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MINI-REVIEW

The applied side of antimicrobial peptide-inducible promoters from *Firmicutes* bacteria: expression systems and whole-cell biosensors

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Abstract The cell envelope is an essential bacterial structure that consists of the cytoplasmic membrane, the cell wall, and—in Gram-negative bacteria—the outer membrane. Because of its crucial functions, it represents a prime antibiotic target. Monitoring and maintaining its integrity are therefore keys to survival, especially in competitive environments where antibiotics represent one means of suppressing the growth of competitors. Resistance against external antibiotic threat, as well as auto-immunity against self-produced antibiotics, is often mediated by two-component systems (2CSs). They respond to antibiotic threat by inducing gene expression that results in the production of specific resistance determinants. The underlying transcriptional control is exhibited at the level of specific target promoters, which usually share a number of relevant features: They are tightly controlled and only induced in the presence of specific (sets of) antibiotics. This induction is dose dependent and often very sensitive, that is, it occurs well below inhibitory antibiotic concentrations. Because of these characteristics, a number of well-characterized cell envelope stress-inducible promoters have been developed for two different applied purposes: first, as whole-cell biosensors for antibiotic detection and mechanism-of-action studies, and second, as antibiotic-inducible expression systems for biotechnological purposes. The current state of research in both fields will be discussed in this review, focusing on 2CS-regulated promoters from *Firmicutes* bacteria that are induced to mediate resistance against antimicrobial peptides (AMPs) targeting the cell envelope.

Keywords Whole-cell biosensor · Expression system · Cell wall antibiotic · Signal transduction · Two-component system · Inducible promoter

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Introduction

Life in the microbial world requires the ability to respond to (changes of) numerous environmental parameters in order to adequately adjust the cell's physiology accordingly at any given time. The complexity of such responses is a direct function of the complexity of the habitat a microbe lives in and often necessitates intricate regulatory networks that perceive, integrate, and compute numerous stimuli at the same time (Pinto and Mascher 2016). In addition to numerous abiotic parameters, bacteria living in densely populated environments such as the soil biosphere must also be prepared to face the fierce competition for the limited resources available. One aspect of this competition is the production of antimicrobial compounds that are released to the environment to suppress the growth of competitors. As a consequence, success in such an environment requires the ability to respond to and resist antibiotic threats from other microorganisms by mounting appropriate countermeasures.

The cell envelope is a prime target for many antibiotics, especially antimicrobial peptides (AMPs), due to its numerous essential functions: It protects the cell from its environment, acts as a molecular sieve and communication interface, and—most importantly—counteracts the internal osmotic pressure. Ensuring its integrity is therefore crucial for the survival. Accordingly, bacteria exposed to microbial competition have evolved designated cell envelope stress responses (CESR) that

monitor and maintain the integrity of the envelope by mounting protective measures in the presence of cell envelope damage caused, for example, by the presence of antibiotics (Jordan et al. 2008; Raivio 2005; Revilla-Guarinos et al. 2014). Mechanistically, such responses are very often mediated by specific signal transducing two-component systems (2CSs) that link the perception of an extracellular stimulus (sensed by a membrane-anchored histidine kinase) with a corresponding cellular response, mediated by a cytoplasmic partner protein, the response regulator (Schrecke et al. 2012). As a consequence, specific target genes are upregulated that encode designated resistance determinants that either inactivate/remove the antimicrobial compounds or counteract the cellular damage caused by their presence. Related mechanisms also apply for mounting the auto-immunity in case of antibiotic production (Gebhard 2012).

The cellular purpose of such CESR, that is, protecting the cell efficiently before a significant damage occurs, provides these systems with very interesting features that inspired the development of genetic tools for applied purposes. The target promoters are usually tightly regulated and almost shut off in the absence of inducing conditions. As soon as the presence of cell envelope-targeting antibiotics is perceived (and way before harmful concentrations can accumulate), the 2CSs need to react in a fast, sensitive, and dose-dependent manner to the perceived stimulus in order to protect the cell adequately. Hence, the threshold response concentrations of such systems (that is, the lowest concentration at which a specific induction of CESR-target promoters can be observed) are well below the minimal inhibitory concentration of the antibiotics that serve as inducers. Moreover, the regulated promoters show highly dynamic dose-response behaviors, often reaching over 100-fold induction range between minimal threshold concentrations and the saturation of the response. This tight regulation and high dose-dependent dynamics lead to the development of a number of AMP-inducible expression systems for Gram-positive model organisms that are widely used both in research and biotechnological applications.

Due to the specificity of stimulus perception, most CESR-sensing 2CSs are very selective with respect to the range of antibiotics that induce a response. Once the antibiotic spectrum has been carefully characterized, their target promoters—if fused to a suitable reporter gene—therefore provide ideal candidates for the development of highly antibiotic-specific whole-cell biosensors. Such reporter strains can then be used to identify antibiotic producers or characterize the mechanism of action of novel antimicrobial compounds.

This mini review aims at illustrating the potential of these two different applications of CESR systems for a specific group of cell wall-active compounds, AMPs, derived from low G + C Gram-positive bacteria (phylum *Firmicutes*).

AMP-inducible expression systems for the *Firmicutes*: "NICE? SURE, I LIKE it!"

For many years, Gram-positive bacteria such as *Lactococcus lactis* and *Bacillus subtilis* have been extensively used as biotechnological workhorses for both small- and large-scale production of heterologously expressed proteins for industrial and pharmaceutical purposes (Gaspar et al. 2013; Morello et al. 2008; Schallmey et al. 2004; van Dijl and Hecker 2013). These organisms share a couple of important features including growth to high cell densities, genetic stability, ease of genetic manipulation, and the GRAS (*g*enerally *r*ecognized *a*s *s*afe) status, thereby allowing a broad range of applications in the biotechnological and even food industry. Additionally, *B. subtilis* is equipped with a versatile and efficient protein secretion machinery, which enables the purification of proteins directly from the culture supernatant (Bongers et al. 2005; Mierau and Kleerebezem 2005).

For high-level protein production, expression systems based on AMP-inducible promoters have become important tools in both *L. lactis* and *B. subtilis*. Here, we will provide an overview and highlight characteristic features of three AMP-inducible 2CSs, which have been used to control recombinant protein production (Table 1).

The NICE system (*NI*sin *C*ontrolled gene *E*xpression) is a versatile expression platform initially established for lactococci and later adapted to other Gram-positive microorganisms, such as lactobacilli or enterococci (Table 1 and Fig. 1b). The 34 residue-long lantibiotic nisin is produced by several strains of *L. lactis* and is used as a natural preservative in the food industry (Delves-Broughton et al. 1996). It causes cell death of other Gram-positive bacteria by a highly specific interaction with the lipid II precursor of cell wall biosynthesis, which is used as an anchor site to ultimately assemble small pores in the cell membrane that lead to cell death (Hasper et al. 2004). The genes involved in regulation, expression, and synthesis of nisin are encoded in the *nis* gene cluster of *L. lactis* (Kuipers et al. 1993) (Fig. 1a). Their expression is controlled by three promoters responsible for the synthesis and maturation of nisin (P_{nisA}), the regulation of the *nis* gene cluster (P_{nisR}), and the auto-immunity against nisin (P_{nisF}), respectively (Fig. 1a). The production of nisin occurs in a cell density-dependent manner through quorum sensing-controlled autoinduction in *L. lactis* (Kleerebezem 2004; Kuipers et al. 1995). This autoregulatory mechanism is mediated by the nisin-responsive 2CS NisRK. This system has been adopted to control the production of the recombinant protein GusA of *Escherichia coli* from the strictly NisRK-dependent, nisin-inducible promoter, P_{nisA}, demonstrating that induction and expression of *gusA* occurs in a dose-dependent manner (Kuipers et al. 1995). Based on these very promising initial experiments, the NICE system was subsequently

Table 1 Overview of the AMP-inducible protein expression systems NICE, SURE, and LIKE

	NICE system[a]	SURE system[b]	LIKE system[c]
Inducer	Nisin	Subtilin	Bacitracin, vancomycin, etc.
Producing strain	*Lactococcus lactis* NZ9700	*Bacillus subtilis* ATCC6633	–
Concentration range	0.1–5 ng ml^{-1}	0.1–0.5 % (vol/vol)	30 μg ml^{-1} (Bac)
Host strains	Lactococci, Lactobacilli, Enterococci, Bacilli	*Bacillus subtilis*	*Bacillus subtilis*
Signal transduction	NisRK (2CS)	SpaRK (2CS)	LiaRS (2CS)
2CS expression vectors	Replicative	Integrative	Not required
2CS expression	Low constitutive	Dually controlled, low	Low constitutive
Target promoter	P_{nisA}	P_{spaS} (wild type or mutated)	P_{liaI} (optimized RBS)
Basal activity	Low	Low	Very low
Gene expression	Strictly 2CS-controlled, dose-dependent, highly dynamic		
Expression range	Up to 1000-fold	About 80–110-fold	Up to1000-fold
Large-scale production	Yes	Not tested so far	Not tested so far
Availability of components			
Inducing AMPs	Commercial	Culture supernatant	Commercial
2CS expression vectors	Yes	Yes	Not required
Protein expression vectors	Yes (replicative vectors for transcriptional, translational fusions, or protein secretion)	Yes (replicative vectors for transcriptional or translational fusions)	Yes (integrative and replicative expression vectors)
Specific expression strain	No	No	Yes
Sources	MoBiTec (commercial)	MoBiTec (commercial)	BGSC (user fee only)

[a] Reviewed by (Mierau and Kleerebezem 2005); [b] (Bongers et al. 2005); [c] (Toymentseva et al. 2012)

developed to tightly control gene expression in *L. lactis* and other Gram-positive organisms. This system uses nisin as an inducer and utilizes the 2CS NisRK to strictly control the activity of P_{nisA} as a target promoter for recombinant protein production (de Ruyter et al. 1996; Eichenbaum et al. 1998; Kuipers et al. 1995) (Fig. 1b).

Twenty years after its initial description, the NICE system is used for a broad range of applications (e.g., food industry, protein secretion) at both the laboratory and industrial scale (Maischberger et al. 2010; Mierau and Kleerebezem 2005; Mierau et al. 2005a; Mierau et al. 2005b; Mironczuk et al. 2012; Zhang et al. 2015). The system was patented by NIZO food research BV, and a variety of *L. lactis* strains expressing NisRK are commercially available (Table 1) that are commonly used as cell-based "biofactories" to produce proteins of interest (Bernaudat et al. 2011; Mierau and Kleerebezem 2005; Morello et al. 2008). A number of replicative vectors are available to fuse genes of interest to P_{nisA} and overexpress proteins in *L. lactis* by adding subinhibitory concentrations of nisin (0.1–5 ng ml^{-1}) to exponentially growing cultures (Mierau and Kleerebezem 2005). The NICE system thereby allows up to 1000-fold induction of expression levels, which can be easily adjusted based on the linear dose-response behavior towards its inducer nisin (Zhou et al. 2006).

Importantly, the NICE system could also be transferred to other Gram-positive hosts, for example, *B. subtilis*, streptococci, or enterococci. For this purpose, the 2CS NisRK needs to be provided to the chosen host on a separate plasmid, in addition to the P_{nisA}-fused gene of interest (Eichenbaum et al. 1998) (Fig. 1b). In some cases, the growth of the host organism was affected by either maintaining the two-plasmid system or by the sensitivity towards the inducer, nisin (Zhou et al. 2006). Moreover, codon-usage incompatibilities also occurred in some Gram-positive microorganisms, such as *Leuconostoc lactis*. Therefore, the overexpression conditions, media composition, cell density, amount of nisin, or expression temperature need to be carefully adjusted to accommodate the respective host organism and protein to be produced (Eichenbaum et al. 1998; Zhou et al. 2006).

The SURE system (*SU*btilin *R*egulated gene *E*xpression) of *B. subtilis* shares many features with the NICE system of *L. lactis* (Table 1 and Fig. 1c). It is induced by the lantibiotic

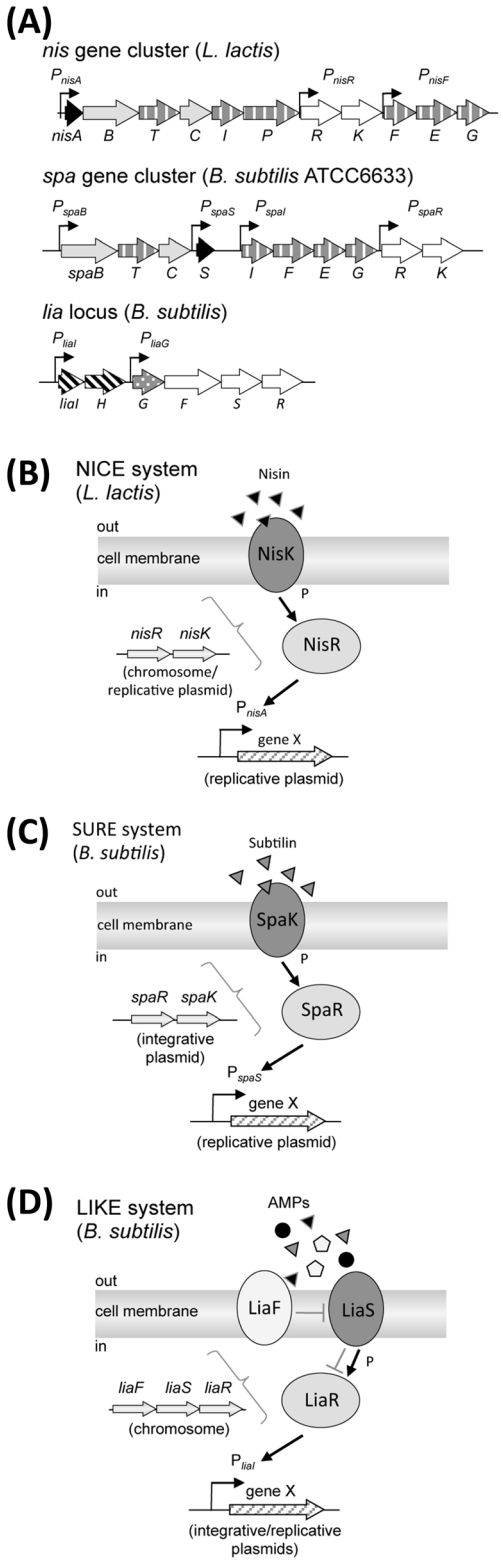

Fig. 1 Expression systems based on AMP-inducible promoters. (**a**) Genomic organization of the nisin gene cluster in *L. lactis*, the subtilin gene cluster, and the *lia* locus in *B. subtilis* (adapted from (Cortes 2014; Kleerebezem 2004; Wolf et al. 2010). The structural genes of nisin (*nisA*) and subtilin (*spaS*) (marked in *black*) are target genes of NisR and SpaR, respectively. Genes involved in peptide biosynthesis and transport or immunity are highlighted in *grey* and vertically hatched, respectively. Regulation genes are indicated in *white*. Incline hatched genes indicate target genes of LiaR. Genes of unknown function are highlighted in *grey dotted*. Schematic representation of the NICE system (**b**), the SURE system (**c**), and the LIKE system (**d**). Antibiotic-responsive 2CSs are expressed either directly from chromosomal genes or need to be provided on replicative/integrative plasmids. In response to inducing antibiotic concentrations, the histidine kinases NisK, SpaK, or LiaS (marked in *dark grey*) autophosphorylate and subsequently transfer a phosporyl group (*P*) to their cognate response regulator NisR, SpaR, or LiaR (highlighted in *light grey*), respectively. The corresponding target promoters control the expression of the desired gene-of-interest and are provided on replicative and/or integrative plasmids. See text for details

subtilin, a small 32 amino acid-long peptide lantibiotic that shares significant structural and mechanistic similarities with nisin (Breukink and de Kruijff 1999; Kleerebezem 2004; Siezen et al. 1996). Likewise, the regulation and expression of subtilin in *B. subtilis* is related to that of nisin in *L. lactis* (Siezen et al. 1996) (Fig. 1a). The genes encoding the functions for the biosynthesis, maturation, and modification of subtilin as well as those for the regulation and the autoimmunity against subtilin are located in the *spa* gene cluster on the chromosome of *B. subtilis* ATCC6633 (Fig. 1a). Transcription of the monocistronic structural gene encoding subtilin, *spaS*, is controlled by the P_{spaS} promoter. All remaining genes of the *spa* locus are transcribed as polycistronic mRNAs from three independent promoters (Kleerebezem 2004) (Fig. 1a). With the exception of P_{spaR}, responsible for expression of the 2CS SpaRK, all other promoters of the *spa* locus are auto-regulated by SpaRK in a subtilin-dependent manner (Kleerebezem et al. 2004; Stein et al. 2003). Of these, P_{spaS} is the strongest subtilin-inducible promoter within the *spa* gene cluster (Kleerebezem et al. 2004). In contrast to the constitutive expression of *nisRK* in *L. lactis*, the promoter mediating expression of the homologous 2CS SpaRK in *B. subtilis* ATCC6633, P_{spaR}, is subject to a complex growth phase-dependent regulation. It is dually controlled by AbrB and the alternative sigma factor H (σ^H) during the transition from the exponential to the stationary growth phase (Stein et al. 2002).

For developing the SURE system, the *spaRK* operon, encoding the subtilin-responsive 2CS, and its target promoter P_{spaS} were transferred from the subtilin producer strain ATCC6633 into the laboratory reference strain 168 (Bongers et al. 2005) (Fig. 1c). The β-glucuronidase GusA from *E. coli* and the green fluorescent protein GFP could be overproduced successfully both under control of the wild type or a mutated P_{spaS} after the addition of subtilin as an inducer. The mutated *spaS* promoter contains a perfect direct repeat (the *spa* box), which is assumed to enhance recognition and binding of the response regulator SpaR to the promoter sequence (Bongers et al. 2005). Under non-inducing conditions (in the absence of subtilin), both versions

of P_{spaS} were only slightly active, thereby ensuring a tight transcriptional control. As a potential disadvantage of the SURE system, its inducer subtilin is not commercially available. But gene expression can easily be induced by adding 0.1–0.5 % (vol/vol) subtilin-containing supernatants from cultures of the producer strain *B. subtilis* ATCC6633 (Bongers et al. 2005). Higher amounts of supernatant do not increase protein yields significantly due to increasing growth inhibition (Bongers et al. 2005). Of course, the exact amount of supernatant to be added has to be adjusted with each new batch of subtilin-containing supernatant.

So far, the SURE system—which is commercially available from the same supplier as the NICE system, MoBiTec (Göttingen, Germany)—consists of an integrative vector for expressing SpaRK and replicative expression vectors. The latter allow expressing a gene of interest either transcriptionally or translationally placed under the control of either the wild-type P_{spaS} or mutated P_{spaS}, resulting in an 110-fold or 80-fold overexpression (relative to background activities), respectively (Table 1 and Fig. 1c). It is recommended to determine the choice of which promoter/fusion to use for each target protein individually (Bongers et al. 2005).

The LIKE system Recently, the existing bioengineering toolbox for *B. subtilis* was extended by a novel AMP-inducible gene expression system, termed the LIKE system (from the German, *LI*a-*Kontrollierte Expression*) (Toymentseva et al. 2012) (Table 1 and Fig. 1d). Here, gene expression and protein production are based on the *liaI* promoter, which is controlled by LiaRS (Fig. 1a). This 2CS responds to a variety of structurally unrelated AMPs (e.g., bacitracin, lantibiotics, or vancomycin) interfering with the lipid II cycle of cell wall biosynthesis, resulting in a strong activation of P_{liaI} (Mascher et al. 2004). The genes encoding the LiaRS are embedded in the *lia* locus of *B. subtilis*, which consists of the genes *liaIHGFSR* that are transcribed from two independent promoters, P_{liaG} and P_{liaI} (Fig. 1a) (Jordan et al. 2006; Mascher et al. 2004). The constitutive *liaG* promoter is responsible for expressing the 2CS LiaRS and two additional regulatory genes, *liaG* and *liaF*, at basal level in the absence of inducing conditions (Jordan et al. 2006). P_{liaI} is strictly LiaR dependent and virtually "shut off" during exponential growth in the absence of AMP stress, due to direct repression through the transition state regulator AbrB (Jordan et al. 2007). Additionally, the inhibitory membrane protein LiaF, which is co-expressed with *liaSR*, keeps the histidine kinase LiaS strictly in its phosphatase state in the absence of any stimulating signal, thereby efficiently inactivating LiaR and preventing the induction of P_{liaI} (Jordan et al. 2006) (Fig. 1d). In the presence of inducing compounds such as bacitracin, LiaF releases the inhibitory grip on LiaS, which switches into the kinase-ON state, autophosphorylates, and subsequently transmits the signal via a phosphoryl group transfer to the response regulator LiaR (Fig. 1d). As a consequence, LiaR is activated and binds to its target promoter, P_{liaI}, resulting in a fast and strong transcriptional activation of *liaIH* expression, which is enhanced up to 1000-fold, depending on the inducing conditions (Mascher et al. 2004; Toymentseva et al. 2012).

The low basal activity and tight regulation of P_{liaI}, together with a choice of inducers of different potency, make the LIKE system an interesting alterative to the other two systems for engineering protein production processes (Toymentseva et al. 2012) (Fig. 1d). Due to the native regulation, only one vector is needed to introduce the gene of interest fused to P_{liaI}. A replicative (multi-copy but potentially less stable) and an integrative (single-copy and stable) expression vector (pLIKE-rep and pLIKE-int, respectively) are freely available from the Bacillus Genetic Stock Center (BGSC). Both vectors carry an optimized Shine-Dalgarno sequence to enhance translation efficiency (Toymentseva et al. 2012) (Table 1). While these vectors work perfectly fine in all standard *B. subtilis* wild-type strains, the LIKE system also provides three specialized production hosts (derivatives of strain 168), in which the *liaIH* operon and parts of its genomic neighborhood have been deleted, to avoid the metabolic/translational burden of the strong native production of LiaI and LiaH. As with the SURE system, the ideal combination of strains and plasmids needs to be empirically determined for each protein to be produced.

In addition to the AMP-inducible expression just described, an existing LIKE-based production strain can easily be converted to a strong constitutive overexpression host by simply deleting the *liaF* gene encoding the LiaRS-specific inhibitor protein (Toymentseva et al. 2012). The dynamics of P_{liaI} activation are yet another unique feature of the LIKE system. In response to the inducer, the promoter strongly responds instantly but only transiently for about 60–90 min. This could be an advantage for producing toxic proteins, while it may limit the overall yield of otherwise "well-behaving" proteins. The LIKE system therefore provides unsurpassed degrees of freedom to engineer an optimized overproduction strain.

In summary, all three AMP-inducible expression systems are suitable for scientific and industrial applications but have their pros and cons (summarized in Table 1). While the NICE system has already been adapted to different *Firmicutes* bacteria, it has so far been mostly used in *L. lactis*, due to the overall more robust behavior in its native host (Mierau and Kleerebezem 2005). If proteins should be produced in *B. subtilis*, the SURE or LIKE system is recommended. Both provide stable and versatile expression platforms with a choice of different vectors and strains (Fig. 1c–d). Since in each case, the 2CS (LiaRS and SpaRK) responsible for the regulation of the gene expression is or can be integrated in the genome of *B. subtilis*, both systems only require a single expression vector. The SURE and the LIKE systems also offer a

highly dynamic protein expression range dependent of the used AMP and its concentration for induction (Bongers et al. 2005; Toymentseva et al. 2012).

Since the components of the NICE system are located on two independent replicative plasmids (harboring *nisRK* and P_{nisA}, respectively), it is also possible to combine it with the SURE or the LIKE system in a single *B. subtilis* production strain to co-express two different proteins independent from one another (e.g., for protein-protein interaction studies in vivo). In a NICE-SURE combination, the production would be independently controlled by the separate addition of nisin and/or subtilin. A NICE-LIKE combination would also be possible but could only be induced simultaneously by nisin, since P_{liaI} strongly responds to both nisin and subtilin (Staroń et al. 2011). A LIKE-SURE combination in *B. subtilis* should also be feasible. Again, both expression platforms would then be simultaneously induced by subtilin.

Whole-cell biosensors for antimicrobial discovery

Combatting the ever-increasing threat of antibiotic-resistant pathogens is one of the most urgent medical challenges of the twenty-first century. The true dimension of the global antimicrobial resistance dilemma has recently been highlighted by the first global surveillance report published by the World Health Organization (WHO 2014). It underscores the dire need for identifying novel classes of antimicrobial compounds as new defense lines against antibiotic-resistant bacteria (Fischbach and Walsh 2009; Walsh and Wencewicz 2014), especially of the ESKAPE group, which comprises the leading multi-resistant bacterial pathogens (Bassetti et al. 2013; Boucher et al. 2009; Pendleton et al. 2013).

One crucial step in identifying and characterizing new antimicrobial compounds, irrespective of their source, is to narrow down their mechanism of action in an easy and straightforward manner. This requires sensitive, specific, and also robust (bio) assays that are adjustable to different sample types (e.g., extracts but also living producer strains) and should ideally also be suitable for high-throughput screening. In this context, whole-cell biosensors have gained increasing attention in recent years. Given the focus of this review, we will solely discuss *Firmicutes* biosensors specific for (the action of) AMPs that interfere with envelope integrity. For a broader coverage on other applications of whole-cell biosensors, including monitoring (waste) water, food, and soil samples for contaminations, readers are referred to a number of excellent recent overview articles (Chen and Rosen 2014; Fernandez-Lopez et al. 2015; He et al. 2016; Park et al. 2013; Ron 2007; Virolainen and Karp 2014).

In a nutshell, whole-cell biosensors usually consist of a modified microbial reporter strain, in which a compound- or target-specific inducible promoter is fused to a suitable reporter gene (Fig. 2a). With respect to antibiotic-specific biosensors, most promoters used for such purposes have initially been identified in the course of global transcriptomic or proteomic profiling efforts aimed at studying the bacterial response to antibiotic challenge (Wecke and Mascher 2011). From the Gram-positive bacteria, *B. subtilis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus* have been particularly well studied with regard to their response against numerous antimicrobial compounds. Since the latter two are pathogens with limited genetic accessibility, only *B. subtilis* qualifies for the development of whole-cell biosensors, given its GRAS status, ease of genetic manipulations, and the availability of numerous suitable reporter genes. Moreover, it was subjected

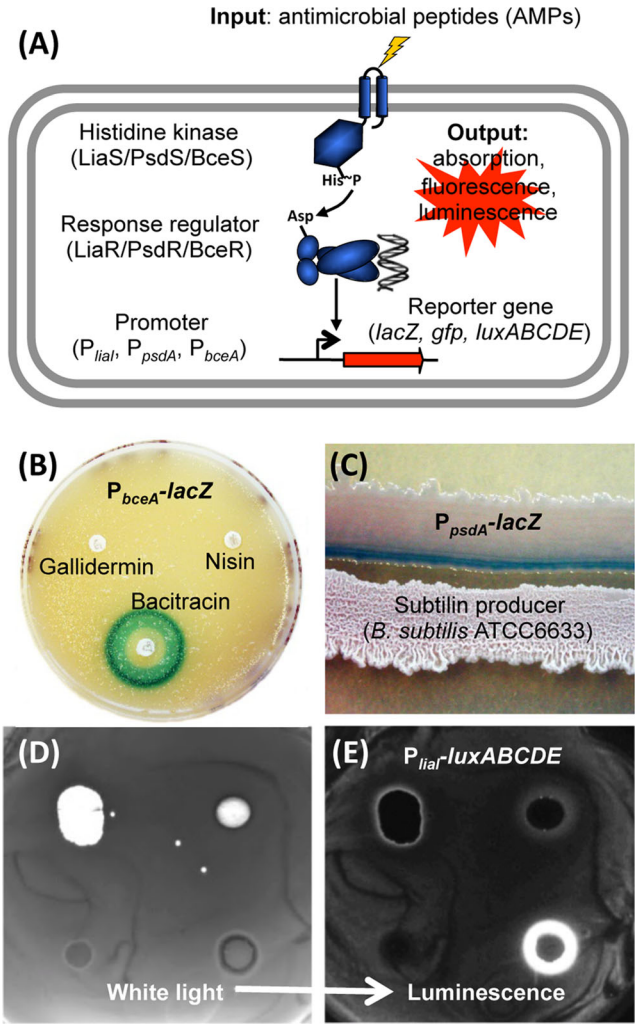

Fig. 2 Whole-cell biosensors based on AMP-inducible promoters. **a** Schematic illustration of the components required for AMP-inducible whole-cell biosensors. Plate-based applications of whole-cell biosensors derived from P_{bceA}-*lacZ* (**b**), P_{psdA}-*lacZ* (**c**), and P*liaI-lux* (**d**, **e**) for screening antimicrobial compounds (**b**, **d**, **e**) or producers (**c**). The whole-cell biosensor is either provided as a lawn on the plates (**b**) or streaked out directly next to a potential producer strain (**c**, **d**, **e**). The formation of *blue zones* (**b**, **c**) or glowing halos (in the luminescence channel of **e**) on the plates indicate the presence of AMPs. See text for details

to the largest range of antimicrobials, thereby providing the most comprehensive dataset for the identification of suitable promoters (Wecke and Mascher 2011). Initially, the profiles derived from these studies identified sets of genes or proteins as reliable markers for a specific antimicrobial mechanism of action (MOA) (Bandow et al. 2003; Hutter et al. 2004b). But such transcriptomic or proteomic profiling—while powerful in later stages of characterizing novel antimicrobials—are too labor and cost intensive to be directly applied for initial large panel screens of extracts or producer strains. For this purpose, individual promoters identified from such profiling efforts were studied and evaluated in great detail over the last 10 years.

Choice of promoters For the development of whole-cell biosensors, potential promoters ideally need to fulfill five criteria: (i) Their activity should be virtually switched off in the absence of inducing conditions, since a low basal activity is an absolute prerequisite for a robust and specific biosensor. (ii) In the presence of suitable triggers, they should exhibit a strong and highly dynamic response. The simple rule here is: the higher the fold change of induction over background, the better. (iii) The response should be sensitive, that is, occur well below the antibiotic concentration at which cells start taking damage. If induction only occurs at or near the minimal inhibitory concentration of the antimicrobial compound, the whole-cell biosensor will always be at the brink of death before it fires, which does not help in the development of a robust system. (iv) The response should be specific for a certain, ideally well-defined group of compounds or conditions to allow for a precise readout. (v) The resulting biosensors should be robust, that is, ideally work under very different experimental conditions (e.g., a range of different media and growth conditions) both in liquid culture and in plate-based assays. Such robustness results from a combination of all four previous criteria, but is also an intrinsic property of the underlying signal transduction and gene regulation.

While the criteria listed above argue in favor of very specific biosensors, it should be noted that more general biosensors that cover a broader group of unrelated compounds do have their merit as initial screening tools. In that light, a panel of whole-cell biosensors developed for *B. subtilis* as markers for inhibition of the major biosynthetic pathways targeted by many antibiotics (fatty acid, protein, DNA, RNA, and cell wall biosynthesis) can be viewed as ideally suited for the first screen (Hutter et al. 2004a; Urban et al. 2007). More compound- or MOA-specific biosensors from within one of the general pathways can then be applied for the subsequent stages of the detailed characterization. The cell wall-specific biosensors from these two panels are listed in Table 2 and will be described below.

Choice of reporter genes As with the promoters, again a number of criteria apply for choosing a suitable reporter gene. (i) The output of a reporter system should ideally be nil in the absence of inducing conditions to ensure a highly dynamic output upon induction. Intrinsic background can result from leakiness of the promoter or intrinsic activities/properties of the host cell. The first is linked to the choice of promoters, and the second can sometimes be very difficult to circumvent and may have an important role in deciding on what reporter gene to use. (ii) The reporter should provide a clear and reproducible correlation between input and output levels over a wide range of antibiotic concentrations. (iii) The reporter should be fast and robust, to allow for reproducible measurements. (iv) The reporter assay should ideally facilitate automated protocols to increase the throughput and reduce experimental errors due to manual handling of samples.

Irrespective of availability and preferences for a given organism, three major groups of well-established transcriptional reporters can be distinguished. (i) Enzymes that convert a chromophoric substrate into a colored product that can be monitored qualitatively by eye or quantitatively by measuring the absorption. The β-galactosidase is without doubt the best-known reporter of this category. (ii) Fluorescent proteins, such as GFP, that can be excited by a specific wavelength to then emit light of a different wavelength. (iii) Luminescent reporters that emit photons as a result of a biochemical reaction. The substrate can either be provided during the assay (e.g., firefly luciferase) or can be metabolically recharged, as is the case for the bacterial luciferase system derived from *Photorhabdus luminescence*.

The β-galactosidase is without doubt the most classical and still widely used reporter system (Miller 1972). The obvious advantages of using β-galactosidase are its simplicity, robustness, good dynamic range, low background, and reliable input-output correlation. Moreover, a direct agar plate-based screening of bioactive extracts or even directly potential producers strains is easily possible without the need of any specialized equipment (Fig. 2b, c) (Mascher et al. 2004; Staroń et al. 2011). On the downside, generating the readout for the β-galactosidase requires performing an assay, thereby precluding a direct online monitoring of promoter activity.

Fluorescent proteins such as GFP represent the least suitable of the three reporters when it comes to developing whole-cell biosensors. While allowing a direct readout that can be monitored online in growing cultures, the primary drawback of fluorescence reporters is the challenge of eliminating sources of intrinsic fluorescence that would otherwise increase the background and thereby dramatically reduce the threshold sensitivity and hence overall dynamic range of the reporter output. Especially complex media components—which are essential for the growth of many antibiotic producers—are a notorious source of background fluorescence that cannot be easily replaced.

Table 2 Promoters used for the development of cell wall antibiotic-specific (AMP-inducible) whole-cell biosensors in *B. subtilis*

Promoter	Regulator	Dynamic range (fold induction)[a]	Reporter gene	Antibiotics	Ref.[b]
P_{ypuA}	σ^M	2–5	β-Galactosidase, firefly luciferase	Bla, D-cyc, Nis, Mer, Ram, Van, Tun, PmB, MP196, Tri	(Urban et al. 2007; Wenzel et al. 2014)
P_{ywaC}	σ^M, σ^W (σ^V)	5–15	Bacterial luciferase	Fos, Bac, Ram, Van	(Czarny et al. 2014; D'Elia et al. 2009)
P_{ytrA}	YtrA	3–10	β-Galactosidase	Bac, Van, Ram, Ris	(Hutter et al. 2004a; Salzberg et al. 2011)
P_{ywoB}	YtrA	2–4	β-Galactosidase	(Bac), (Van), Ris, (Ram)	(Hutter et al. 2004a; Salzberg et al. 2011)
P_{liaI}	LiaFSR	400–500	β-Galactosidase, bacterial luciferase	Bac, End, Ram, Van, Dur, Gal, Nis, Sub, Tri	(Mascher et al. 2004; Staroń et al. 2011)
P_{bceA}	BceRS	100–300	β-Galactosidase, bacterial luciferase	Bac, Ple, Act, Mer	(Schneider et al. 2010; Staroń et al. 2011)
P_{psdA}	PsdRS	100–1000	β-Galactosidase, bacterial luciferase	End, Act, Gal, Nis, Sub	(Staroń et al. 2011)
P_{spaS}	SpaRK	30–100	β-Galactosidase	Sub	(Burkard et al. 2007)

Act actagardine, *Bac* bacitracin, *Bla* β-lactams, *End* enduracidin, *D-cyc* D-cycloserine, *Dur* duracidin, *Gal* gallidermin, *Mer* mersacidin, *MP196* small cationic peptide, *Nis* nisin, *Ple* plectasin, *PmB* polymyxin B, *Ram* ramoplanin, *Ris* ristocetin, *Sub* subtilin, *Tri* triton X-100, *Tun* tunicamycin, *Van* vancomycin

[a] The values for the maximum induction ranges are all based on data derived from *lacZ*-based biosensors

[b] only major references for each biosensor are listed

In many respects, bioluminescence combines the advantages of both reporter systems described above with hardly any true drawback, other than the need for specialized equipment to monitor the readout (Waidmann et al. 2011; Wilson and Hastings 1998). It is virtually without background, thereby ensuring a superb signal-to-noise ratio and hence unsurpassed dynamic range. It allows both online monitoring in any type of liquid cultures (irrespective of media components) and plate-based screens with cell extracts or even producing cultures (Fig. 2d, e) (Kobras et al. 2016; Radeck et al. 2013). Moreover, the output of bacterial bioluminescence reporters derived from *P. luminescence* exhibit a very short half-life of less than 5 min, thereby allowing an online monitoring of both transcription activation and promoter switch-off almost in real time (Radeck et al. 2013). Combined, these properties make bacterial luciferase reporter systems the preferred choice for the development of whole-cell biosensors.

***B. subtilis* biosensors and their applications** Over the years, eight different promoters have been developed into cell envelope stress-specific whole-cell biosensors (Table 2), of which only four fulfill all criteria discussed above. P_{ypuA} is controlled by the extracytoplasmic function (ECF) σ factor σ^M (Eiamphungporn and Helmann 2008) and responds to a cell wall-specific inducer spectrum, for example, antibiotics interfering with cytoplasmic (D-cycloserine), membrane-anchored (tunicamycin, nisin, bacitracin), and extracellular steps (β-lactams) of cell wall biosynthesis, and also membrane-perturbating agents, such as Triton X-100 or polymyxin B (Urban et al. 2007). Since P_{ypuA} is not induced by any antibiotic interfering with other major antibiotic targets, it would provide an almost ideal initial screening tool to identify cell wall antibiotics, if it would not be for its very poor performance. While it is specifically induced by cell wall antibiotics only, the maximum dynamic range is only twofold above background, thereby precluding the development of a robust biosensor or its application in semi-quantitative, for example, plate-based screens. P_{ywaC} represents a second ECF-controlled biosensor (Table 2). While its inducer range is more restricted compared to P_{ypuA} (mostly antibiotics targeting membrane-anchored steps of cell wall and also teichoic acid biosynthesis, such as bacitracin, vancomycin, or ramoplanin), the superior performance of this *lux*-based reporter system result in a more robust biosensor, with an overall dynamic

range of about tenfold over background (Czarny et al. 2014; D'Elia et al. 2009).

Two additional biosensors are based on the response of the transcriptional regulator YtrA to cell envelope damage. The corresponding target promoters, P_{ytrA} and P_{ywoB}, are induced in the presence of the chemically related glycopeptide antibiotics vancomycin and ristocetin (Salzberg et al. 2011). While the initial results from transcriptome studies demonstrated an about 50-fold increase in mRNA levels after the addition of these compounds (Hutter et al. 2004a), the derived promoter-*lacZ* fusions only offered a twofold to sixfold dynamic range (Hutter et al. 2004b; Salzberg et al. 2011), thereby limiting their application potential.

In contrast to these three CESR-inducible promoters, which are controlled either by an ECF σ factor or a one-component system, a number of CESR-inducible promoters controlled by 2CSs could be identified as suitable candidates and were subsequently characterized further. The first CESR-specific promoter to be developed as a whole-cell biosensor was the LiaRS-controlled promoter P_{liaI} (Mascher et al. 2004), which was already introduced for the LIKE expression system. It primarily and strongly responds to peptide antibiotics interfering with the membrane-anchored steps of cell wall biosynthesis, such as the bacitracin, enduracidin, ramoplanin, vancomycin, and a whole range of lantibiotics including duramycin, gallidermin, nisin, and subtilin (Mascher et al. 2004; Staroń et al. 2011). The name "Lia" was chosen to reflect the initially identified inducer spectrum, which was comprised of *L*ipid II-*i*nteracting *a*ntibiotics (Table 2) (Mascher et al. 2004). Subsequently, it was discovered that P_{liaI} is also strongly induced by membrane-perturbating agents such as daptomycin or rhamnolipids (Wecke et al. 2011; Wecke et al. 2009), indicative for a more general monitoring of disturbed membrane-trafficking processes, including the lipid II cycle. This hypothesis is also supported by the (much weaker!) induction of P_{liaI} by rather unspecific stressors, including protein oversecretion, alkaline shock, and detergents, which also affect membrane integrity (Hutter et al. 2004a; Marciniak et al. 2012; Wiegert et al. 2001). Recent evidence indicates that the LiaRS system responds with high sensitivity (that is, long before growth inhibition is detectable) to a specific but so far unknown aspect of the damage caused by the above stress conditions (Radeck et al. 2016; Wolf et al. 2012). This might explain the observed inducer spectrum, which is very broad regarding the chemistry and MOA of the inducing compounds, yet well defined with respect to the processes damaged by them: interference with membrane trafficking processes.

The initial biosensor was based on the *lacZ* reporter (Mascher et al. 2004) and was patented in the USA as a screening tool for antibacterial compounds (Helmann and Mascher 2007). Subsequently, this *lacZ*-based biosensor could also be adapted to a semi-automated microtiter plate-based assay (Burkard and Stein 2008) and additionally to a stable endospore-based low-tech filter variant (termed "Sposensor") for semi-quantitative on-site analyses in natural settings in the absence of any available instrumentation (Fantino et al. 2009). More recently, a *lux*-based whole-cell biosensor was also developed that demonstrated a superior performance and allows for quantitative online measurements both in liquid cultures and on agar plates (Kobras et al. 2016; Radeck et al. 2013). In contrast to the three promoters above, P_{liaI}-based biosensors fulfill all core criteria described above, irrespective of the reporter gene used: they are sensitive, specific, and robust, with a very low basal activity and a high dynamic range.

A similar overall behavior has been described for related AMP-specific biosensors that are also covered by the above-mentioned patent (Helmann and Mascher 2007) and were further developed as whole-cell biosensors in subsequent years. P_{bceA} and P_{psdA} are two closely related promoters that are controlled by two paralogous 2CS, BceRS and PsdRS, respectively (Staroń et al. 2011). Both 2CS share an unusual mode of signal transduction in that their histidine kinases, BceS and PsdS, recruit ABC transporters (BceAB and PsdAB) that function as both AMP-specific sensors (= input) and resistance pumps (= output) (Dintner et al. 2011; Rietkötter et al. 2008; Staroń et al. 2011). This novel flux-sensing mechanism—in which a transporter basically uses a 2CS to adjust its own cellular concentration in response to the concentration of a harmful AMP substrate—enables a highly dynamic yet fine-tuned produce-to-demand regulation (Fritz et al. 2015). At the transcriptional level, this mechanism expresses itself by the strictly dose-dependent activation of the respective target promoters, making P_{bceA} and P_{psdA} ideally suited for the development of whole-cell biosensors.

Both P_{bceA} and P_{psdA} respond to a specific range of AMPs interfering with the lipid II cycle of cell wall biosynthesis. P_{psdA} predominantly responds to positively charged lantibiotics, such as the pore-forming nisin or subtilin and the non-pore former gallidermin. In addition, this promoter is also induced by the negatively charged lantibiotic actagardine and also the cyclic lipopeptide enduracidin (Staroń et al. 2011). The latter is particularly remarkable, since a closely related compound, the cyclic lipoglycopeptide ramoplanin, is not recognized at all. P_{bceA}, on the other hand, has initially been described as a specific bacitracin sensor, and the regulated transporter, BceAB, also provides high-level resistance against this compound (Mascher et al. 2003; Ohki et al. 2003). But subsequent studies identified a number of additional, rather unrelated AMPs that also function as strong inducers of the Bce response, including the related lantibiotics actagardine and mersacidine as well as the defensin plectasin (Schneider et al. 2010; Staroń et al. 2011).

The exact determinant of recognition of and discrimination between different AMPs by these two promoters is still

puzzling. On the one hand, the two systems can discriminate between highly similar compound pairs such as mersacidine-actagardine (Bce) or enduracidin-ramoplanine (Psd). On the other hand, the Bce system is able to respond to compounds as different as bacitracin, actagardine, and plectasin. Moreover, while the spectrum of the two biosensors is mostly well separated, making them a highly synergistic biosensor pair, they nevertheless both respond to a single compound, the lantibiotic actagardine. But together, these two related biosensors cover a broad range of diverse AMPs that all bind some intermediate of the lipid II cycle. With the exception of plectasin and actagardine, all compounds recognized by the concerted action of these two biosensors are also detected by the P_{liaI}-derived biosensor (Staroń et al. 2011). The strength of P_{bceA} and P_{psdA} over the latter biosensor is their superior dose-response behavior. For all but the pore-forming lantibiotics nisin and subtilin, both promoters show a gradual increase in reporter output over two to three orders of magnitude in inducer concentrations between threshold concentrations (= first significant response) and saturating concentration (= maximum response), which normally occurs still below the inhibitory concentration. In contrast, P_{liaI} shows a much narrower response window between threshold and saturation of hardly more than one order of magnitude in AMP concentrations (Mascher et al. 2004).

Finally, the *spaS* promoter, which was already used for the SURE expression system described above, has also been developed into a whole-cell biosensor, based on a P_{spaS}-*lacZ* reporter gene fusion (Burkard et al. 2007). While the behavior of this promoter with respect to the overall dynamics and robustness are well in agreement with the core criteria defined above, its very narrow inducer spectrum restricts its use for the search of a single compound, subtilin (Table 2). This precise response is not surprising, since SpaRK 2CS and its target promoter P_{spaS} are involved in the autoregulatory circuitry orchestrating the self-induced expression within the biosynthetic locus for this lantibiotic (Kleerebezem et al. 2004; Stein et al. 2003). Hence, it can only be used for identifying novel subtilin producers, e.g., from strain collections or new isolates. But it will not be useful for the identification of new AMPs.

Taken together, the best AMP-specific biosensors are derived from 2CS-controlled CESR-inducible promoters, which combine a low basal activity with a highly dynamic, dose-dependent response at sublethal AMP concentrations. Their inducer range, while not fully understood, is specific, yet broad enough to allow for the identification of novel compounds within a certain range of chemical structures. In contrast, AMP-inducible promoters controlled by other signaling devices, such as 1CSs or ECFs, exhibit a significantly lower dynamic range, thereby reducing their value as biosensors. 2CS-controlled promoters from AMP-biosynthetic loci, while being highly dynamic, are usually restricted to a single inducing compound, for example, the product of this biosynthesis, and are hence not useful for the screening and identification of novel antimicrobial agents.

Conclusions and outlook

This review aimed at summarizing the current state of the art with respect to applying AMP-inducible promoters from low G + C Gram-positive bacteria for two purposes: (i) as expression systems for the production of (heterologous) proteins and (ii) as whole-cell biosensors for antimicrobial research. Despite the fact that both fields are already well developed, there is still an increasing demand in tools to facilitate and streamline the underlying efforts towards producing valuable proteins or identifying and characterizing novel antimicrobial compounds, respectively. While the three expression systems described herein are well established and powerful, each has its specific limitations and none is perfect. Likewise, the available whole-cell biosensors so far only show a good coverage and performance for identifying and characterizing antibiotics interfering with the membrane-anchored steps of cell wall biosynthesis (P_{liaI}, P_{bceA}, P_{psdA}). Additional biosensors that are specific for either the intracellular or the extracellular steps are urgently needed, since the options currently available (P_{ypuA}, P_{ytrA}, P_{ywoB}) show an overall poor performance (Table 2).

Despite these minor limitations, we hope that we could provide convincing evidence of the applied potential of *Firmicutes* bacteria in general and their AMP-inducible promoters in particular. Especially the Gram-positive model organism *B. subtilis*, with its powerful genetics, GRAS status, and biotechnological relevance, plays a central role. In the age of Synthetic Biology, this organism is currently being developed as a major chassis for applied purposes (Liu et al. 2013). The underlying efforts include (i) large-scale genome reduction projects, which result in more robust and simpler organisms for applied purposes (Juhas et al. 2014); (ii) the development of standardized genetic tools such as the *Bacillus* BioBrick Box (Radeck et al. 2013), which streamlines and simplifies the work with this already well-accessible organism; and (iii) because of the above, *B. subtilis* is a preferred heterologous host for large-scale production of (especially secreted) proteins or for the implementation of novel regulatory circuitries and metabolic pathways (Liu et al. 2013). The tools and applications described herein are but some of the pieces of this fascinating puzzle.

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