

# Common Regulators of Virulence in Streptococci

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**Abstract** Streptococcal species are a diverse group of bacteria which can be found in animals and humans. Their interactions with host organisms can vary from commensal to pathogenic. Many of the pathogenic species are causative agents of severe, invasive infections in their hosts, accounting for a high burden of morbidity and mortality, associated with high economic costs in industry and health care. Among them, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Streptococcus suis* are discussed here. An environmentally stimulated and tightly controlled expression of their virulence factors is of utmost importance for their pathogenic potential. Thus, the most universal and widespread regulators from the classes of stand-alone transcriptional regulators, two-component signal transduction systems (TCS), eukaryotic-like serine/threonine kinases, and small noncoding RNAs are the topic of this chapter. The regulatory levels are reviewed with respect to function, activity, and their role in pathogenesis. Understanding of and interfering with transcriptional regulation mechanisms and networks is a promising basis for the development of novel anti-infective therapies.

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## 1 Introduction

Streptococcal species can colonize and live in animals and humans and are present in almost all body compartments. They mostly live in benign and commensal relationship with their hosts. However, several pathogenic species are known, which act as zoonosis pathogens or represent exclusive human pathogens. Infections are multifaceted and occur in all age groups ranging from newborns to adults and the elderly. The pathogenic streptococcal species reviewed in this chapter can cause severe and life-threatening invasive diseases in humans. They are responsible for substantial mortality and morbidity. In the era of spreading antibiotic resistance, these species are studied intensively to understand their pathogenesis mechanisms, including the regulatory machinery in control of virulence factor expression. This knowledge will help to discover novel targets for innovative therapies.

*Streptococcus pyogenes* is an exclusive human pathogen, which is transmitted from person to person and causes diseases ranging from mild superficial and self-healing to severe invasive diseases. Sepsis, toxic shock like syndrome and necrotizing fasciitis are the most frightening disease characteristics (Cunningham 2000; Carapetis et al. 2005). Antibiotic treatment is still the therapy of choice and is mandatory to prevent post-streptococcal autoimmune sequelae.

*Streptococcus pneumoniae*, commonly referred to as the pneumococcus, is known to cause a high burden of human disease and death. *S. pneumoniae* can switch from commensal and asymptomatic stage to infectious stage, thereby infecting the lung, blood, and brain. Otitis media and pneumonia are the most frequent infections caused by these bacteria (Mitchell and Mitchell 2010; Gamez and Hammerschmidt 2012).

Like the other streptococci mentioned above, *Streptococcus agalactiae* is also a common asymptomatic colonizer of healthy adults. As an opportunistic pathogen, it is able to defend itself against all host immune system functions, and to cause severe invasive diseases and tissue damage in unborn and newborn children as well as adults (Rajagopal 2009).

For a long time, *Streptococcus suis* was considered a classical swine pathogen responsible for high economic losses in the swine industry worldwide. *S. suis* associated diseases include meningitis, septicemia and endocarditis (Fittipaldi et al. 2012). Late in the 1960s first cases of human infections were reported and since then *S. suis* gained increasing importance as zoonosis pathogen also affecting humans.

Common to all these severe and invasive disease causing pathogens is environmentally driven, coordinated, and fine-tuned regulation of expression of their armament of virulence factor genes. The activity of the most prevalent stand-alone transcriptional regulators, two-component regulatory systems (TCS), eukaryotic-like serine/threonine kinases and, a rather just recently appreciated level, small noncoding RNAs (sRNAs) is the topic of this review.

Numerous so-called stand-alone transcriptional regulators influence the coordinated expression of virulence factors in all streptococci discussed here. Some of them have been extensively studied, especially in *S. pyogenes* (Kreikemeyer et al. 2003; McIver 2009; Fiedler et al. 2010a). In this review, we will focus on those stand-alone regulators that can be found and are involved in virulence regulation in more than one of the pathogenic streptococcal species, such as the members of the RofA-like protein (RALP) family of regulators, Mga and orthologous proteins, as well as several regulators primarily responsible for controlling metabolism.

A common way for bacteria to adapt to environmental conditions is mediated by two-component signal TCS (Hoch 2000; Stock et al. 2000), which are encoded in varying numbers on the chromosomes of these species. In *S. pyogenes*, the genome sequencing era allowed identification of 11 up to 13 such systems (Kreikemeyer et al. 2003; Fiedler et al. 2010a), in *S. pneumoniae* 13 systems are present (Paterson et al. 2006), in *S. agalactiae* up to 20 TCS have been reported, depending on the strain investigated (Glaser et al. 2002; Tettelin et al. 2002), and in *S. suis* 15 TCS were predicted from comparative genomics analyses (Chen et al. 2007). Common to almost all TCS is the basic composition of two proteins. A membrane-associated sensor histidine kinase (HK) is responsible for receiving external signals and autophosphorylates a conserved histidine residue. The phosphate group is subsequently transferred by the HK to a conserved aspartate residue in the cognate response regulator (RR). The RR undergoes a conformational change enabling DNA binding and regulation of gene expression (Stock et al. 2000; Hoch 2000). There are variations of this common activation theme. In several studies, response regulators were found which are independent of their cognate histidine kinases for phosphorylation. Eukaryotic-like serine/threonine kinase/phosphatase systems have been discovered and studied, which either independently regulate pathogen physiology and virulence, or which are networking with TCS systems (Burnside and Rajagopal 2011). For all stand-alone

**Table 1** Distribution and function of stand-alone regulators, TCS, and STK/STP systems in different pathogenic streptococci

Regulator system	<i>S. pyogenes</i>	<i>S. agalactiae</i>	<i>S. pneumoniae</i>	<i>S. suis</i>
Mga	Mga locus: <i>emm</i> and <i>emm</i> -like genes  Allelic variants associated with preferred site of infection ( <i>mga-1</i> : throat; <i>mga-2</i> : skin, generalists) Central in regulatory network	Presence/function unknown	Designated MgrA, Mga <sup>spn</sup> Pilus ( <i>rtrA</i> islet) Crucial for development of pneumoniae in mouse model	Presence/function unknown
LuxS	Hemolysis (SLS), SpeB secretion, capsule biosynthesis  <i>fasX</i> , <i>emm</i>	Presence reported, function unknown	Biofilm formation, pneumolysin/autolysin production Competence	Biofilm formation, capsule biosynthesis,  Hemolysis, host cell adherence
CcpA	Binds to P <sub><i>mga</i></sub> , transcriptional activation of <i>mga</i> <i>sag</i> operon, <i>speB</i>		Also designated RegM Capsule biosynthesis, surface enolase, $\beta$ -galactosidase, <i>nanA/B</i> , <i>sodA</i> , <i>pcpA</i>	Capsule biosynthesis, <i>eno</i> , <i>ofs</i> , <i>cpsA2</i>
CodY	Activates expression of <i>mga</i> , <i>fasX</i> , <i>rofA</i> , and <i>rivR</i>	Presence/function unknown	Deletion in wild types fatal	Presence/function unknown
RALP	Represses expression of <i>covRS</i> and <i>sptRS</i> Important regulators of pilus protein expression, encoded on pathogenicity like-islands Act in species-specific and within species even strain-specific regulatory circuits Frameshift mutations occur bistable phenotypes bistable phenotypes		<i>pcpA</i> (adherence)	Presence/function unknown

(continued)

**Table 1** (continued)

Regulator system	<i>S. pyogenes</i>	<i>S. agalactiae</i>	<i>S. pneumoniae</i>	<i>S. suis</i>
MsmR	Adversely controls pilus gene transcription together with RALP	Positive control of pilus gene expression on pilus island 1	MgrA (Mga family) regulator adjacent to pilus island found Presence of MsmR-like regulator unknown	Presence/function unknown
CovRS	Networking with other TCS and sRNAs	Crucial TCS for virulence (rat and mouse infection models)	Presence/function unknown	Orphan response regulator CovR present
	Heat, acid, salt stress	General stress response		Acts as global negative regulator and repressor of virulence
	Most important virulence-associated TCS	Bidirectional activity		
	Spontaneous mutants allowing switch from pharyngeal to invasive lifestyle			
	Unidirectional activity			
CiaHR	In control of virulence factors like hemolysis, MSCRAMMs, DNAses	Promotion of intracellular survival	Also designated TCS05	Important for host cell adherence
		Resistance to host innate immune functions	Involved in natural competence	Crucial for survival in macrophages
			Stress response	Important for mortality in mouse and pig infection models
			Antibiotic resistance	
			Spontaneous mutants in CiaH found in clinical isolates	
		Important for survival in blood and brain		

(continued)

**Table 1** (continued)

Regulator system	<i>S. pyogenes</i>	<i>S. agalactiae</i>	<i>S. pneumoniae</i>	<i>S. suis</i>
Ihk/Irr	Important for growth and survival in blood, saliva, phagocytes, and macrophages	Presence/function unknown	Presence/function unknown	Important for virulence in mice Host cell adherence Macrophage killing Oxidative stress survival In control of cellular metabolism
VicRK	Fine tuning together with CovRS			
STK/STP		Crucial for murein biosynthesis, cell division, lipid integrity, exopolysaccharide synthesis, biofilm phenotype, virulence factor expression Eukaryotic-like serine/threonine kinase/phosphatase systems with regulatory connections to TCS Play important roles in pathogen physiology and virulence		Presence/function unknown

regulators and TCS discussed in this review, designations and known or putative functions are summarized in [Table 1](#).

In addition to the known protein based transcriptional regulation, the role of sRNAs in the control of bacterial gene expression became increasingly evident. The high number of regulatory RNAs identified in different bacterial species was unexpected (Brantl 2009; Narberhaus and Vogel 2009; Waters and Storz 2009) and the variability in length, structure, and mode of action of the different RNAs is very striking (Gottesman and Storz 2011). Bacterial regulatory RNAs influence the expression of genes involved in processes as diverse as stress response, sugar metabolism, and surface composition (Vanderpool and Gottesman 2005; Gottesman et al. 2006; Heidrich et al. 2006; Gorke and Vogel 2008; Gogol et al. 2011; Sharma et al. 2011). Thus, it is not surprising that pathogens employ regulatory RNAs for the tightly controlled expression of virulence factor genes and in some cases for the fine tuning of conventional, protein-mediated gene regulation (Livny et al. 2006; Papenfort and Vogel 2010). Although the regulatory character is shared by this class of RNAs, the basic properties between subtypes vary immensely. There is an intriguing diversity of regulatory mechanisms. Some *cis*-regulatory RNAs are located on untranslated regions (UTRs) of a coding transcript and act as RNA-thermometers or riboswitches (Klinkert and Narberhaus 2009; Bastet et al. 2011). Another group consists of small sRNAs, which are transcribed independently and function via *cis*- and *trans*-antisense base pairing. Of those, *cis*-acting sRNAs are encoded on the opposite strand of their respective target gene. Consequently, they show a high sequence complementarity to their target RNA, which guarantees very specific binding. Recently, differential sequencing analysis revealed a high antisense transcriptional activity in several model organisms, including *Helicobacter pylori* and *S. pyogenes*, which points to the importance of *cis*-regulatory elements in bacteria in general with implications for specific involvement in bacterial virulence regulation (Sharma et al. 2010; Deltcheva et al. 2011). Compared to their *cis*-acting counterparts, *trans*-acting sRNAs exhibit only a short and imperfect complementarity to their target RNAs, allowing them to control several different target genes. These sRNAs are usually regulated in response to environmental stimuli. Some *trans*-acting sRNA molecules act as repressors of translation and destabilize mRNA transcripts but others activate and stabilize the target mRNAs (Thomason and Storz 2010; Storz et al. 2011). Although the conventional point of view regarded *trans*-acting sRNAs as inhibitory antisense regulators, to date a significant number of sRNAs activating bacterial gene expression is known (Frohlich and Vogel 2009). Furthermore, regulatory mechanisms include the stabilization as well as the destabilization of target transcripts (Podkaminski and Vogel 2010). Later in this chapter, we will focus on the structure and function of regulatory RNAs in streptococci. We will give an overview of already characterized sRNAs known to mediate virulence factor regulation in streptococcal diseases, but we will also summarize recent whole-genome screens for sRNAs in pathogenic streptococci.

## 2 Streptococcal Stand-Alone Transcriptional Regulators

### 2.1 Multiple Gene Regulator of Group A Streptococci—Mga and Orthologous Regulators

One of the best characterized and probably most important stand-alone virulence regulators in *S. pyogenes* is Mga. Initially, Mga was identified as positive regulator of the expression of the M-protein encoding *emm* gene (Caparon and Scott 1987; Okada et al. 1993; Podbielski et al. 1995). Nowadays, it is known that Mga is a global transcriptional activator in the exponential growth phase (Kreikemeyer et al. 2003). The Mga regulon of *S. pyogenes* comprises multiple genes encoding for virulence factors involved in host cell adhesion (e.g., M- and M-like proteins, fibronectin- and collagen-binding proteins) and immune evasion (e.g., C5a peptidase and other complement inhibitors) (Hondorp and McIver 2007; Fiedler et al. 2010a). Genomic analysis revealed the serotype-dependent presence of two allelic variants of the *mga* gene in *S. pyogenes*, *mga-1* and *mga-2* (Haanes et al. 1992; Hollingshead et al. 1993). The genomic presence of either version is correlated to the *S. pyogenes* strain's M-protein class, serum opacity factor production, and especially to the structure of the adjacent *emm* gene region, i.e., the absence or presence of *emm*-related genes upstream and downstream of the respective *emm* gene (Bessen and Hollingshead 1994; Hollingshead and Bessen 1995; Bessen and Lizano 2010). It has been proposed that the allelic *mga* variants are indicative of tissue tropism of *S. pyogenes* strains, with *mga-1* primarily associated with throat infecting strains and *mga-2* associated with skin infecting or “generalist” strains (Bessen et al. 2005; Bessen and Lizano 2010).

Genes directly activated by Mga are referred to as the Mga core regulon and comprise the virulence genes *emm*, *scpA* (C5a peptidase), *sclA* (collagen-like protein), *sic* (secreted inhibitor of complement), *fba* (fibronectin binding protein), and *sof* (serum opacity factor) as well as the *mga* gene itself (Ribardo and McIver 2006). Furthermore, there are several virulence genes that can be regulated by Mga indirectly, such as the capsule biosynthesis (*has-*) operon or the cysteine protease gene *speB* (Ribardo and McIver 2006; Hondorp and McIver 2007). It was also shown that Mga acts as a transcriptional repressor of genes related to sugar metabolism such as the mannose/fructose phosphotransferase system component IIA (Ribardo and McIver 2006; Hondorp and McIver 2007).

For activation of the core regulon genes, a direct binding of Mga in the promoter region of the respective genes is necessary (McIver et al. 1995, 1999; Podbielski et al. 1995; Almengor and McIver 2004). The mechanism of transcriptional activation by Mga binding seems to vary for different genes, depending on the position of the binding sites in relation to the transcriptional start site. Usually, the Mga binding site overlaps at least in part with the -35 region and is thus located in close proximity to the transcriptional start site of the respective genes. Mga bound to these proximal sites probably stabilizes the binding of the RNA polymerase by direct interaction. For some genes (*sof-sfbX*, *sclA*), the Mga



binding site is located further upstream, indicating that binding of Mga to these distal sites rather activates transcription via DNA binding than via direct interaction of Mga with the RNA polymerase. Finally, upstream of the *mga* gene there is both, a distal and a proximal Mga binding site combining both activation mechanisms (McIver et al. 1995, 1999; Almengor and McIver 2004; Almengor et al. 2006). Mga activity displays growth phase association with a maximum in the exponential phase (McIver and Scott 1997; Ribardo and McIver 2003). The transcription of the *mga* gene is not entirely depending on autoregulation but is influenced and fine-tuned by numerous other transcriptional regulators of *S. pyogenes*, such as the regulators of the RALP-family, the sugar metabolism regulator CcpA, the CovR repressed response regulator TrxR and the sugar metabolism regulator MsmR (Podbielski et al. 1999; Beckert et al. 2001; Almengor et al. 2007; Kreikemeyer et al. 2007; Kratovac et al. 2007; Leday et al. 2008; Fiedler et al. 2010a).

Since Mga is involved in the activation of many of the major *S. pyogenes* virulence factors, the deletion of the *mga* gene leads to a dramatic loss of virulence of *S. pyogenes* in vitro and in animal models. Mga-defective mutants are more sensitive to phagocytosis, exhibit a decreased adherence to human skin tissue sections, an attenuated virulence in intraperitoneal and skin infection mouse models, and show a reduced ability to bind human serum and matrix proteins (Perez-Casal et al. 1993; Kihlberg et al. 1995; Luo et al. 2008; Fiedler et al. 2010b).

Mga orthologous regulators were found in *S. pneumoniae* (Hemsley et al. 2003; Solano-Collado et al. 2012) and other streptococci, such as *S. dysgalactiae*, *S. equi*, *S. gordonii*, and *S. mitis* (Vahling and McIver 2006). No Mga-like regulators have been described in *S. agalactiae* and *S. suis*. In *S. pneumoniae* the Mga-like regulator is designated MgrA (TIGR4 strain) or Mga<sub>Spn</sub> (R6 strain) (Hemsley et al. 2003; Solano-Collado et al. 2012). It has been shown that MgrA is involved in virulence, i.e., by repression of the genes of the pilus-encoding *rlrA* pathogenicity islet (Hava and Camilli 2002; Hemsley et al. 2003). Since MgrA/Mga<sub>Spn</sub> can be found in all currently sequenced pneumococcal strains while the *rlrA* pathogenicity islet is only present in some of them, it is improbable that the *rlrA* pathogenicity islet genes are the major target of MgrA/Mga<sub>Spn</sub> regulation (Hoskins et al. 2001; Tettelin et al. 2001; Lanie et al. 2007). Transcriptional activation of the operon downstream of the *mga*<sub>Spn</sub> gene in *S. pneumoniae* R6 by Mga<sub>Spn</sub> binding to two binding sites in the promoter region of this operon has been shown. The function of the respective operon is not known to date (Solano-Collado et al. 2012). Although the regulatory mechanisms and the role of the *S. pneumoniae* Mga-like regulators in pathogenesis are not very well understood, it is apparent that they can function as transcriptional activator and repressor as it has also been shown for Mga of *S. pyogenes*. It is likely that the Mga orthologous regulator(s) play a crucial role in development of full virulence in *S. pneumoniae*, as it has been shown that the presence of MgrA is required for development of pneumonia in a mouse model (Hava and Camilli 2002; Hemsley et al. 2003).

## 2.2 *LuxS* and AI-2 Dependent Quorum Sensing

LuxS is an enzyme of the activated methyl cycle (AMC) and catalyzes the reaction from S-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione which can spontaneously convert into an active furanosyl borate diester designated autoinducer 2 (AI-2) (Schauder et al. 2001; Zhu et al. 2004). AI-2 has originally been described to be involved in cell density dependent gene regulation in *Vibrio harveyi* (Surette et al. 1999). Nowadays, it is known that AI-2 is produced by numerous gram-negative and gram-positive species and serves the intra- and interspecies quorum sensing (Schauder et al. 2001; Federle and Bassler 2003; Federle 2009). In many pathogenic bacteria, LuxS/AI-2 has been associated with regulatory processes in virulence (Vendeville et al. 2005; Antunes et al. 2010). In *S. pyogenes*, *S. pneumoniae*, and *S. suis* the impact of LuxS/AI-2 on virulence regulation has been investigated to some extent. Also in *S. agalactiae* the presence of *luxS*/AI-2 has been described but a detailed analysis is still lacking (Ou et al. 2005; Ouyang et al. 2006).

In *S. pyogenes*, there is evidence that LuxS/AI-2 is influencing virulence factor expression in a growth phase dependent manner (Lyon et al. 2001). *S. pyogenes luxS* deletion mutants exhibit, in addition to growth deficiencies, an increased transcription of the streptolysin S precursor gene *sagA* accompanied by enhanced hemolytic activity (Lyon et al. 2001). Furthermore, a decreased secretion of the immunoglobulin degrading cysteine protease SpeB can be observed. Transcription of *speB* or secretion of SpeB might depend on LuxS and seems to be *S. pyogenes* strain specific (Lyon et al. 2001; Marouni and Sela 2003). It has been shown that capsule biosynthesis as well as the transcription of the sRNA gene *fasX*, the *emm3* gene and the immunoglobulin-binding protein-encoding *sib* gene can be influenced by LuxS in *S. pyogenes* in a serotype or strain dependent manner (Marouni and Sela 2003; Siller et al. 2008). LuxS deficient mutants are more efficiently internalized into epithelial cells and show better growth in acidic environments or in the presence of human serum (Siller et al. 2008).

LuxS has been proposed to be the key regulator for early biofilm formation in *S. pneumoniae* (Joyce et al. 2004; Romao et al. 2006; Vidal et al. 2011; Trappetti et al. 2011). Biofilm formation in LuxS deficient mutants is drastically hampered and autolysin and pneumolysin production is decreased. While LuxS activates the transcription of the pneumolysin (*ply*) and autolysin (*lytA*) genes (Joyce et al. 2004; Vidal et al. 2011), the *com*-operon genes involved in the regulation of the genetic competence of *S. pneumoniae* are repressed by LuxS (Romao et al. 2006; Trappetti et al. 2011). Consequently, LuxS deficient mutants proved to be less virulent in nasopharyngeal mouse models and were outcompeted by the wild-type strain when co-administered in an intraperitoneal mouse model (Stroehrer et al. 2003; Joyce et al. 2004).

In *S. suis*, the deletion of *luxS* was shown to lead to decreased biofilm formation, capsule biosynthesis, adherence to epithelial cells, hemolytic activity and hydrogen peroxide tolerance (Cao et al. 2011; Wang et al. 2011). In a *luxS* deficient mutant of

a *S. suis* serotype 2 strain, the transcription of several virulence-associated genes (e.g. genes for fibronectin/fibrinogen-binding protein FbpS, muramidase released protein Mrp, or extracellular factor EF) was decreased compared to the parental strain (Wang et al. 2011). In zebra fish or piglet models, *luxS* deficient *S. suis* mutants were described to be severely attenuated in virulence (Wang et al. 2011; Cao et al. 2011).

In *S. pyogenes*, *S. suis*, and *S. pneumoniae* *luxS* is transcribed as a monocistronic mRNA. In batch cultures, the highest *luxS* expression level can be observed during early exponential growth while maximum AI-2 secretion is reached in the transition phase. Hence, the *luxS* gene expression is apparently not depending on (or correlating with) AI-2 levels (Siller et al. 2008; Han and Lu 2009; Vidal et al. 2011). Although it has been shown that *luxS* transcription is induced by iron in *S. pneumoniae* or repressed by the CovR (CsrR) response regulator in *S. pyogenes*, the regulatory mechanisms controlling *luxS* expression and AI-2 production in streptococci are not well understood (Marouni and Sela 2003; Trappetti et al. 2011). Anyway, phenotypes caused by the deletion of *luxS* might not necessarily be associated with the lack of AI-2 production but could be caused by the accumulation of toxic intermediates of the AMC such as S-adenosylhomocystein, as recently described for *Streptococcus sanguinis* (Redanz et al. 2012). Here, transcriptome analysis revealed that in a *luxS* deficient mutant 216 genes were differentially expressed in comparison to the wild-type strain. When this strain was complemented with an alternative route of the AMC, preventing the accumulation of S-adenosylhomocystein, only nine genes showed altered transcription in comparison to the wild type (Redanz et al. 2012). This experimental approach dissects the AI-2 effects and the AMC effects and clearly demonstrates that biofilm formation of *S. sanguinis* is not depending on AI-2 quorum sensing but on an intact AMC. This demonstrates the importance of distinguishing between the quorum sensing and metabolic or toxic effects of *luxS* deletions. Especially in terms of biofilm formation, an impact of AI-2/LuxS has been described not only for *S. pyogenes*, *S. pneumoniae*, and *S. suis* but also for several streptococci residing in the human oral cavity such as *S. mutans*, *S. anginosus*, *S. gordonii*, or *S. intermedius* (Blehert et al. 2003; Yoshida et al. 2005; Petersen et al. 2006; Ahmed et al. 2008). Doubtlessly, LuxS is crucial for biofilm formation in streptococci, but it is necessary to reconsider the role of AI-2 dependent quorum sensing in the above-mentioned context.

### ***2.3 Regulators in Control of Metabolism***

Streptococcaceae have a relatively small genome but need to be able to quickly adapt to changing nutritional conditions in the course of infection. Consequently, complex regulatory mechanisms are applied to allow efficient use of the nutrients available at the respective site of infection. Transcriptional changes as a consequence of varying nutritional conditions not only affect metabolism but also

virulence-related genes. One global regulator responsible for regulation of metabolism and virulence in streptococci CcpA is the central sugar metabolism regulator. CcpA is primarily responsible for C-catabolite repression (CCR), which means repression of genes involved in catabolism of sugars less favorable than glucose. This is achieved by binding of CcpA to C-responsive elements (*cre*) in the promoter of the respective genes (Price et al. 2011). In *S. pyogenes*, apart from sugar utilization associated genes also numerous virulence-related genes are repressed by CcpA either directly or indirectly (Kinkel and McIver 2008; Shelburne et al. 2008; Kietzman and Caparon 2011). The impact of CcpA on virulence gene regulation is more pronounced under nutrient limitation (Shelburne et al. 2008). One of the major interfaces between CcpA and virulence in *S. pyogenes* is the control of *mga* expression by direct binding of CcpA to at least one *cre* element in the promoter of the *mga* gene (*Pmga*). Binding of CcpA to this *cre* element increases *mga* transcription and therefore indirectly induces the expression of Mga-regulated genes (Almengor et al. 2007). Furthermore, CcpA apparently represses the streptolysin S (*sag* operon) genes (Shelburne et al. 2008; Kinkel and McIver 2008; Kietzman and Caparon 2010). Whether this is due to direct binding to the promoter upstream of the *sagA* gene or to an indirect regulatory effect is controversially discussed (Shelburne et al. 2008; Kinkel and McIver 2008; Kietzman and Caparon 2010). Furthermore, direct regulation of *speB* expression by CcpA has been demonstrated (Kietzman and Caparon 2010). Interestingly, data on the contribution of CcpA to virulence in vivo are contradictory. Two studies have been published on the effect of a *ccpA* deletion in the *S. pyogenes* M1 strain MGAS5005 on virulence in a CD-1 mouse model. While one of the studies found the mutant to be less virulent (Shelburne et al. 2008) the other group observed a hypervirulent phenotype (Kinkel and McIver 2008).

Much less is known about the contribution of CcpA on virulence gene regulation in other streptococci. In *S. suis* and *S. pneumoniae* as well as in *S. pyogenes* CcpA was shown to be involved in regulation of the capsule biosynthesis (Giammarinaro and Paton 2002; Shelburne et al. 2008; Willenborg et al. 2011). Furthermore, CcpA regulates the transcription of the plasminogen-binding surface enolase (*eno*) gene and the  $\beta$ -galactosidase gene which is crucial for the colonization of the nasopharynx by *S. pneumoniae* (Iyer et al. 2005; Kaufman and Yother 2007; Carvalho et al. 2011). A comprehensive transcriptome study on *S. pneumoniae* wild-type and CcpA deletion strains under several nutritional conditions additionally revealed a CcpA mediated repression of the expression of neuraminidase encoding genes *nanA/B*, the superoxide dismutase gene *sodA* and the choline-binding protein gene *pcpA* (Carvalho et al. 2011). In *S. suis*, a recent publication described CcpA to be involved in capsule biosynthesis, expression of surface enolase and transcriptional activation of virulence factor encoding genes *ofs* and *cpsA2* (Willenborg et al. 2011). Generally, the role of CcpA in virulence regulation is extremely complex, since CcpA activity is strongly influenced by the availability of sugars in the environment of the bacteria. Hence, the impact of a *ccpA* deletion on virulence gene expression might be different in the presence of glucose than in the presence of less-favorable sugars in the growth medium.

Another metabolic regulator commonly involved in virulence gene regulation is the branched chain amino acid (BCAA) activated CodY, which is induced under nutritional deprivation conditions and can exclusively be found among the Firmicutes (Sonenshein 2005; Stenz et al. 2011). With a helix-turn-helix motif the dimeric CodY with one BCAA bound to each monomer can bind to conserved palindromic DNA sequences called CodY boxes. Common targets of CodY regulation in streptococci and other bacteria are i.e., oligopeptide permeases (*opp*) (Malke et al. 2006; Malke and Ferretti 2007; Hendriksen et al. 2008; Stenz et al. 2011) and the *codY* gene itself (Guedon et al. 2005). Additionally, several virulence factors are directly or indirectly regulated by CodY in streptococci. In *S. pyogenes*, CodY has been shown to activate the expression of *mga*, *fasX*, *rofA*, and *rivR* and to repress the expression of *covRS* and *sptRS* (Malke et al. 2006; Malke and Ferretti 2007; Kreth et al. 2011). Since all these genes are encoding for important *S. pyogenes* virulence regulators, the effect of CodY on virulence gene expression, although indirect, is very comprehensive. A deletion of *codY* in *S. pyogenes* consequently leads to decreased expression of the genes encoding for M-protein, streptokinase, streptolysin O, NAD-glycohydrolase, C5a peptidase, SpeH and others while an increased transcription of e.g., capsule biosynthesis genes can be observed (Malke et al. 2006; Malke and Ferretti 2007; Kreth et al. 2011). Although CodY binds in the promoter region of its own gene, neither in the promoter regions of the regulator nor of the virulence factor genes, CodY binding motifs have been detected (Malke and Ferretti 2007; Kreth et al. 2011). Hence, the mechanism by which CodY influences the expression of the regulator genes is not known to date. It has been speculated that at least the regulation of CovRS (see Sect. 3.1) could depend on the intracellular potassium level, which is influenced by the impact of CodY on the expression of potassium uptake system genes (Malke and Ferretti 2007). Especially, the interplay between CodY and CovRS has been investigated in more detail by analysis of *codY*, *covR*, and *codY-covR* deletion mutants (Kreth et al. 2011). It has been postulated that the expression of the above-mentioned virulence genes in *S. pyogenes* are well balanced by the actions of CcpA (integrating information on sugar availability via PTS), CodY (integrating amino acid nutrition information), and CovRS directly acting at the promoter of many virulence factors to adapt to either invasive or noninvasive phenotypes depending on the nutritional conditions. CodY is postulated to counteract the activity of CovRS by stimulating expression of those genes repressed by CovRS (Kreth et al. 2011), leading to a rather invasive phenotype when nutritional conditions are unfavorable.

In *S. pneumoniae*, the investigation of CodY regulatory effects is complicated by the fact that a deletion of *codY* is fatal unless an ectopic copy of the gene is present (Caymaris et al. 2010). Originally, it had been published that CodY is activating the expression of *pcpA*, a choline-binding protein necessary for successful adherence of the bacteria to nasopharyngeal cells (Hendriksen et al. 2008). The *S. pneumoniae* D39 *codY* deletion strain used in the study of Hendriksen et al. (2008) has later been sequenced and it has been postulated that mutations in genes for the ferric uptake iron permease (*fatC*) and an oligopeptide permease (*amiC*)

allowed for tolerance of the *codY* deletion in that strain (Caymaris et al. 2010). The data indicate that in *S. pneumoniae* CodY action to certain extent controls the ability of the bacteria to adhere to host cells.

To the best of our knowledge, the role of CodY in *S. suis* and *S. agalactiae* has not been investigated yet. For an overview on the involvement of CodY in virulence gene regulation in *S. mutans* and other gram-positive bacteria, we refer to a recent review published by Stenz et al. (2011).

Next to CcpA and CodY, there are many other metabolic regulators involved in virulence gene regulation in streptococci, such as the regulatory tagatose-1,6-bisphosphate aldolase LacD.1 in *S. pyogenes* (Loughman and Caparon 2006, 2007), the methionine transport regulator MtaR in *S. agalactiae* (Shelver et al. 2003; Bryan et al. 2008) or the stringent response amino acid metabolism regulator RelA in *S. pyogenes* and *S. pneumoniae* (Malke et al. 2006; Kazmierczak et al. 2009), to name only a few. Apart from the direct or indirect effects of metabolic regulators on virulence gene expression, tight regulation of metabolic processes in response to the changing nutritional conditions encountered by streptococci in the course of infection is crucial for the general fitness of the bacteria and therefore essential for successful infection of the host.

## **2.4 Transcriptional Regulators of Streptococcal Pilus Genome Regions**

Two classes of transcriptional regulators, which are encoded on the chromosomes of several streptococcal species, gained special attention during the last couple of years. The RALP-family of RofA/Nra regulators is present and active in *S. pyogenes*, *S. agalactiae*, *S. pneumoniae* whereas no information is available in *S. suis*. The second family is the MsmR-like stand-alone transcriptional regulator family, which belongs to the large group of AraC/XylS-type regulators. This family is present and active in *S. pyogenes* and *S. agalactiae*. No orthologous genes have been reported in *S. pneumoniae* and *S. suis* yet.

As the most intriguing and unique feature of these regulators, presence of the genes of both regulator families in so-called pathogenicity island-like chromosomal regions can be noted. Such regions encode all necessary proteins enabling streptococci to form long pilus-appendages on their surface. Pili are now recognized to play important and pivotal roles in infections caused by streptococci. The genetic makeup of these discrete genomic regions, their size, their composition, aspects of expression, assembly, and function in virulence have recently been reviewed on a comparative basis (Kreikemeyer et al. 2011). Briefly, in *S. pyogenes* nine different pilus region variants can be found among clinical isolates (FCT1–FCT9), *S. pneumoniae* isolates encode two pilus variant regions (PI-1 and PI-2), in *S. agalactiae*, the pilus proteins are encoded in three related genomic regions (island-1, island-2a, and island-2b), and recently *S. suis* was shown to encode four

distinct genomic regions encoding pilus proteins (*srtBCD*, *srtE*, *srtF*, and *srtG*) (Telford et al. 2006; Takamatsu et al. 2009; Kreikemeyer et al. 2011). However, these important virulence factors are not limited to *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and *S. suis*. There is a growing list of streptococcal species for which pilus genome islands and gene clusters have been identified and surface-localized pilus expression was proven. Recently, *Streptococcus gallolyticus*, a causative agent of infective endocarditis also associated with colon cancer, was shown to encode three separate pilus loci, named *pil1-pil3* (Danne et al. 2011). Also *S. oralis*, *S. mitis*, and *S. sanguinis*, all members of the Mitis group of streptococci, harbor a pilus-encoding genomic region and express variable pili on their surface (Zahner et al. 2011), resembling the genetic organization of the PI-2 islet of *S. pneumoniae*.

From the virulence point of view, all pili play a similar role in the respective species during the infection process. In case of *S. pyogenes*, numerous studies have shown their importance for attachment to pharyngeal cells, human tonsillar epithelium, and keratinocytes and suggested a role in autoaggregation and biofilm formation. In *S. agalactiae*, pili are important for adherence to human brain microvascular endothelial cells and cervical epithelial cells, and have important functions in biofilm formation and resistance against cationic antimicrobial peptides as well as for survival in phagocytes. Mutational analyses revealed a function for *S. pneumoniae* pili in many pathogenic processes, including adhesion to respiratory cells. Interestingly, mutants in the *S. suis srtF* pili cluster were not attenuated in host cell adherence and a murine model of *S. suis* sepsis, suggesting that pili structures are dispensable for critical steps of *S. suis* pathogenesis and infection (Fittipaldi et al. 2010). The role of pili in the Mitis group of streptococci needs to be elucidated, whereas in *S. gallolyticus* pili are critical for collagen binding, biofilm formation, and virulence in experimental endocarditis (Zahner et al. 2011; Danne et al. 2011).

Since pilus proteins of many streptococcal species are apparently immunogenic—they might even represent excellent candidates for vaccine development—it is critical for streptococcal pathogens to tightly control expression of the pilus-encoding genes. Within the pilus gene clusters, this is achieved by aforementioned stand-alone transcriptional regulators of the RALP- and XylS/AraC-families. However, also stand-alone regulators and two-component signal TCS encoded outside of the core pilus genomic regions are implicated in pilus gene regulation, but are discussed in other sections of this review.

In *S. pyogenes* pilus islands, the RALP-family regulator RofA/Nra and the XylS/AraC-family regulator MsmR, both encoded within the FCT regions, adversely control pilus gene transcription (Nakata et al. 2005; Kreikemeyer et al. 2007). Of note, *S. pyogenes* serotype-dependent regulatory circuits have been reported (Luo et al. 2008), further complicating the attribution of common functions of these regulators. External signals to which pilus expression is responsive include temperature, anaerobic atmosphere, and pH (Granok et al. 2000; Nakata et al. 2009; Manetti et al. 2010). In *S. agalactiae*, three RALP-family regulators have been described and investigated: (I) RogB, which is present 284 bp upstream

of island-2a but is absent from island-2b, has been described to positively control transcription of *fbsA*, encoding a *S. agalactiae* fibrinogen-binding protein, negatively regulates capsular polysaccharide gene cluster expression (Gutekunst et al. 2003), and acts as a positive transcriptional regulator of the PI-2a encoded genes (Dramsi et al. 2006), (II) Rga which is located outside of the pilus region and is involved in transcriptional control of the *secA2* locus (Mistou et al. 2009) and was recently identified as the major transcriptional activator of the PI-2a island in *S. agalactiae* (Samen et al. 2011; Dramsi et al. 2012), (III) Gbs1426, which is more distantly related to RogB and Rga. In *S. agalactiae*, the *sag0644* gene, present 380 bp upstream of the island 1, is the respective pilus region AraC/XylS-type regulator with 72 % identity toward the *S. pyogenes* MsmR regulators present in *S. pyogenes* FCT regions 3, 4, 7, and 8 (Nakata et al. 2005). Deletion of this gene in *S. agalactiae* decreased production of pili, suggesting a positive role in pilus expression. Of note, *S. agalactiae* pilus region PI-2b does not encode any RALP- or AraC/XylS-type regulator.

In *S. pneumoniae*, almost nothing is known on regulation of genes encoded in PI-1, and PI-2 regulation is only partially studied. Both pilus genomic islands are flanked by regulator-encoding genes (Scott and Zahner 2006; Telford et al. 2006). One gene encodes the transcriptional regulator RlrA, which belongs to the RALP-family, and which was shown to be required for colonization, lung infection, and systemic infection. RlrA is positively autoregulated, and controls expression of six genes within the PI-2 island, acting on four different promoters (Hava et al. 2003; Kreikemeyer et al. 2011). Downstream of both *S. pneumoniae* pilus islands MgrA is encoded. This transcriptional regulator belongs to the Mga family, best described in *S. pyogenes*. MgrA activity is also important for development of pneumonia and this regulator acts as transcriptional repressor of the same four promoters controlled by RlrA (Hemsley et al. 2003). MgrA is discussed into more detail elsewhere in this review. It should be noted that *S. pneumoniae* is the only species, in which MgrA is encoded so close to the pilus genomic regions. In *S. pyogenes*, the Mga gene is more distantly located to the FCT regions, although there is a clear regulatory circuit connection of Mga and RALP regulators in *S. pyogenes*, which is absent in *S. pneumoniae*. Moreover, also metal-dependent regulators were implicated in pilus gene control. For example, the PsaR (Mn<sup>2+</sup>-dependent) and MerR (metal-dependent) regulator activities converge in the pilus island control (Rosch et al. 2008).

In the *S. suis* pilus genome islands no transcriptional regulators have been characterized into detail. Only a MerR metal-responsive regulator with homology to *S. agalactiae* MerR-type regulators has been annotated in the SrtE-*S. suis* pilus island (Takamatsu et al. 2009).

Two common aspects regulating pilus gene expression need to be mentioned. First, during investigation of clinical isolates many point mutations in pilus genes were identified leading to either altered pilus protein composition or nonpilated variants. In *S. agalactiae*, variability in the expression of pili 1 and 2a among isolates are due to frameshift mutations in the aforementioned regulatory genes. One-base-pair deletions inactivating the *rogB* gene, or in-frame-stop codon



mutations inactivating the *sag0644* gene were reported. In a *S. suis* strain carrying a *srtF* pilus cluster, a nonsense mutation at the 5' end of the gene encoding the minor pilin subunit was detected (Fittipaldi et al. 2010).

Second, in *S. pyogenes* and *S. pneumoniae*, a rather bistable expression mode was found. Nakata and colleagues reported higher expression levels of the *S. pyogenes* pilus backbone protein FctA at 30 °C compared to 37 °C (Nakata et al. 2009). Moreover, at 37 °C only 20 % of all *S. pyogenes* cells expressed pili on their surface. This number increases to 47 % at 30 °C, which is a clear indication of a bistable expression mode. In *S. pneumoniae*, the bistable expression of type I pili is dependent on the native *rlrA* promoter, as a nonexpressing population reverts to the previous bimodal distribution, whereas the expressing population retains the same high level expression (Basset et al. 2011). Other studies also reported the positive feedback loop being under control of the transcriptional regulator RlrA expression (De Angelis et al. 2011; Basset et al. 2012).

Together, there are many common themes among transcriptional regulators, their action on pilus gene transcription and their regulatory circuits among different streptococcal species.

## 3 Streptococcal Two-Component Signal Transduction Systems

### 3.1 The CovRS/CsrRSS system

The CovRS/CsrRS TCS is the best characterized in *S. pyogenes* and *S. agalactiae*. In *S. pyogenes*, it was first discovered by several groups as a major regulator of capsule gene expression, streptolysin S production, and cysteine protease SpeB expression (Levin and Wessels 1998; Heath et al. 1999; Bernish and van de Rijn 1999). This system is functionally connected to *S. pyogenes* general stress response, including growth in heat, acid environments, and under salt stress. CovRS is also important for growth under iron starvation, in the presence of antimicrobial peptides, and plays a role in the serotype-dependent *S. pyogenes* biofilm formation and keratinocyte adherence (Dalton et al. 2006; Froehlich et al. 2009; Sugareva et al. 2010). Moreover, it is apparently the major sensor of *S. pyogenes* for Mg<sup>2+</sup> (Gryllos et al. 2007).

Apart from the above-mentioned *S. pyogenes* virulence factors and functions, many more are directly or indirectly under control of this important TCS. A total of 15 % of all genes encoded on the *S. pyogenes* chromosome are repressed by CovR, including its own gene. The activity of CovR on most promoters is direct and involves the consensus binding sequence ATTARA, mutation of which relieved CovR repression. Promoter occlusion is the primary mechanism of repression by CovR (Gusa and Scott 2005). Phosphorylation of CovR is critical to its functional activity and leads to dimerization (Gao et al. 2005; Gusa et al. 2006). However, a

subcutaneous mouse infection model revealed that CovR is independent of its cognate sensor kinase CovS to get phosphorylated and exert its control functions, suggesting other sources for phosphotransfer (Dalton et al. 2006). Eukaryotic-type serine/threonine kinases, discussed in the next section, are donors for phosphotransfer. Of note, the HK CovS itself was found to inactivate the RR CovR, thereby allowing *S. pyogenes* to grow under the above-mentioned stress conditions. Thus, the CovRS system has a Janus-like behavior. CovS acts as a kinase to activate the CovR response regulator, which subsequently acts as gene repressor. However, CovS, upon environmental stimulation, can act as a phosphatase inactivating CovR to permit gene transcription from respective promoters. The importance, functional activity and the Janus-like behavior of this system in *S. pyogenes* have recently been reviewed (Churchward 2007).

Although CovRS is among the most important TCS in *S. pyogenes*, it is not a master regulator per se, but is rather integrated into existing regulatory networks and is connected and linked to other *S. pyogenes* stand-alone regulators and sRNAs (Kreikemeyer et al. 2003; Fiedler et al. 2010a). Roberts and Scott investigated the link between CovRS and the Mga regulon (described elsewhere in this review) and found CovRS to repress RivR, a RALP-family regulator (Ralp4). Adjacent to *rivR* these authors identified *rivX*, encoding a small regulatory RNA. RivR enhanced transcription by Mga in vivo and in vitro and the *rivRX* locus was involved in pathogenesis in a mouse soft tissue infection model (Roberts and Scott 2007). Another *S. pyogenes* TCS, termed TrxSR, which is homologous to the *S. pneumoniae* HK07/RR07 system, was found as another CovR repressed system (Leday et al. 2008). TrxR activates transcription of Mga-regulated virulence genes, and thus, TrxR defines a new pathway by which CovR affected virulence via control over Mga. A study conducted by Shelburne and colleagues established partially overlapping transcriptomes of Cov and CcpA mutants, proved that both regulator proteins bound to promoters of co-regulated genes, and identified attenuated phenotypes of the mutants in a myositis model (Shelburne et al. 2010). Moreover, CovRS activity was critical for *S. pyogenes* growth in human blood, during pharyngitis in cynomolgus macaques and mouse soft tissue infection. Pioneering work of Sumbly and colleagues linked CovRS function, observed in vitro and from animal studies, to the real situation in clinical isolates (Sumbly et al. 2006). Two fundamentally different transcriptome profiles could be detected during comparison of nine clinical isolates. The pharyngeal transcriptome differed by 10 % from the invasive transcriptome. Complete genome sequencing of an invasive transcriptome isolate uncovered a 7-bp frameshift mutation in the CovS encoding gene. Based on these initial observations, further studies revealed that different in vivo-induced *covR* mutations led to different transcriptomes and that the CovS-mediated regulation of CovR activity is critical for *S. pyogenes* cycling between pharyngeal and invasive phenotypes (Trevino et al. 2009). Although also wild-type bacteria undergo extensive transcriptional reprogramming under in vitro and in vivo infection conditions, this rapid host adaptation is not sufficient to survive in the host. Rather the hypervirulent *cov* mutant strains are able to outcompete the wild type, and thus it can be concluded that mutations and rapid reprogramming

are critical steps allowing *S. pyogenes* to switch infectious phenotypes and to move between niches. This puts sociomicrobiological aspects into the focus of infection research (Aziz et al. 2010).

What are the functions of the CovRS system in *S. agalactiae*? The genes for the CovRS orthologous system in *S. agalactiae* are part of a seven-gene operon and mutation led to dramatic phenotypic changes (Lamy et al. 2004). Although the mutant adhered much better to epithelial cells, the survival in human serum was attenuated. Moreover, as also reported for *S. pyogenes*, the *S. agalactiae* CovRS system is critical for virulence, as proven by neonate rat sepsis and mouse infection models (Lamy et al. 2004; Jiang et al. 2005). A total of 76 genes are repressed whereas 63 were positively regulated. Transcriptome comparison of several strains uncovered a conserved 39 gene core regulon (Jiang et al. 2008). Moreover, there was a significant overlap of the acid stress transcriptome in *S. agalactiae* with the CovRS regulon (Santi et al. 2009), and CovRS as well as pH-regulated *S. agalactiae* adherence to host cells of vaginal, cervical and respiratory origin (Park et al. 2012).

In contrast to CovRS in *S. pyogenes*, the *S. agalactiae* system can act bidirectional and is not an exclusive repressor system (Jiang et al. 2008). Direct binding of phosphorylated CovR to many of the target gene promoters has been reported (Lamy et al. 2004; Jiang et al. 2008). However, the binding site differs from the consensus sequence reported for *S. pyogenes* CovR, although both share a high AT content. It can be concluded that the general classes of genes under control of both systems are identical in *S. pyogenes* and *S. agalactiae*. It is currently unknown, whether *S. agalactiae* CovR is dependent on CovS activity, but there is clear evidence linking CovRS and serine/threonine kinase systems also in *S. agalactiae* (discussed in Sect. 4).

It is obvious that the CovRS system is central to the virulence of both streptococcal species. Many similar functions have been elucidated, but depending on the pathogens primary niche in the host, both systems also underwent a pathogen-specific evolution and adaptation. It remains to be established if point mutations in the CovRS system in *S. agalactiae* also occur in a disease specific manner in clinical isolates and if such mutants would be as hypervirulent as their *S. pyogenes* counterparts.

No CovRS orthologs were found in *S. pneumoniae*. In *S. suis* exclusively CovR was identified as an orphan response regulator, lacking the adjacent CovS histidine kinase. Virulence studies characterized the *S. suis* CovR as a globally acting negative modulator of virulence, as in a mutant most phenotypes changed in the direction of higher virulence potential (Pan et al. 2009).

### 3.2 The CiaHR System

The CiaHR TCS is widespread among streptococci. Genes encoding this TCS are found on the chromosomes of *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and also *S. suis*. The amino acid sequence identity of CiaH and CiaR from different species

ranges between 48–71 and 77–85 %, respectively (Riani et al. 2007). Most data on Cia function have been compiled from studies in *S. pneumoniae*. An increased cefotaxime resistance and impaired natural competence was noted in a *S. pneumoniae* CiaH mutant (Guenzi et al. 1994). The influence on competence involves sensing of  $\text{Ca}^{2+}$  and oxidative stress (Zahner et al. 2002). A key connection between the *S. pneumoniae* CiaHR regulon, which has been defined in at least three different strains, and competence is the CiaHR control of HtrA expression. This surface expressed serine protease is down-regulated in CiaHR mutants (Paterson et al. 2006). A restoration of *htrA* expression in the Cia mutant background alone restored natural competence (Sebert et al. 2005). *S. pneumoniae* Cia regulons comprise roughly 20 genes and apart from competence genes also stress response genes and operons were identified (Paterson et al. 2006), suggesting natural competence as *S. pneumoniae* stress factor, which is balanced by Cia activity. Of note, many other virulence genes are found in the Cia regulon, which makes it difficult to clearly define its role in virulence. Further studies have revealed that the CiaH acts as kinase/phosphatase, that CiaR needs to be phosphorylated, that CiaR can obtain phosphates independent from CiaH, and that CiaR acts directly on 15 promoters identified by transcriptional mapping (Halfmann et al. 2007, 2011). Those promoters belong to genes involved in teichoic acid biosynthesis, sugar metabolism, chromosome segregation, and protease maturation. Five of those promoters were identified upstream of small noncoding RNAs (Marx et al. 2010). Matching the observation made in the CovRS TCS in *S. pyogenes*, clinical *S. pneumoniae* isolates, particularly those with spontaneous  $\beta$ -lactame resistance, apparently acquired mutations in the CiaH encoding gene. This suggested that *ciaH* alleles, which overstimulate the CiaR regulon expression, are present in clinical isolates (Muller et al. 2011). Although not studied into such a detail as in *S. pneumoniae*, it is apparent that the *S. pyogenes* Cia system has different functions. As *S. pyogenes* is not naturally competent and lacks large parts of the *com* operon genes, regulation of competence, and as a consequence, also major regulation of stress response is not attributed to *S. pyogenes* CiaHR. The CiaH sensor mutants in two *S. pyogenes* serotype M49 strains allowed identification of up to 120 genes as Cia-controlled, with equal numbers up- and down-regulated (Riani et al. 2007). Among those, genes encoding proteins for divalent cation transport, PTS systems, hemolysins, hyaluronidase, matrix-protein-binding factors, and DNAses were identified. Divergent to observations in *S. pneumoniae*, metal ions did not affect Cia expression significantly, antibiotic resistance was not affected, and only a small number of six stress response genes were differentially transcribed in the *S. pyogenes* *ciaH* mutant (Riani et al. 2007). In *S. suis*, *ciaHR* mutants revealed a decreased adherence toward HEp-2 and PIEC cells, a higher susceptibility toward killing by RAW2647 macrophages, and were attenuated in mice and pig animal models of infection (Li et al. 2011). Not unexpected, CiaR was found to promote intracellular survival and resistance to innate immune defences in *S. agalactiae*. A *ciaR* mutant had a reduced survival in neutrophils, human macrophages and brain microvascular endothelial cells (BMCE) (Quach et al. 2009). In mouse infection models, the mutant was attenuated and showed

decreased survival in blood and brain compared to the wild type (Quach et al. 2009). In summary, particularly in *S. pneumoniae* CiaHR is crucial for natural competence and general virulence, whereas in the other species virulence control is the major function of Cia.

### 3.3 The *Ihk/Irr* System

The *Ihk/Irr* TCS of *S. pyogenes* is highly important for the pathogenesis of these bacteria and is thus reported to be active during *S. pyogenes* growth in many body fluids and host niches. As human saliva is one of the first liquids encountered after *S. pyogenes* droplet-mediated entry into the host oral cavity, growth, and persistence in saliva was tested. The most important TCS in hierarchy in saliva is the SptRS TCS. Among many others, also the genes encoding the *Ihk/Irr* TCS were up-regulated during *S. pyogenes* growth in saliva (Shelburne, III et al. 2005). Moreover, a role for *Ihk/Irr* could be established during *S. pyogenes* growth in human blood, conditions under which up to 75 % of the genes were differentially transcribed, and during phagocytosis by neutrophils, under which varying sets of genes are time-dependently and differentially transcribed (Voyich et al. 2003; Graham et al. 2005). A more recent study discovered 145 differentially transcribed genes during a 2-h *S. pyogenes* persistence in macrophages, of which many belong to metabolic and energy dependent processes. *Ihk/Irr* was again established as a key regulator important for the early processes in *S. pyogenes*-macrophage interaction (Hertzen et al. 2012). A major shift of response regulator activity from time point 2–6 h during persistence was noted, in which *Ihk/Irr* was down-regulated and the CovRS system was up-regulated. This hinted toward a TCS driven and fine-tuned temporal expression shift in *S. pyogenes* intracellular life cycle (Hertzen et al. 2012). In *S. suis* serotype 2, an *Ihk/Irr* ortholog was recently characterized (Han et al. 2012). Deletion of this system notably attenuated virulence in mice. Mutants showed reduced adherence to host cells, an increased susceptibility to macrophages killing, and a decreased survival under oxidative stress conditions. Of note, the important *S. suis* virulence factors suilysin, autolysin, and muramidase-released protein encoding genes were not under *Ihk/Irr* control. Rather cell metabolism and superoxide dismutase were repressed in the mutant (Han et al. 2012). It is conceivable, that in the other streptococcal species discussed here, other TCS have similar functions although they cannot be classified as *Ihk/Irr* orthologs based on sequence homology.

### 3.4 The *VicRK* System

The *VicRK* system is noteworthy as it is present and conserved in many Firmicutes and for a long time was considered essential for growth in all organisms after many failed mutation attempts. In the literature, there are a couple of synonymous names,

like YycFG, MicAB, and WalRK. An unconditional *vicR* insertional mutant generated in *S. pyogenes* allowed insight into functional aspects of this system (Liu et al. 2006). The mutant grew well in rich lab media, but was severely attenuated in growth in nonimmune human blood and serum, had attenuated virulence, and was unstable in mice. However, as phagocytosis and killing was normal, a general influence on evasion of host defences was excluded. Among Vic-controlled genes, those involved in cell-wall hydrolysis (*pcsB*), phosphotransferase systems, osmo-protectant transporters, and nutrient uptake were identified by transcriptomics (Liu et al. 2006). A view across the species barriers linked the essentiality in some organisms to VicRK exerted control over functions like murein biosynthesis, cell division, lipid integrity, exopolysaccharide synthesis, biofilm formation, and also virulence factor expression. However, the signals stimulating the Vic-system are largely unknown (Winkler and Hoch 2008). In *S. pneumoniae*, the essentiality of the Vic-system could only be bypassed by a constitutive PcsB cell-wall hydrolase expression, suggesting that control over cell-wall biosynthesis and osmosis is the critical function (Winkler and Hoch 2008). Recently, also in *S. suis* in vivo swine infection the VicRK system was found up-regulated together with its target gene *pcsB* (Li et al. 2010). Since not many virulence factor encoding genes were found to be directly controlled by the VicRK TCS, it cannot be designated a general virulence regulator. However, considering that central cell-wall turnover and metabolic functions are also directly linked to fitness of the pathogens in vivo there is a clear link of VicRK to virulence. Apparently, homologs and orthologs were also found in Actinobacteria like *Streptomyces*, *Mycobacterium*, and *Corynebacterium* species, which places the Vic-system in the focus as excellent target for novel antimicrobial therapy strategy developments (Winkler and Hoch 2008). First structure-based virtual screenings of potential inhibiting compounds from the SPECS library have been done in *S. pneumoniae*, and interesting target compounds with activity against *S. pneumoniae* were identified in vitro and also in a mouse sepsis infection model. These compounds decreased mortality in mice and had no general cytotoxic effect (Li et al. 2009).

## 4 Eukaryotic-Type Serine/Threonine Kinases in Streptococci

Results discussed in the paragraphs above have already indicated that some response regulators can be phosphorylated independently of their cognate kinases, suggesting a potential link to other TCS within the same organism. Just recently, a whole new level of signal recognition and signal processing has been discovered in prokaryotes thanks to advances in genetic strategies and genome sequencing approaches. This includes eukaryotic-like serine/threonine kinases (STKs) which were found to be linked to adjacent phosphatases (STPs). An in-depth review focussing on discovery and functional analysis of these systems in various species has been published (Burnside and Rajagopal 2011). What places these signaling

and response systems into the research focus is the fact that their eukaryotic counterparts are excellent targets for therapy. Many STK inhibitors are approved by the United States Food and Drug Administration (USFDA) or at various stages in clinical trials. Thus, the prospect that such inhibitors also work for the bacterial STK/STP systems is promising. The STKs in *S. pyogenes*, *S. pneumoniae*, and *S. agalactiae* have been identified at nearly the same time (Rajagopal et al. 2003; Echenique et al. 2004; Jin and Pancholi 2006). The *S. pyogenes* STK and STP (SP-STK and SP-STP) were characterized as functional manganese-dependent kinase and phosphatase enzymes (Jin and Pancholi 2006). An altered cell-shape, incomplete cell separation, a loosely associated electron-dense layer, and a tendency to settle during growth in laboratory media were the initial phenotypes after mutation of SP-STK. Increases in doubling time and hemolysis activity were noted. For the virulence of *S. pyogenes*, the SP-STK was found to be critical for host cell adherence and resistance to phagocytic killing. As a mediator of SP-STK activity on gene regulation, phosphorylation of a 10 kDa histone-like protein was suggested (Jin and Pancholi 2006). The SP-STP was subsequently shown to be involved in phosphorylation of the *S. pyogenes* VicRK and CovRS TCS (Agarwal et al. 2011). In *S. agalactiae*, mutants defective in STK and STP were attenuated in a neonatal rat sepsis model (Rajagopal et al. 2003). STK expression is vital for resistance to human blood, neutrophils, and oxidative stress. Transcription of the *S. agalactiae*  $\beta$ -hemolysin is mediated via threonine phosphorylation of the CovR response regulator, which improved CovR repression (Rajagopal et al. 2006). This verified a regulatory connection between TCS and STK/STP systems in these pathogenic streptococcal species. STP mutation in *S. agalactiae* allowed identification of more phenotypes and up to 294 differentially transcribed genes were found. Phosphopeptide enrichment methods allowed identification of 35 STK/STP phosphorylated peptides as part of 27 different proteins (Burnside et al. 2011). In *S. pneumoniae*, pleiotropic functions are under control of a single STK encoded on the chromosome, including virulence, competence, antibiotic resistance, growth and stress response (Echenique et al. 2004), and functional links to active TCS systems were found (Agarwal et al. 2012). Direct STK phosphorylation of target proteins involved in ion transport, cell division, and RNA-polymerization was proven. Currently, the role of the phosphatase is not known. However, it is tempting to speculate that the *S. pneumoniae* phosphatase functionally resembles *S. pyogenes* and *S. agalactiae* phosphatases. Together, these streptococcal serine/threonine kinase–phosphatase systems play an important role in the physiology and virulence of the species discussed here. They apparently have rather conserved functions in all streptococci. Currently, not much is known about the signals that initiate their activity and where in the regulatory hierarchy in conjunction with stand-alone transcriptional regulators and TCS they play their major role. The success story using inhibitors in their eukaryotic counterparts to fight human diseases raises hope that novel inhibitors counteracting bacterial STK/STP systems will be developed and serve as innovative therapies in combating severe bacterial infections.

## 5 Noncoding Regulatory RNAs

### 5.1 *Cis-Regulatory RNAs*

Diverse *cis*-regulatory systems controlling virulence factor genes have been described in gram-positive pathogens including RNAIII in *Staphylococcus aureus* (Novick et al. 1993) and a thermosensor located in the 5'-UTR of the *prfA* mRNA in *Listeria monocytogenes* (Johansson et al. 2002). One of the first examples for a regulatory RNA discovered in *S. pyogenes* was the untranslated 500 bases RNA of the streptococcal pleiotropic effect locus (*pel*). The region contains the structural gene *sagA* coding for a precursor of the streptococcal hemolysin SLS. The locus was initially detected following transposon mutagenesis of a *S. pyogenes* M49 serotype strain. It was found to positively regulate the genes of important streptococcal secreted and surface virulence factors. Loss of the 500 bases transcript decreased transcription of *emm* and *speB* genes and reduced secretion of streptokinase. Whether a protein encoded by the locus or an untranslated RNA was responsible for the regulatory effects was not clear at the time (Li et al. 1999). In a later study, it could be shown that *pel*-dependent virulence factor regulation was mediated by a 459-bases untranslated RNA molecule (Pel). It was demonstrated that regulation by Pel occurs at both transcriptional (e.g. *emm* and *sic*) and post-transcriptional (e.g. SpeB) levels (Mangold et al. 2004). Strain specificity of Pel function is indicated by the fact that in a *sagA*-deficient mutant with an M6 background, *emm* transcription was not affected (Biswas et al. 2001). Similar results have been obtained in *S. pyogenes* M1 and M18 Tn916 *sagA* mutant strains (Betschel et al. 1998). Additionally, *pel* deletion mutant analysis of four MIT1 *S. pyogenes* isolates did not confirm any regulatory function of the Pel sRNA in this serotype (Perez et al. 2009).

Expression of ribosomal protein genes (*rplJ-rplL*) in *E. coli* is regulated by an autogenous control mechanism involving the 5'-untranslated region of the mRNA. Ribosomal proteins L10 and L12 bind to the leader region of the L10 operon and inhibit translation (Johnsen et al. 1982). The secondary structure of the leader RNA is important for the formation of a stable complex (Christensen et al. 1984). A family of putative ribosomal protein leader autoregulatory structures was also found in *B. subtilis* and other Firmicutes. For the genus *Streptococcus*, 393 members of the L10-leader RNA family from 385 species are listed in Rfam (Gardner et al. 2009). Recently, the expression of a L10-leader sRNA, was demonstrated in different growth phases of *S. mutans* (Xia et al. 2012). Growth phase and pH dependency of expression indicate a regulatory role for the L10-leader in the acid adaptive response in this cariogenic bacterium. Expression of L10-leader RNAs and other ribosomal leader RNAs, including L13-, L20-, and L21-leader RNA, was also observed in *S. pyogenes* by tiling array analyses (Perez et al. 2009; Patenge et al. 2012) and in *S. pneumoniae* by tiling array analysis and RNAome sequencing (Kumar et al. 2010; Acebo et al. 2012; Mann et al. 2012).



## 5.2 FMN-Riboswitch

Riboswitches are regulatory elements found in 5'-untranslated regions of prokaryotic mRNAs. They are acting in *cis* by controlling expression of their downstream genes through a metabolite-induced alteration of their secondary structure. Doing so, riboswitches play a prominent role in bacterial metabolism control (Nudler and Mironov 2004). The flavin mononucleotide (FMN) riboswitch is a metabolite-dependent riboswitch that directly binds FMN. The element controls expression of genes that encode for FMN biosynthesis and transport proteins (Vitreschak et al. 2002). A link to virulence-related gene expression comes from *L. monocytogenes*, where the riboflavin analog roseoflavin targets an FMN-riboswitch and blocks *L. monocytogenes* growth, but also stimulates virulence gene expression and infection (Mansjo and Johansson 2011). Furthermore, two riboswitches acting as noncoding RNAs *in trans* control expression of the virulence regulator PrfA in *L. monocytogenes* (Loh et al. 2009). Metabolite-driven *trans*-regulatory sRNAs form a new class of regulatory non-coding RNAs in bacteria. Expression of FMN-riboswitches has also been detected in streptococci in several recent sRNA expression screens (Perez et al. 2009; Kumar et al. 2010; Patenge et al. 2012; Mann et al. 2012). To date, no involvement of FMN-riboswitches in virulence gene expression has been documented in streptococci. Nevertheless, like other riboswitches, FMN-riboswitches may represent interesting novel drug targets (Blount and Breaker 2006). The natural antibacterial compound roseoflavin binds to FMN-riboswitches and has been shown to inhibit bacterial growth and to regulate gene expression in *B. subtilis* and *L. monocytogenes* (Lee et al. 2009; Mansjo and Johansson 2011). The identification of compounds that trigger FMN-riboswitch function in streptococci may be a promising alley for the development of novel antimicrobial drugs.

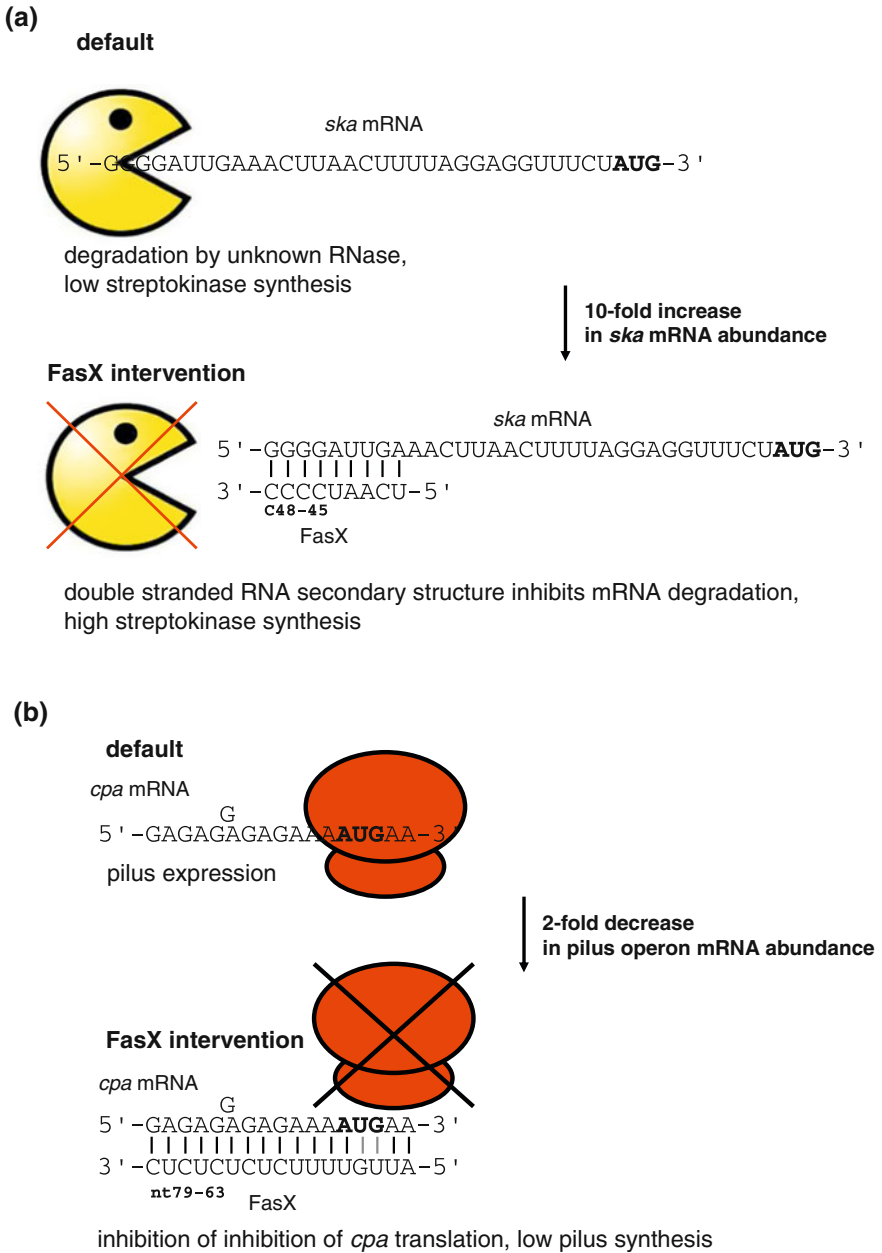
## 5.3 Trans-Antisense sRNAs

sRNAs working by *trans*-antisense binding to their target mRNAs tend to interact with conventional TCS in streptococci. Several examples of sRNA-TCS networks will be described in this chapter.

The small regulatory RNA FasX was one of the first characterized sRNAs in streptococci. It was initially identified during the analysis of the two-component type regulator Fas (fibronectin/fibrinogen binding/hemolytic activity/streptokinase regulator) in *S. pyogenes* (Kreikemeyer et al. 2001). The *fasBCA* operon in *S. pyogenes* encodes two potential sensor kinases and one response regulator. It is expressed in a growth phase-dependent manner and controls the production of several secreted virulence factors such as streptokinase and streptolysin S. Downstream of the *fasBCA* transcriptional unit, an independently transcribed

gene, *fasX*, was found to encode a short RNA molecule. Expression of *fasX* depends on the RR FasA. Gene replacement of *fasX* resulted in a phenotype similar to *fasBCA* or *fasA* knock-out mutations with prolonged expression of extracellular matrix-protein-binding adhesins and reduced expression of secreted virulence factors. Complementation of the *fasX* deletion mutant, with *fasX* expressed *in trans* from a plasmid, restored the wild-type *fasBCA* regulation pattern (Kreikemeyer et al. 2001). From these data, it was concluded that FasX, a non-translated RNA, is the main effector molecule of the Fas regulon. Accordingly, *S. pyogenes* carrying a *fasX* deletion induced a reduced response of host epithelial cells in terms of cytokine production, apoptosis, and cytotoxicity parameters (Klenk et al. 2005). FasX enhances streptokinase activity in *S. pyogenes*. Stimulation of streptokinase gene (*ska*) expression by FasX is achieved by binding to the 5' end of the *ska* mRNA and thereby increasing the stability of transcript (Ramirez-Pena et al. 2010). Lack of FasX-*ska*-mRNA interaction in *fasX* mutants led to decreased transcript levels and consequently to a decreased streptokinase protein abundance (Ramirez-Pena et al. 2010). Recently, it has been shown that FasX also controls pilus gene expression by pairing to the extreme 5' end of the pilus biosynthesis operon transcript. In this case, the resulting RNA-RNA interaction reduces the stability of the mRNA, while at the same time inhibiting translation of at least the first gene in the pilus biosynthesis operon. As a consequence of down-regulated pilus expression, adherence to host epithelial cells by *S. pyogenes* was reduced (Liu et al. 2012). Virulence gene regulation by FasX works by classical antisense binding to target mRNAs, but diverse mechanisms could be identified as depicted in Fig. 1: in one example sRNA-mRNA interaction stabilizes the target (*ska* expression, Fig. 1a), in the other it destabilizes the target (pilus biosynthesis control, Fig. 1b). This is particularly striking, because up-regulation of Ska as well as pilus down-regulation represent activities related to the transition of *S. pyogenes* from the colonization stage of infection to the dissemination phase.

Another link between a two-component system and sRNAs became evident recently in *S. pneumoniae*. The *ciaRH* regulatory network plays a major role in the maintenance of cell-wall integrity. Penicillin-binding protein independent resistance to beta-lactam antibiotics is conferred by the *ciaRH* regulatory system (Zahner et al. 2002). Genes controlled by the *ciaRH* system are involved in cell-wall biochemistry. Bacteria carrying mutations in *ciaH* failed to develop genetic competence due to repression of the *comCDE* operon region (Mascher et al. 2003). *ciaRH* mutants were hypersusceptible to a variety of lysis-inducing conditions (Mascher et al. 2006). In two recent studies, a new level of regulation has been introduced to the *ciaRH* network in *S. pneumoniae*. Among the genes regulated by CiaR, five sRNA genes have been identified, designated *cia*-dependent small RNAs (csRNAs). All csRNAs identified so far, show a high sequence similarity and share the same mfold-predicted secondary structure presenting with two stem loops, separated by a stretch of unpaired bases. Deletion mutants lacking csRNA4 and csRNA5, respectively, showed enhanced stationary phase autolysis (Halfmann et al. 2007). The mechanism of csRNA4/5 function is not known yet, but it does not seem to involve interference with LytA and LytC production. Genes for



**Fig. 1** Schematic of the differential outcome of FasX binding. The start codons of the respective target mRNAs are indicated with bold letters. **a** Stabilization of *ska* transcripts by interaction with FasX, pac man: unknown RNase molecule. **b** Destabilization of pilus transcripts and inhibition of *cpa*-mRNA translation following interaction with FasX, mushroom: ribosome

csRNAs have been detected on the basis of CiaR binding site presence over a wide range of streptococcal genomes, including *S. mitis*, *S. oralis*, *S. sanguinis*, and *S. pyogenes*. Expression of csRNA genes has been demonstrated by Northern blot analyses and suggests that genes for csRNAs belong to the regulon of the response regulator CiaR in all streptococcal species (Marx et al. 2010).

Another excellent example of a regulatory cascade that involves the function of an sRNA is the *rivRX* (RofA-like protein IV regulator R/X) operon in *S. pyogenes*. The global transcriptional regulator CovR represses *mga* and *rivRX*. RivR activates *mga* expression and its mechanism has been studied in a *covR*<sup>-</sup> background (Roberts and Scott 2007). RivR belongs to the RofA family of transcriptional regulators (described in Sect. 2.4) and contains a DNA-binding site. The protein seems to interact with Mga in order to enhance *mga* expression. The genes *rivR* and *rivX* are co-transcribed but mediate distinct pathways of Mga regulon activation. The *rivX* sequence contains two putative ORFs. Nonsense mutations in the potential start sites revealed that the *rivX* transcript rather than peptides encoded by *rivX* are responsible for the regulatory phenotype. From primer extension analysis, it was concluded that RNA processing could lead to the production of RivX following co-transcription of *rivR* and *rivX* explaining the independent function of the two genes. RivX stimulation of *mga* expression and Mga-activated gene expression depended on the presence of Mga protein. There was no stabilization of *mga* transcript observed. The mechanism of RivX function seems to involve a direct or indirect interaction with the *mga* transcript leading to enhancement of Mga translation (Roberts and Scott 2007). The RivR/X system works as integrator of the signals provided by the global CovR and Mga regulatory networks. This cross-talk allows the pathogen to respond to a broad variety of external stimuli with the fine-tuned expression of appropriate virulence factors.

#### 5.4 sRNA Interaction with Proteins

Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci are well-known to provide an adaptive RNA-based immune system in bacteria and archaea. CRISPR protects the cells from horizontal gene transfer originating from phage and plasmid DNA (Marraffini and Sontheimer 2010). Differential RNA sequencing identified a CRISPR/Cas locus in *S. pyogenes* SF370 (M1 serotype) (Deltcheva et al. 2011) encoded by the system II (Nmeni/CASS4 subtype) (Haft et al. 2005). This locus encodes the *trans*-activating CRISPR RNA (tracrRNA), which is, in concert with RNAase III and the CRISPR-associated Csn1 protein, responsible for the maturation of CRISPR RNA (crRNA) (Deltcheva et al. 2011). Following crRNA maturation, the cleavage of substrate DNA needs to be initiated. Very recently, it could be shown that the DNA endonuclease Cas9 from the type II CRISPR system in *S. pyogenes* was guided by a dual RNA molecule to its target DNA. The RNA–RNA complex consisted of the activating tracrRNA and the targeting crRNA, which contained a sequence complementary to the DNA

substrate (Jinek et al. 2012). In an independent study, it could be shown that the Cas9-crRNA complex of the *S. thermophilus* CRISPR/Cas system was able to cleave in vitro an artificial DNA substrate containing a sequence complementary to crRNA (Gasiunas et al. 2012). Bacterial CRISPR/Cas systems seem to be functionally conserved over a wide range of phyla. It could be shown that the *S. thermophilus* CRISPR/Cas system provided immunity in *E. coli* (Sapranaukas et al. 2011). An excellent description of the function of the CRISPR system in streptococci is included in a recent review about sRNAs by Le Rhun and Charpentier (Le Rhun and Charpentier 2012).

Formerly regarded as a house keeping RNA, the 4.5S RNA, a component of the bacterial signal recognition particle (SRP), represents another untranslated RNA with influence on streptococcal virulence (Trevino et al. 2010). While the 4.5S RNA gene is not essential for streptococcal growth under laboratory culture conditions, it proved to be essential for *S. pyogenes* to cause lethal infections in a murine bacteraemia model of infection. Mutation of the 4.5S RNA gene resulted in an altered secretome, including a reduction in secretion of the hemolysin streptolysin O and the SpeB protease. Moreover, remodeling of the *S. pyogenes* transcriptome was observed following 4.5S RNA mutation. More detailed assessment of gene expression upon loss of the 4.5S RNA gene revealed that differences in abundance of *grab*, *speB*, *spy0430*, and *slo* were *covS*-dependent. A further link of the 4.5S RNA gene to virulence was the strong reduction of growth in human saliva of *S. pyogenes* mutants affected in the gene and decreased virulence of these strains in a murine soft tissue infection model.

## 5.5 Bioinformatics Prediction Tools for sRNAs

Novel bioinformatics tools and whole-genome expression analyses employing tiling arrays or next generation sequencing helped to study the function of sRNAs in gram-positive pathogens (Mraheil et al. 2010). As knowledge about the role of regulatory RNAs in gram-positive bacteria is rising, new tools are being developed for the analysis of RNA structure and function, e.g., a database focusing on sRNA data from gram-positive bacteria (Pischmarov et al. 2012).

One of the most prominent bioinformatics prediction tools invented for sRNAs was the *sRNA identification protocol using high throughput technology* (SIPHT) tool, which has been used for many bacterial species (Livny et al. 2005; Livny and Waldor 2007). However, comparison of the prediction results with the actual in vivo expression of sRNAs, often revealed a low overlap between the different screening methods (Perez et al. 2009; Arnvig and Young 2009; Mraheil et al. 2011). This phenomenon is due to a combination of limitations of the prediction programs and the fact that not all sRNAs are expressed under all conditions. Today, the development of sRNA prediction software with improved properties is still ongoing. Several recently published bioinformatics tools have been used for

the identification of putative sRNAs in streptococci (Raasch et al. 2010; Sridhar et al. 2010; Pichon et al. 2012).

### 5.6 Whole-Genome sRNA Expression Screens

In recent years, whole-genome sRNA screens in streptococci employing either tiling array or next generation sequencing approaches, revealed an unexpected number of potential sRNAs (Tsui et al. 2010; Kumar et al. 2010; Mraheil et al. 2011; Beaume et al. 2011; Chen et al. 2011). Based on a previous bioinformatic prediction of putative sRNAs (Livny et al. 2006) expression of 40 putative sRNAs was tested in *S. pneumoniae* strain D39 by Northern analysis (Tsui et al. 2010). Nine new pneumococcal sRNAs were identified and the previously reported CcnA sRNA (Halfmann et al. 2007) was confirmed. However, functional characterization of deletion mutants and ectopic overexpression constructs of three of the candidate sRNA genes did not reveal strong effects on the phenotypes tested in this study. In a whole-genome approach, a total of 50 sRNAs from the intergenic regions of *S. pneumoniae* TIGR4 were identified using high-resolution genome tiling arrays (Kumar et al. 2010). In a deep sequencing approach, 88 regulatory sRNAs were identified in the TIGR4 strain of *S. pneumoniae* (Acebo et al. 2012). Of those, three housekeeping sRNAs were detected, several riboswitches and other *cis*-regulatory RNAs and 68 novel sRNAs. One of the novel candidates seems to be involved in the modulation of competence regulation in *S. pneumoniae* (Acebo et al. 2012). In another recent RNAseq study in *S. pneumoniae* TIGR4, 89 sRNAs were detected, 56 of which were novel. Tn-seq analysis testing relative fitness of bacterial mutants during infection predicted a high number of sRNAs involved in pneumococcal pathogenesis (Mann et al. 2012). Attenuated fitness was predicted during infection of the nasopharynx for 26 sRNAs, in the lung for 28 sRNAs, and in the bloodstream for 18 candidates. Fitness predictions were confirmed by individual targeted deletions in a subset of sRNAs. The high number of sRNA genes involved in pathogenesis underlines the overall importance of sRNAs in streptococcal virulence.

A whole-genome intergenic tiling array screen of *S. pyogenes* MIT1 identified approximately 40 sRNAs that were expressed during the exponential growth phase in cells cultivated in THY complex medium (Perez et al. 2009). There was a high conservation of sRNA genes among *S. pyogenes* strains, but the expression of the individual genes was found to be strain dependent. A targeted deletion within the *pel* region did not confirm the phenotype reported from other *S. pyogenes* strains. Expression of 16 candidate genes was confirmed by Northern blot analyses. Together with a former bioinformatics prediction (Livny et al. 2006), the number of putative sRNAs in *S. pyogenes* amounts to 75. In a whole-genome intergenic tiling array screen in *S. pyogenes* M49, 55 putative sRNAs were identified. A total of 42 sRNAs were novel, whereas 13 RNAs had been described before. The sequences of most of the candidates were conserved over streptococcal genomes.

However, comparison of the sRNA expression data to the above-mentioned analysis of the MIT1 strain and to two in silico screening methods revealed a low overlap between the different approaches (Patenge et al. 2012). Thus, the investigation of several conditions and the combination of screening tools will be necessary to gain a comprehensive understanding of the abundance of sRNAs in *S. pyogenes*. It is to be expected that further analyses of *S. pyogenes* genomes by RNAseq will lead to the identification of more sRNA genes.

## 6 Conclusions

The existing and still increasing richness of information on streptococcal transcriptional regulators, two-component signal transduction systems, eukaryotic-like serine/threonine kinase/phosphatase systems, and noncoding regulatory RNAs summarized in this review underscores the importance to decipher the regulatory networks acting in these pathogenic bacteria. A detailed understanding of virulence and pathogenicity mechanisms is critical for the development of novel antibacterial therapies. This review highlighted the presence of many orthologous/homologous regulators and regulatory mechanisms across streptococcal species, of which many have similar functions and are central for the pathogenesis of all the species. In parallel, many have different functions or have undergone a pathogen-specific alteration, including strain- or serotype-specific adaptation. This raises the question whether developing interference strategies targeting regulators and regulatory networks is a promising goal that should be pursued further. In the opinion of the authors, this question can be answered with “yes”. What is required to develop such interference strategies/therapies? (I) The complete operons/regulons of interesting candidates need to be identified. (II) Knowledge needs to be transferred from in vitro to in vivo experiments. (III) Particularly the species-specific regulators/regulatory mechanisms should be kept in focus, as they allow a selective targeting without harmful effects on related species or the residual bacterial flora. In the opinion of the authors the goal of interference therapy should be species-specific virulence/pathogen attenuation rather than killing, as such a strategy allows the immune system of the host to cope with infections and develop immunity. Such novel agents need to be accompanied by available antibiotic approaches. Particular noncoding regulatory RNAs provide a wealth of potential targets, which fulfill above criteria. What are possible means to interfere with their function? In the author’s lab antisense-PNA (peptide nucleic acids) technology is currently explored for specific sRNA-activity interference.

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