaptation or from selection of aurantimycin A-resistant suppressors, we repeated this experiment and used wild type and Δ*lftS* cells grown in the presence of 625 ng/ml aurantimycin A to re-inoculate fresh culture medium containing increasing concentrations of aurantimycin A (Fig. S3). Surprisingly, both inoculums exhibited an increased aurantimycin A resistance after re-incoculation (Fig. S3). This indicates that both the wild type and the Δ*lftS* mutant had acquired inheritable aurantimycin A resistance through suppressor mutation(s). Remarkably, the MIC of these suppressors (2.5 mg/ml aurantimycin A, Fig. S3) was identical to that of the Δ*lftR* mutant

(Tab. 2), suggesting that LftR may have been inactivated. These results confirm that LftS is required for de-repression of P_{lieAB} but also show that determination of aurantimycin A MICs (Tab. 2) is masked by formation of suppressors.

Next, we wondered whether aurantimycin A resistance is due to LieAB-dependent transport of aurantimycin A. As aurantimycin A has been described forming pores in artificial membranes, we studied its effect on *L. monocytogenes* lysis by monitoring the optical density of bacterial cultures in Tris-buffer. These experiments revealed that aurantimycin A induces lysis of *L. monocytogenes* wild type cells, which in turn would hardly lyse in plain buffer (Fig. 5). Similar lysis curves were observed with strains LMSH26 (Δ*lftR*) and LMSH35 (Δ*lftR* Δ*lieAB*) suggesting that LieAB does not confer protection against aurantimycin-induced lysis under this condition (Fig. 5). Since LieAB requires energy for transport, glucose was added as energy source. With energy available, lysis of the Δ*lftR* mutant was clearly diminished while this effect was abrogated in the Δ*lftR* Δ*lieAB* double mutant (Fig. 5). This result indicates energy-dependent transport of aurantimycin in a LieAB-dependent manner.

DISCUSSION

We here show that LftR regulates a very specific set of *L. monocytogenes* genes. With *lieAB* and *lftRS*, there are only four main target genes of LftR (Fig. 6). These two operons are heavily overexpressed in the Δ*lftR* mutant. The induction values of the remaining five de-repressed genes found here are low and presumably represent unspecific side effects. The genes found to be downregulated in the Δ*lftR* mutant LMKK42 were all σ^B-dependent due to a second site mutation in *rsbT*. This mutation most likely explains the daylight-dependent swarming phenotype that we observed earlier with this strain (Kaval *et al.*, 2015). Daylight-dependent coordination of swarming is known to be a σ^B-dependent trait (Tiensuu *et al.*, 2013) and it was the only phenotype reported for this mutant that was not corrected by *lieAB* deletion (Kaval *et al.*, 2015). A new Δ*lftR* mutant without unwanted second site mutations was isolated and this mutant still showed de-repression of P_{lieAB} (Fig. 3B). These results confirm the LftR-dependence of the *lieAB* promoter and exclude the possibility that *lieAB* overexpression can only be observed in a *lftR* mutant when *rsbT* is mutated simultaneously. We can also exclude the possibility that the invasion defect observed with the first Δ*lftR* mutant (Kaval *et al.*, 2015) is explained by the *rsbT* mutation for two reasons: First, this phenotype was cured by deletion of *lieAB* and second, deletion of *lieAB* also cures the invasion defect of a *lftRS* double mutant, which was constructed independently and neither showed the σ^B-dependent swarming phenotype (Kaval *et al.*, 2015) nor did contain any unwanted second site mutations (data not shown). The mechanism(s) explaining how LieAB overproduction affects the invasion process of *L. monocytogenes* into eukaryotic cells remain unknown. What now became clear is that these genes are rather expressed during life in the environment, where *L. monocytogenes* is exposed to aurantimycin A produced by streptomycetes also inhabiting the soil. Whether the *lieAB* genes are expressed during

infection at all, still needs to be studied, but this seems rather unlikely.

Aurantimycin A is composed of a cyclic hexadepsipeptide core fused to a lipophilic polyketide side chain (Gräfe *et al.*, 1995) (Fig. S2). Its synthesis depends on a biosynthetic gene cluster with 36 open reading frames found in *S. aurantiacus* that encodes enzymes for precursor synthesis, several non-ribosomal peptide synthetase and polyketide synthase modules, putative tailoring enzymes and the ArtJK ABC transporter (Zhao *et al.*, 2016). The most probable function of the ArtJK ABC transporter is to export aurantimycin from *S. aurantiacus* cells. ArtJK is remarkably similar to LieAB (54% identity between ArtJ and LieA and 44% identity between ArtK and LieB), which supports the idea that LieAB acts as an exporter for aurantimycin A. Particularly low MICs of aurantimycin A (0.007-0.08 µg/ml) have been reported for several Gram-positive bacteria, including *B. subtilis*, *Staphylococcus aureus* and streptococci, whereas Gram-negative bacteria are generally insensitive to aurantimycins (Gräfe *et al.*, 1995). The LieAB transporter is clearly important for the considerably higher aurantimycin A resistance of *L. monocytogenes*. When *lieAB* is deleted, listerial aurantimycin A resistance drops down to almost the same level as in these other Gram-positive bacteria, suggesting the lack of similar aurantimycin A resistance mechanisms in these organisms.

Aurantimycin A was reported to increase the electrical conductivity through an artificial phospholipid membrane indicating that aurantimycin A could form membrane pores (Grigoriev *et al.*, 1995), consistent with aurantimycin-induced lysis of *L. monocytogenes* cells (this study) and erythrocytes (Gräfe *et al.*, 1995). However, this raises the question as to how LieAB would remove aurantimycin A from the membrane. How ABC transporters can export membrane-bound substrates is also not answered in better studied systems such as the BceAB ABC transporter of *B. subtilis* (Clemens *et al.*, 2017), which contributes to detoxification of bacitracin (Ohki *et al.*, 2003), but some evidence for direct expulsion from the membrane to

the outside through ABC transporters exists for other antimicrobial peptides such as epidermin (Otto *et al.*, 1998). Another open question is to what extent aurantimycin A would accumulate inside the cytoplasm, where it could be sensed by LftR. The planar depsipeptide ring of aurantimycin A could be suited as an LftR substrate since the multidrug binding site in the homologous PadR-like repressor LmrR is a flat pore lined with hydrophobic amino acid side chains and also interacts with planar polycyclic compounds (Madoori *et al.*, 2009). A direct interaction of LftR with aurantimycin A could then lead to relief of repression of the *lieAB* and *lftRS* operons through the induction of conformational changes in LftR (Fig. 6) as described for the interaction of LmrR with its ligands (Madoori *et al.*, 2009). Induction of *lieAB* expression by aurantimycin A would lead to an accumulation of LieAB, and thereby to the export of aurantimycin A from the cell or its expulsion from the membrane so that ligand-free LftR could again act as a repressor (Fig. 6).

How LftS contributes to this process also remains unknown. LftS did not affect repression of the *lieAB* genes but is required for their inducer-dependent de-repression. The LftS protein (12.8 kDa) is entirely made up of a DUF1048 domain, a globular domain of unknown function that forms dimers *in crystallo* (PDB: 2O4T). Possibly, LftS acts in aurantimycin A sensing or in removal of LftR from its operator sites. Further studies, involving co-crystallization of LftR and/or LftS with or without aurantimycin A, are needed to clarify this and despite of the unsolved issues discussed, we have identified the first set of bacterial genes conferring and regulating resistance to aurantimycin A. We have to assume that the presence of these genes in *L. monocytogenes* reflects the frequent contact of this bacterium with competitors in the soil and contributes to survival of *L. monocytogenes* during life in the environment.

EXPERIMENTAL PRODECURES

Bacterial strains and growth conditions

All strains used in this study are listed in Table 3. *L. monocytogenes* strains were grown in BHI broth or on BHI agar plates at 37°C. Antibiotics and other supplements were used when required at the following concentrations: erythromycin (5 µg mL^{-1}), kanamycin (50 µg mL^{-1}) and X-Gal (100 µg mL^{-1}). Aurantimycin A was either included as test substance in the natural compound collection (see below) or purchased from Enzo Life Sciences (Lörrach, Germany). *Escherichia coli* TOP10 was used as the standard cloning host (Sambrook *et al.*, 1989).

General methods, manipulation of DNA and oligonucleotide primers

E. coli transformation and plasmid DNA isolation were performed using standard methods (Sambrook *et al.*, 1989). *L. monocytogenes* transformation was carried out as described elsewhere (Monk *et al.*, 2008). Enzymatic DNA manipulation was done in accordance with the manufacturer´s protocols. QuikChange mutagenesis was used for restriction free modification of plasmids (Zheng *et al.*, 2004). All oligonucleotide sequences are listed in Table 4.

Plasmid and strain construction

For construction of pBP117, plasmid pBP116 was constructed first. To this end, the *gltAB* promoter of *B. subtilis* 168 and the *lacZ* gene from pAC5 were amplified using oligonucleotides IW1/FC271 and FC269/FC270, respectively. The P_{gltAB} fragment was cut using EcoRI and the *lacZ* PCR product was digested with EcoRI/XhoI. Both fragments were simultaneously ligated to pIMK cut with EcoRI/XhoI. Blue colonies were isolated on LB plates containing kanamycin and X-Gal after transformation into *E. coli* XL1-blue. Plasmid

DNA was isolated from a blue colony, yielding pBP116. Plasmid pBP116 was then cut with EcoRI to remove the P*gltAB* fragment and religated, yielding pBP117. Loss of the *gltAB* promoter was confirmed by DNA sequencing.

For construction of the P_{lieAB}-*lacZ* fusion, the *lieAB* promoter was amplified from chromosomal DNA with the oligonucleotides SAH88/ SAH99. The obtained PCR product was used to insert the P_{lieAB} fragment into plasmid pBP117 by restriction free cloning (van den Ent & Löwe, 2006), resulting in plasmid pSAH10. Plasmid pSAH11, carrying a P_{lftRS}-*lacZ* fusion was constructed in the same way, but primers SAH79 and SAH100 were used instead.

The *gpsB* and *divIVA* promoters were amplified using the primer pairs SAH138/SAH139 and SAH140/SAH141, respectively, and the obtained PCR products were used as primers to introduce both promoter fragments into pBP117 by restriction free cloning. This procedure resulted in plasmids pSAH18 and pSAH19.

All derivatives of pBP117 were introduced into *L. monocytogenes* by electroporation and kanamycin resistant clones were selected. Integration of plasmids into the chromosomal *attB* site of the $tRNA^{Arg}$ locus was verified by PCR.

Plasmid pSAH13 was constructed for deletion of the *sigB* gene. To this end, regions encompassing the *sigB* gene were amplified using the primer pairs SAH101/SAH102 (upstream) and SAH107/SAH104 (downstream) and fused together by splicing by overlapping extension PCR (SOE PCR) with SAH101/SAH104 as the primers. The resulting fragment was cloned into pMAD using NcoI/EcoRI. For reintroduction of *lftS* into the Δ*lftS* mutant, a fragment containing *lftS* was amplified with primers SAH193/SAH194 and inserted into pMAD using restriction free cloning. The resulting plasmid was named pSAH36. Derivatives of pMAD were introduced into the relevant strain backgrounds by electroporation and erythromycin resistant transformants were selected at 30°C. Allelic

exchanges were then performed using a protocol described by others (Arnaud *et al.*, 2004) and verified by PCR.

Genome sequencing

Genomic DNA was isolated using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) as described (Halbedel *et al.*, 2018). Libraries were prepared using the Nextera XT DNA Library Prep Kit and genomes were sequenced on a MiSeq benchtop sequencer as described recently (Rismondo *et al.*, 2017). SNPs were identified using the Geneious software package and the EGD-e genome sequence (NC_003210.1) (Glaser *et al.*, 2001) as the reference.

mRNA isolation

Strains were grown in BHI broth to an OD_{600} of ~0.5 (mid exponential phase). 25 ml of this culture was collected and quenched by adding of 25 ml ice cold killing buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3). After 5 min incubation on ice, cells were harvested by centrifugation.

RNA extraction followed the protocol of Gertz *et al.* (Gertz *et al.*, 1999) with modifications. Cell pellets were resuspended in 1 ml Lysis Buffer I (25% sucrose; 20 mM Tris-HCl pH 8, 0.25 mM EDTA) and 2 µl of lysozyme (100 mg/ml) was added. After incubation for 5 min on ice, the samples were pelleted (15,000 x g for 30 s at 4°C) and resuspended in 300 µl Lysis Buffer II (3 mM EDTA, 200 mM NaCl). This solution was then mixed with 300 µl Lysis Buffer III (3 mM EDTA, 200 mM NaCl, 1% SDS) that had been pre-incubated at 95°C and the mixture was incubated at 95°C for five more minutes. 600 µl phenol/chloroform/isoamylalcohol (25:24:1) was added and vigorously mixed at room temperature for 5 min. Phases were separated by centrifugation (15,000 x g, 5 min), the

aqueous upper phase was collected and the phenol/chloroform extraction was repeated. Afterwards, the aqueous phase was mixed with 600 µl chloroform/isoamylalcohol (25:1), shaken vigorously for 5 min and phases were separated by centrifugation (5 min at 15,000 x g). The chloroform extraction was repeated. Finally, RNA was precipitated by addition of 0.1 x volume 3 M sodium acetate (pH 5.2) and 1.5 volumes 96% ethanol and incubation at -20°C overnight and pelleted by centrifugation (15,000 x g for 15 min at 4°C). The pellet was washed with 70% ethanol and resuspended in 100 µl DEPC treated water.

10 µg total RNA were digested with DNAse using the RNase-Free DNase Set (Qiagen). RNA was then purified using RNA clean & concentrator columns (Zymo Research) for purification for RNA molecules longer than 200 nucleotides. RNA quality was assessed using Agilent Bioanalyzer RNA Nano chips.

rRNA depletion was performed using the Ribo-Zero Bacteria Kit (Illumina) following the manufacturer's instructions. A total of approximately 2 µg purified RNA was treatedd with 10 µl Ribo-ZeroRemoval Solution and RNA concentrations were determined using a Qubit® fluorometer. After rRNA removal the remaining RNA was pelleted by ethanol precipitation following the recommendations in the Ribo-Zero protocol.

RNA sequencing

RNA libraries were prepared using the TruSeq® Stranded mRNA Kit, starting with 19.5 µl Fragment, Prime, Finish Mix being added to the dried RNA pellet after rRNA depletion and ethanol precipitation. From this point on the manufacturers protocol was strictly followed. The RNA libraries were sequenced in paired-end mode with 2 times 76 cycles on the Illumina MiSeq (MiSeq® Reagent Kit v3; 150 cycles). RNA transcripts were quantified by quasi-mapping of the reads to the *L. monocytogenes* EGD-e cDNA, provided by the Ensembl Genomes server (Kersey *et al.*, 2018), using the Salmon software (Patro *et al.*, 2017).

Average expression from three biological replicates of the mutant divided by the average expression from three biological replicates of the wildtype gave the differential expression ratio. Log2-transformed transcript counts from three biological replicates were then used to calculate *P* values using Students *t*-test. Significantly differentially expressed genes were defined as having a *P*-value less than 0.01 and an absolute differential expression factor of more than 2, as well as having an expression level of at least 10 TPM. RNA sequencing raw files are available at the NCBI Geo Server (https://www.ncbi.nlm.nih.gov/geo/) under study accession numbers GSE118775.

Northern blotting

Northern blotting was performed as described by Wetzstein *et al.* (Wetzstein *et al.*, 1992). The *lftS*-specific digoxigenin-labelled RNA probe was generated by *in vitro* transcription with T7 RNA polymerase (Roche Diagnostics) and an *lftS*-internal PCR fragment fused to the T7 RNA polymerase recognition site, obtained using the primers SAH129/SAH130. *In vitro* transcription was performed with the DIG RNA labeling Kit (Roche). Hybridisation and signal detection were carried out using the DIG wash and block buffer set, an anti-digoxigenin antibody conjugated to alkaline phosphatase and the CDP-Star reagent (Roche) according to the manufacturer's instructions.

β-galactosidase reporter assays

To screen substances for their induction of P_{lieAB}-*lacZ* and P_{lftRS}-*lacZ* in diffusion assays, an overnight culture of the respective reporter strain was diluted 1:1000 in 25 ml ice cold BHI broth containing 100 µg/ml X-Gal and then mixed with 25 ml warm BHI agar (1.5%). After solidification, 2 µl of the substances to be screened were spotted on the agar plates and incubated overnight at 37°C.

For determination of β-galactosidase activity, reporter strain cultures were grown in BHI broth until an OD_{600} of 0.5-0.6. Where indicated, aurantimycin A or rhodamine 6G were added to the cultures right from the beginning. Cells were pelleted, washed once with 500 µl H_2O and then resuspended in 1.2 ml Z-Buffer (60mM Na_2HPO_4, 40 mM NaH_2PO_4, 10mM KCl, 1 mM $MgSO_4$, 20 mM 2-mercaptoethanol). Cells were lysed by sonification and cellular debris was removed by centrifugation (12000 x g, 2 min). The resulting supernatant was used to measure protein content (Roti®-Nanoquant) and β-galactosidase activity. For β-galactosidase activity measurement sample were appropriately diluted in Z buffer to a final volume of 1000 µl and incubated at 30°C for 10 minutes. The reaction was started by addition of 200 µl ONPG (4 mg/ml in Z-Buffer) and stopped by adding 500 µl 1M Na_2CO_3 as soon as the first sample turned clearly yellow. Absorption was measured at 420 nm against Z-Buffer incubated with ONPG as the blank and Miller units (MU) were calculated.

MIC determination

The determination of the minimal inhibitory concentration (MIC) was performed in 96-well plates in a total volume of 200 µl. Overnight cultures of the respective strains were used to inoculate 200 µl BHI containing defined concentrations of the antibiotic (geometric dilution series of the antibiotic of interest) at an OD_{600} of 0.05. The microtiter plates were incubated over night at 37°C and examined for growth after 18±2 hours. The MIC was defined as the lowest concentration of antibiotic at which no growth could be observed.

Natural compound collection

The natural compounds used in this study have been compiled as part of the German Centre for Infection Research (DZIF) infrastructure “Natural Compound Library” at the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) in a ready-to-screen library. The

proprietary collection consists of secondary metabolites from myxobacteria, fungi, and streptomycetes, which were collected in natural product screening programs at HIPS, the Helmholtz Centre for Infection Research (HZI), and the University of Tübingen, respectively. At the time of screening, the library contained a total number of 681 purified natural compounds, in particular, 253 from myxobacteria, 340 from streptomycetes, and 88 from fungi. Library compounds were stored in DMSO at -80°C at a stock concentration of 10 mM and were transferred as 1 mM stock solutions in DMSO into conical 96-well plates using an automated liquid handling robot (STARlet; Hamilton Robotics, Bonaduz, Switzerland) in randomized order and encrypted by a barcode system for non-biased screening.

Lysis assay

An overnight culture was used to inoculate 20 ml fresh BHI at an OD_{600} of 0.05. This culture was grown until OD_{600}=0.5 and harvested by centrifugation. The cells were resuspended in 50 mM Tris pH 8.0 to obtain an OD_{600} of 2. 100 µl of this cell suspension were added to a microwell plate containing 100 µl 50 mM Tris pH 8.0 and 2 mg/ml aurantimycin A, where indicated. Optionally, 2.5 g/l glucose was added to this buffer to energize the cells. Lysis was monitored automatically by OD measurements in a plate reader at 5 minute intervals during incubation at 37°C.

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AUTHOR CONTRIBUTIONS

All authors have made major contributions to the conception or design of the study, SaH, JH, MM, JG, SF, and SvH contributed to the acquisition, analysis, or interpretation of the data; SaH and SvH wrote the manuscript.

ABBREVIATED SUMMARY

Listeria monocytogenes is a soil bacterium that can infect humans upon ingestion. Numerous genes are known that are specifically expressed during infection, but less is known about genes important for survival in the soil. We identified the first genetic module regulating and mediating resistance against the depsipeptide antibiotic aurantimycin A. This substance is produced by *Streptomyces* species sharing the soil habitat. Consequently, these genes may support survival of an important human pathogen in its natural reservoir.

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FIGURE LEGENDS

Figure 1: Effect of LftR on *lieAB* and *lftRS* promoter activity.

(A) Scheme illustrating promoter architecture of the *lieAB* and *lftRS* operons. (B) β-galactosidase activity assays in wild type and Δ*lftR* $rsbT^{10C\rightarrow T}$ and Δ*sigB* mutant backgrounds carrying P_{lieAB}-*lacZ* or P_{lftRS}-*lacZ* fusions. Strains LMSH5 (P_{lieAB}-*lacZ*), LMSH6 (Δ*lftR* $rsbT^{10C\rightarrow T}$ P_{lieAB}-*lacZ*), LMSH7 (P_{lftRS}-*lacZ*), LMSH8 (Δ*lftR* $rsbT^{10C\rightarrow T}$ P_{lftRS}-*lacZ*) were cultivated to exponential phase and β-galactosidase activity was measured. Average values and standard deviations are shown (n=3). *L. monocytogenes* strains EGD-e (wt), LMSH16 (*lacZ*), LMSH24 (Δ*sigB* P_{lieAB}-*lacZ*) and LMSH25 (Δ*sigB* P_{lftRS}-*lacZ*) were included for comparison. Significance levels (*t*-test) are indicated by asterisks (** - $P<0.01$, *** - $P<0.001$). (C) Northern blot showing derepression of *lftS* transcription in the Δ*lftR* $rsbT^{10C\rightarrow T}$ mutant (LMKK42). RNA samples from strains LMSH9 (Δ*sigB*), LMKK26 (Δ*lftS*) and LMKK31 (Δ*lftRS*) were included for comparison.

Figure 2: Induction of the *lieAB* and *lftR* promoters by rhodamine dyes.

(A) Agar plates showing induction of P_{lieAB}-*lacZ* and P_{lftRS}-*lacZ* promoter fusions by rhodamine B and rhodamine 6G. Strains LMSH5 (P_{lieAB}-*lacZ*) and LMSH7 (P_{lftRS}-*lacZ*) were poured into X-gal containing BHI agar and 2 µl droplets of rhodamine 6 G, rhodamine B or ethidium bromide solutions (all at a concentration of 2.5 mg/ml) were spotted onto the agar surface. Strain LMSH16 (*lacZ*) was included as negative control. Agar plates were

photographed after 24 h of incubation at 37°C. (B) Quantification of the induction of the P_{lieAB}-*lacZ* and P_{lftRS}-*lacZ* promoter fusions by rhodamine 6G during growth in liquid culture. Strains LMSH5 (P_{lieAB}-*lacZ*) and LMSH7 (P_{lftRS}-*lacZ*) were grown in BHI broth at 37°C containing increasing concentrations of rhodamine 6G and LacZ activity was determined. Strains LMSH6 (Δ*lftR* $rsbT^{I0C\rightarrow T}$ P_{lieAB}-*lacZ*) and LMSH8 (Δ*lftR* $rsbT^{I0C\rightarrow T}$ P_{lftRS}-*lacZ*) were included for comparison. Measurements were repeated three times and average values and standard deviations are shown. Significance levels (*t*-test) are indicated by asterisks (* - $P<0.01$, ** - $P<0.001$).

Figure 3: Induction of the P_{lieAB} promoter by rhodamine 6G and aurantimycin A requires LftS.

(A) Agar plates showing induction of the P_{lieAB} promoter by rhodamine 6G and aurantimycin A. Strains LMSH5 (P_{lieAB}-*lacZ*), LMSH27 (Δ*lftS* P_{lieAB}-*lacZ*) and LMSH49 (*lftS* revertant P_{lieAB}-*lacZ*, designated *lftS*$^+$) were poured into X-gal containing BHI agar and solidified. 2 µl droplets of a 2.5 mg/ml rhodamine 6G or 2.5 mg/ml aurantimycin A solutions were spotted on top of the agar surface and images were taken after incubation at 37°C overnight. (B) β-galactosidase activity in the same set of strains as above during growth in BHI broth containing increasing concentrations of aurantimycin A. Measurements were repeated three times and average values and standard deviations are shown. Significance levels (*t*-test) are indicated by asterisks (* - $P<0.01$, ** - $P<0.001$). Strain LMSH34 (Δ*lftR* P_{lieAB}-*lacZ*) devoid of the $rsbT^{I0C\rightarrow T}$ mutation was included for comparison. (C) Induction of the P_{lieAB}-*lacZ* reporter by aurantimycin A in in Δ*lftRS* and Δ*lieAB* mutants. β-galactosidase activity was measured as described above in strains LMSH5 (P_{lieAB}-*lacZ*), LMSH27 (Δ*lftS* P_{lieAB}-*lacZ*), LMSH64 (Δ*lftRS* P_{lieAB}-*lacZ*) and LMSH65 ((Δ*lieAB* P_{lieAB}-*lacZ*). Average values and standard deviations are shown (n=3).

Figure 4: Contribution of *lftS* and *lieAB* genes to growth in the presence of aurantimycin A. Growth of *L. monocytogenes* strains EGD-e (wt), LMKK26 (Δ*lftS*), LMS160 (Δ*lieAB*) and LMSH66 (Δ*lftS* Δ*lieAB*) in the presence of different aurantimycin A concentrations after pre-adaption to aurantimycin A. For induction of *lieAB* expression, all strains were pre-grown in BHI broth containing a sub-inhibitory concentration of 100 ng/ml aurantimycin A to mid-exponential growth and then used to start cultures, the growth of which is shown here. The experiment was repeated three times and average values and standard deviations are shown.

Figure 5: Lysis of *L. monocytogenes* strains in the presence of aurantimycin A. *L. monocytogenes* strains EGD-e (wt), LMSH26 (Δ*lftR*) and LMSH35 (Δ*lftR* Δ*lieAB*), all devoid of the $rsbT^{10C\rightarrow T}$ mutation, were incubated in buffer and lysis was recorded over time in plain buffer, in the presence of 2 mg/ml aurantimycin A, or in the presence of 2 mg/ml aurantimycin A and 2.5 mg/ml glucose. All experiments were performed in triplicate and average values and standard deviations are shown.

Figure 6: LftRS function in *L. monocytogenes*.

(A) Repressed state. Basal transcription levels of *lftRS* ensure synthesis of LftR for repression of the *lftRS* and *lieAB* operons. (B) Induced state. Transcription of the *lftRS* and *lieAB* operons is induced in the presence of aurantimycin A. Induction requires LftS in a hitherto unknown manner. Massive synthesis of LieAB ABC transporter molecules leads to fast excretion of aurantimycin A out of the cell.

Table 1: *L. monocytogenes* genes deregulated in strain LMKK42 (Δ*lftR rsbT*$^{10C\rightarrow T}$)

locus	function	fold induction LMKK42 vs. wt	P value	σ^B control
upregulated genes				
lmo0979	*lieA* ABC transporter, ATP-binding protein	466±173	0.0003	
lmo0980	*lieB* ABC transporter, membrane component	454±138	0.0005	
lmo0720	*lftS*	187±32.1	$3*10^{-7}$	
lmo0981	transporter	7.1±2.0	0.0065	
lmo0982	peptidase	4.4±1.2	0.0071	
lmo2678	*kdpE* response regulator	2.7±0.5	0.0014	
lmo2679	*kdpD* histidine kinase	2.7±0.4	0.0017	
downregulated genes				
lmo0434	*inlB* internalin	0.4±0.04	0.0071	(Oliver *et al.*, 2009)
lmo0670	hypothetical protein	0.1±0.10	0.0062	(Hain *et al.*, 2008)
lmo1427	*opuCB*	0.1±0.03	0.0065	(Hain *et al.*, 2008)
lmo1425	*opuCD*	0.1±0.02	0.0021	(Hain *et al.*, 2008)
lmo1426	*opuCC*	0.1±0.01	0.0016	(Hain *et al.*, 2008)
lmo1428	*opuCA*	0.08±0.02	0.0041	(Hain *et al.*, 2008)
lmo0669	putative oxidoreductase	0.08±0.03	0.0058	(Hain *et al.*, 2008)
lmo0602	putative transcriptional regulator	0.07±0.02	0.0021	(Hain *et al.*, 2008)
lmo2748	hypothetical protein	0.07±0.01	0.0041	(Hain *et al.*, 2008)
lmo0937	hypothetical protein	0.06±0.02	0.0093	(Oliver *et al.*, 2009)
lmo2230	arsenate reductase	0.05±0.02	0.0095	(Hain *et al.*, 2008)
lmo2213	Isd-type haem degradation protein	0.04±0.02	0.0095	(Mujahid *et al.*, 2013)
lmo0994	hypothetical protein	0.04±0.02	0.0083	(Hain *et al.*, 2008)
lmo0263	*inlH* internalin	0.04±0.03	0.0089	(Hain *et al.*, 2008)
lmo0596	hypothetical protein	0.04±0.01	0.0043	(Hain *et al.*, 2008)
lmo0913	succinate semialdehyde dehydrogenase	0.03±0.02	0.0089	(Hain *et al.*, 2008)

Table 2: MICs of *L. monocytogenes* mutants lacking *lftRS* and *lieAB* genes against rhodamine 6G and aurantimycin A.

strain[1]	genotype	MIC[2]	
		rhodamine 6G [µg/ml]	aurantimycin A [µg/ml]
EGD-e	wild type	2	1.25*
LMKK26	Δ*lftS*	2	1.25*
LMSH26	Δ*lftR*	2	2.5
LMS160	Δ*lieAB*	2	0.156
LMSH35	Δ*lftR* Δ*lieAB*	2	0.156

[1] The Δ*lftR* mutants used here do not contain the *rsbT*$^{10C\rightarrow T}$ mutation.

[2] Minimal inhibitory concentrations were determined three times and representative results are shown.

* These MICs are masked by suppressor formation. See text for details.

Table 3: Strains and plasmids used in this study

name	relevant characteristics	source/ reference
plasmids		
pAC5	*amyE´lacZ cm ´amyE*	(Martin-Verstraete *et al.*, 1992)
pMAD	*bla erm bgaB*	(Arnaud *et al.*, 2004)
pIMK	*neo*	(Monk *et al.*, 2008)
pKK56	*bla erm bgaB* Δ*lftR*	(Kaval *et al.*, 2015)
pSH399	*bla erm bgaB* Δ*lieAB*	(Kaval *et al.*, 2015)
pBP116	P_{gltAB}-*lacZ neo*	this work
pBP117	*lacZ neo*	this work
pSAH10	P_{lieAB}-*lacZ neo*	this work
pSAH11	P_{lftRS}-*lacZ neo*	this work
pSAH13	*bla erm bgaB* Δ*sigB*	this work
pSAH18	P_{gpsB}-*lacZ neo*	this work
pSAH19	P_{divIVA}-*lacZ neo*	this work
pSAH36	*bla erm bgaB lftS*	this work
***B. subtilis* strains**		
168	wild type	lab collection
***L. monocytogenes* strains**		
EGD-e	wild type, serovar 1/2a strain	lab collection
LMKK26	Δ*lftS* (*lmo0720*)	(Kaval *et al.*, 2015)
LMKK31	Δ*lftRS* (*lmo0719-0720*)	(Kaval *et al.*, 2015)
LMKK42	Δ*lftR* (*lmo0719*) $rsbT^{10C\rightarrow T}$	(Kaval *et al.*, 2015)
LMS160	Δ*lieAB* (*lmo0979-lmo0980*)	(Kaval *et al.*, 2015)
LMS168	Δ*lftRS* Δ*lieAB*	(Kaval *et al.*, 2015)
LMSH5	*attB::*P_{lieAB}-*lacZ neo*	pSAH10→EGD-e
LMSH6	Δ*lftR* $rsbT^{10C\rightarrow T}$ *attB::*P_{lieAB}-*lacZ neo*	pSAH10→LMKK42
LMSH7	*attB::*P_{lftRS}-*lacZ neo*	pSAH11→EGD-e
LMSH8	Δ*lftR* $rsbT^{10C\rightarrow T}$ *attB::*P_{lftRS}-*lacZ neo*	pSAH11→LMKK42
LMSH9	Δ*sigB* (*lmo0895*)	pSAH13↔EGD-e
LMSH16	*attB::lacZ neo*	pBP117→EGD-e
LMSH17	*attB::*P_{gpsB}-*lacZ neo*	pSAH18→EGD-e
LMSH18	*attB::*P_{divIVA}-*lacZ neo*	pSAH19→EGD-e
LMSH24	Δ*sigB attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMSH9
LMSH25	Δ*sigB attB::*P_{lftRS}-*lacZ neo*	pSAH11 → LMSH9
LMSH26	Δ*lftR* (*lmo0719*)	pKK56 ↔ EGD-e
LMSH27	Δ*lftS attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMKK26
LMSH34	Δ*lftR attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMSH26
LMSH35	Δ*lftR* Δ*lieAB*	pSH399 ↔ LMSH26
LMSH43	*lftS* revertant	pSAH36 ↔ LMKK26
LMSH49	*lftS* revertant *attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMSH43
LMSH64	Δ*lftRS attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMKK31
LMSH65	Δ*lieAB attB::*P_{lieAB}-*lacZ neo*	pSAH10 → LMS160
LMSH66	Δ*lftS* Δ*lieAB*	pSH399 ↔ LMKK26

Table 4: Oligonucleotides used in this study

name	sequence (5´→3´)
FC269	AAAGAATTCCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCC
FC270	TTTCTCGAGACATAATGGATTTCCTTACGCGAAATACGGGCAGACATGGCCTGCCC
FC271	TTTGAATTCTTGAGCTTTTGGCATTTGATTGTACGTCATAATTCC
IW1	AAAGAATTCGATCAGCGGCTTCTGAAACGTG
SAH79	AGAACTAGTGGATCCCACGCTAATCTTGATGAGAAATGCC
SAH88	GCTCTAGAACTAGTGGATCCCTCGATGAACTTTGTGGTATTC
SAH99	GTTGTAAAACGACGGGGAATTCCATTTGAATACAACCTTCTTTCC
SAH100	AGAACTAGTGGATCCCATTTGAATTCACTCCTCTACTAGTTG
SAH101	CTATCGATGCATGCCATGGTGTTGCTGGGGAGATCGATGC
SAH102	GATTCAACTGCCTTGTTCATTTAATCAGGTTGAGATACTTTTGGCATTC
SAH104	CTGCAGAAGCTTCTAGAATTCAATCCGCGCGAAAAAAACG
SAH107	GAATGCCAAAAGTATCTCAACCTGATGAGGAAGTGGAGTAAATGAACAAGG
SAH129	AAACGAATAGCCGCTTTGCC
SAH130	CTAATACGACTCACTATAGGGAGATCTCGCAATTTCTGACGTTGT
SAH138	CTAGAACTAGTGGATCCCTGACCGAGCGACTCAAGC
SAH139	GTAAAACGACGGGGAATTCCATATAACTCCACCTCTATCTCT
SAH140	CTAGAACTAGTGGATCCCTGCCACTATGCTGTCG
SAH141	GTAAAACGACGGGGAATTCCATATCTAGCACCTCCATAC
SAH193	CTATCGATGCATGCCATGGTCACATCTGACCAAGAAGCAGC
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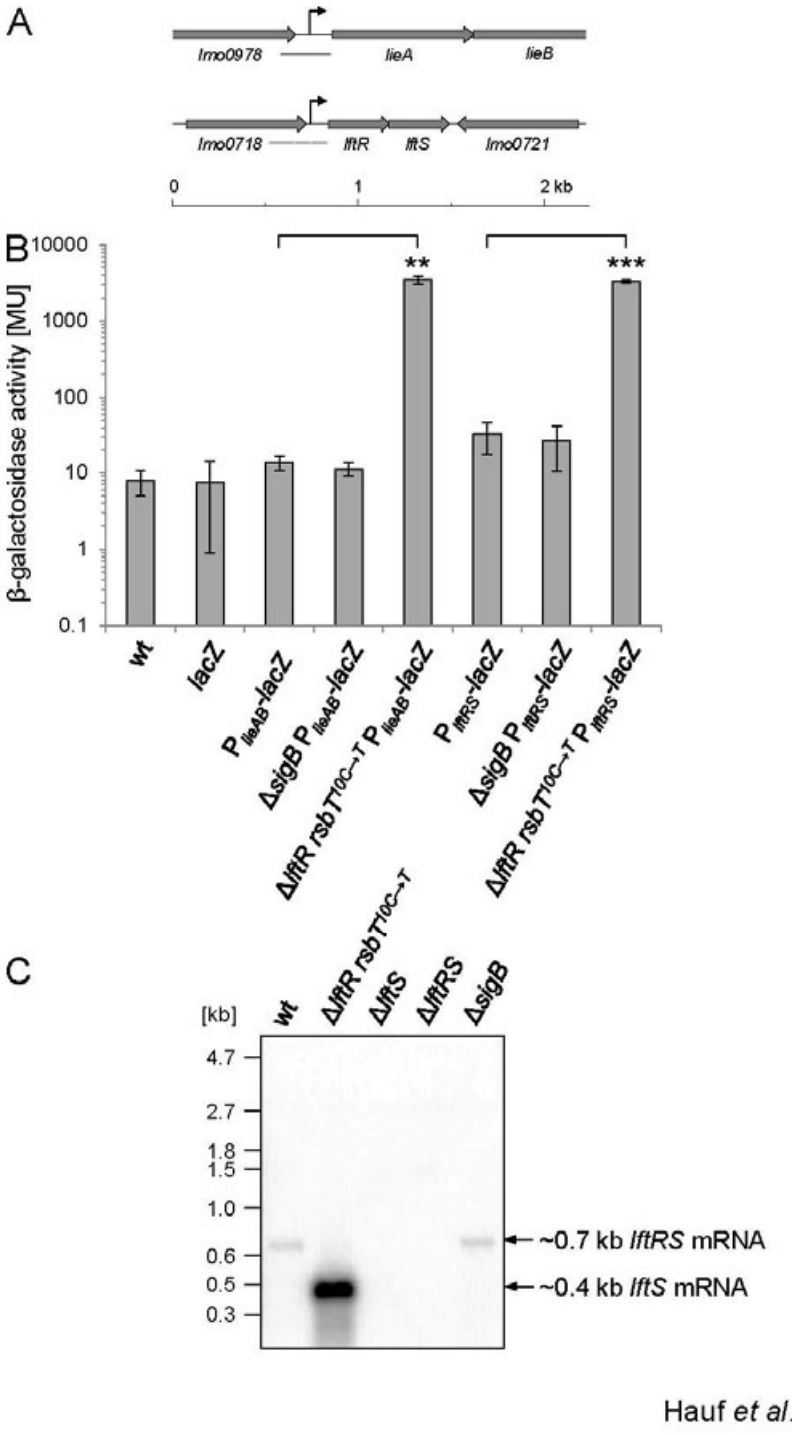
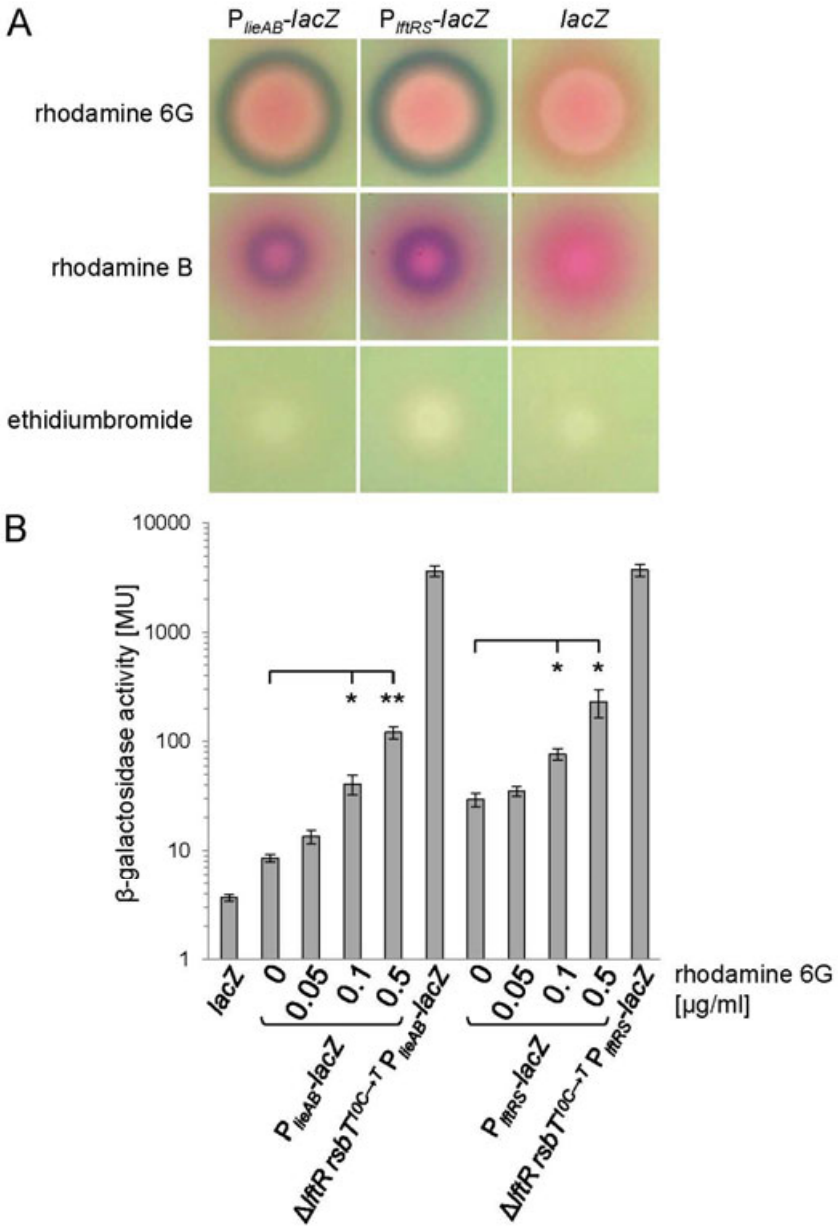
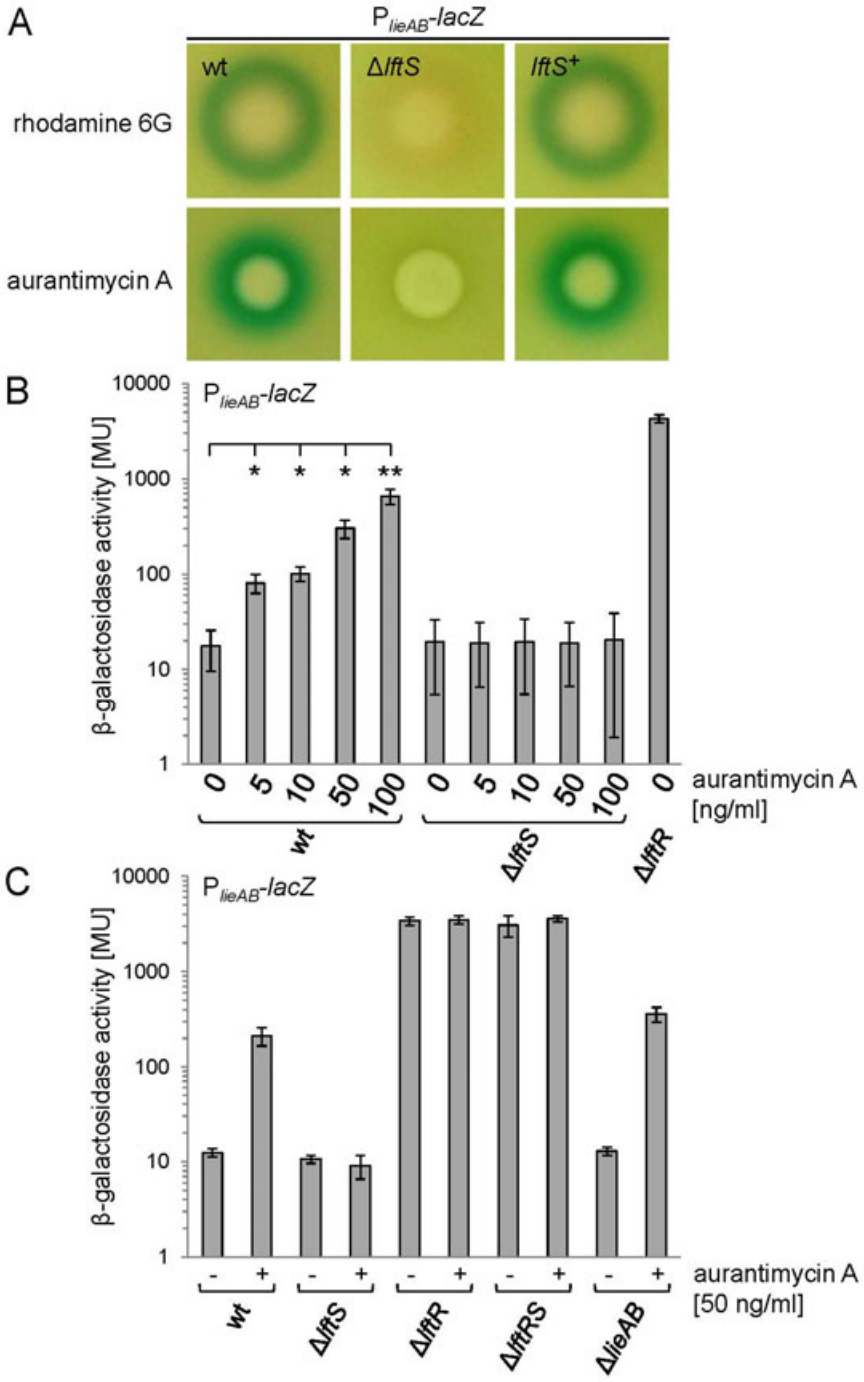


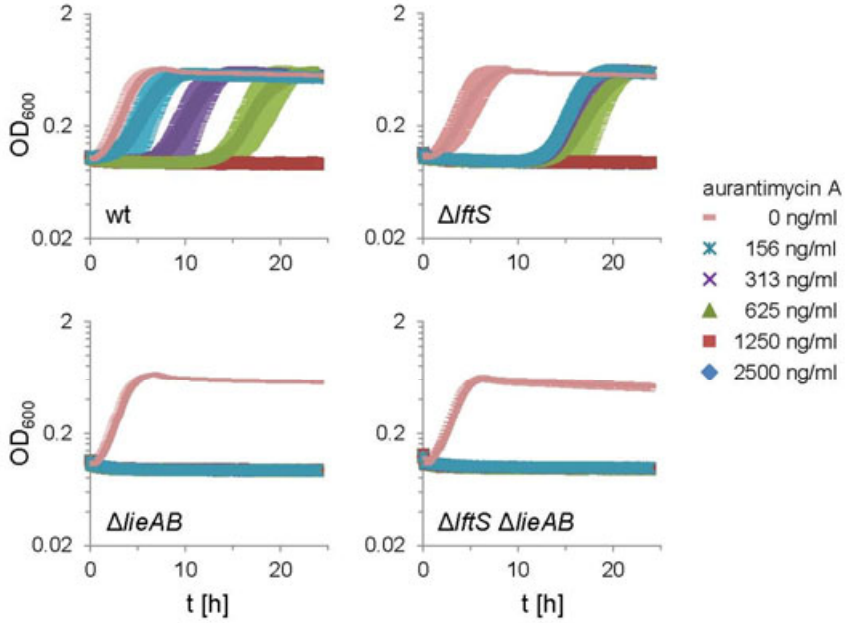
Hauf *et al.*, Figure 1



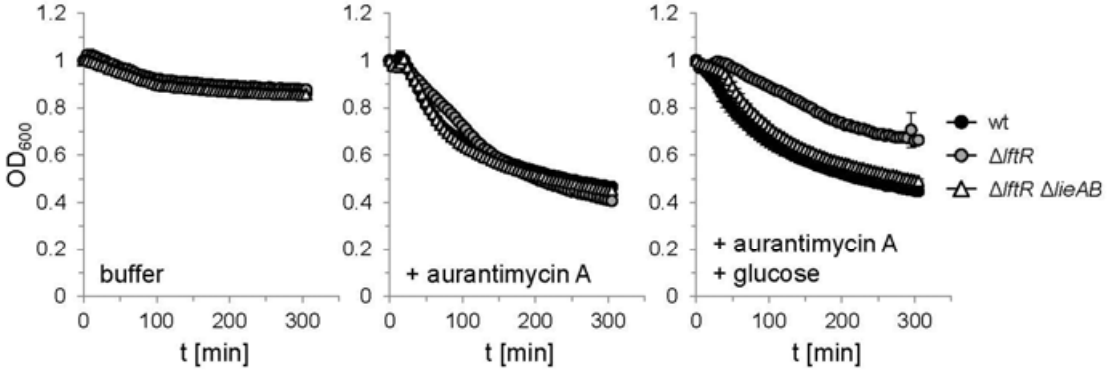
Hauf *et al.*, Figure 2



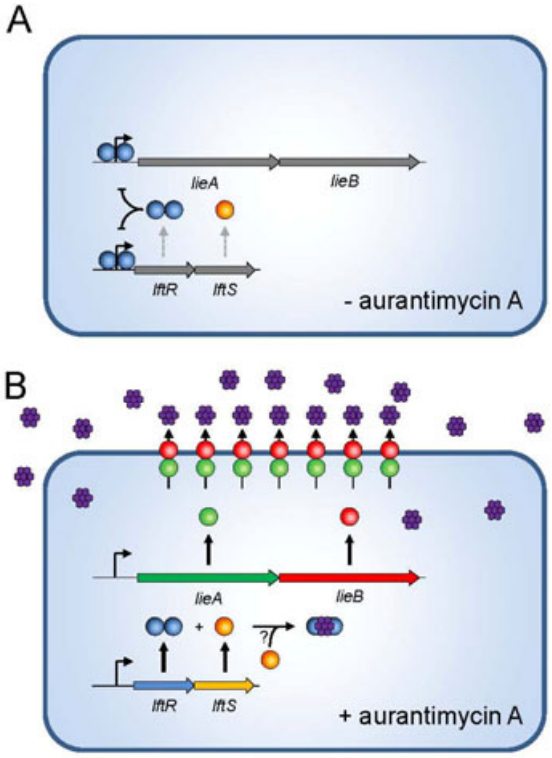
Hauf *et al.*, Figure 3



Hauf *et al.*, Figure 4



Hauf *et al.*, Figure 5



Hauf *et al.*, Figure 6