Regulated intramembrane proteolysis in the control of the *Bacillus subtilis* anti-sigma factor RsiW

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CONTENTS

SUMMARY

The activity of the extracytoplasmic function (ECF)-sigma factor σ^W of the Gram-positive soil bacterium *Bacillus subtilis* is modulated by a specific membrane-bound anti-sigma factor (RsiW). Initiated most likely by cell wall stress, RsiW is degraded by the mechanism of regulated intramembrane proteolysis (RIP). This process involves two site-specific proteolytic cleavage events in the extracytoplasmic part (site-1) and in the transmembrane domain (site-2) of RsiW. In consequence, σ^W is released to interact with the RNApolymerase, and the transcription of σ^W-controlled genes is initiated. In general, regulation of differential gene expression by RIP seems to play a major role in prokaryotic stress responses, pathogenesis and antibiosis. However, in most cases the molecular basis is not understood.

The main objective of this work was to use *B. subtilis* σ^W / RsiW as a model to investigate the mechanism of RIP in detail. In particular, there are significant differences to the inducing stress-signal and the site-1 protease that are described for the well investigated *Escherichia coli* ECF sigma factor σ^E-system.

The basis of this work were different mutants with a defect in RIP of RsiW that were isolated in a transposon screen.

First, PrsW (formerly YpdC) was identified as the site-1 protease. It belongs to a superfamily of potential membrane embedded metalloproteases (MEM) with so far unknown function in bacteria. Further characterization of PrsW in a reconstituted *E. coli* system showed that PrsW cleaves RsiW in a site-specific manner to form a protein truncated for 40 C-terminal extracytoplasmic amino acid residues. The tail specific protease Tsp was shown to further degrade the extracytoplasmic part of this RsiW site-1 cleavage product, which is crucial for subsequent RasP catalyzed site-2 clipping. Several other peptidases seem to be involved in trimming of RsiW downstream of PrsW and upstream of RasP in *B. subtilis*.

Second, the transposon screen revealed that a defect of the ABC-transporter EcsAB impairs RsiW site-2 cleavage by RasP for unknown reasons. It is conceivable that an EcsAB substrate competitively inhibits RasP activity.

In summary, a new model of two proteolytic modules involved in intramembrane proteolysis of RsiW could be established. Each module consists of a site-specific processing peptidase (site-1: PrsW, site-2: RasP) that subjects cleaved RsiW to degradation by unspecific proteases (site-1: Tsp-like, site-2: Clp-proteases).

ZUSAMMENFASSUNG

Die Aktivität des ECF Sigmafaktors σ^W des Gram-positiven Bodenbakteriums *Bacillus subtilis* wird durch den membrangebundenen anti-Sigmafaktor (RsiW) reguliert. Stresssignale der Umwelt, welche vermutlich die Integrität der Zellhülle beeinflussen, initiieren den Abbau von RsiW. Dieser Prozess, auch als regulierte intramembrane Proteolyse (RIP) bezeichnet, umfasst zwei spezifische proteolytische Schnitte im extracytoplasmatischen Teil (Site-1) und in der Transmembrandomäne (Site-2) von RsiW. Daraus resultiert die Freisetzung von σ^Wund durch Wechselwirkung mit der RNA-Polymerase wird die Transkription σ^W-abhängiger Gene initiiert. Die Regulation der differentiellen Genexpression durch RIP scheint bei Prokaryonten eine signifikante Rolle bei Stressantworten, der Pathogenität und der Abwehr antimikrobieller Komponenten zu spielen. Die molekulare Grundlage ist jedoch in den meisten Fällen nicht verstanden.

In dieser Arbeit wurde das σ^W / RsiW System aus *B. subtilis* als Modell verwendet, um den Mechanismus der RIP im Detail zu untersuchen. Offensichtlich liegen signifikante Unterschiede zum induzierenden Stresssignal und der Site-1 Proteolyse im Vergleich zu dem gut untersuchten ECF Sigmafaktor σ^E-System aus *Escherichia coli* vor.

Grundlage dieser Arbeit war ein Transposon-Screen, mit dem Mutanten mit einem Defekt der RIP von RsiW isoliert worden waren.

Zunächst wurde PrsW, früher als YpdC bezeichnet, als die Site-1 Protease identifiziert. PrsW gehört zu einer Superfamilie von potentiellen membran-integrierten Metalloproteasen (MEM), denen in Bakterien bisher keine Funktionen zugewiesen werden konnten. Die weitere Charakterisierung von PrsW in einem rekonstituierten *E. coli*-System zeigte, dass RsiW sequenzspezifisch um 40 C-terminale Aminosäuren verkürzt wird. Die ‚Tail-specific protease' Tsp baut den restlichen extracytoplasmatischen Teil von Site-1 geschnittenem RsiW ab, was für die nachfolgende Site-2 Prozessierung durch RasP entscheidend ist. In *B. subtilis* scheinen verschiedene andere Peptidasen an der weiteren Verkürzung von Site-1 geschnittenem RsiW beteiligt zu sein.

Ferner konnte durch den Transposon-Screen gezeigt werden, dass ein Defekt des ABC-Transporters EcsAB die Site-2 Proteolyse von RsiW durch RasP aus unbekannten Gründen verhindert. Es ist anzunehmen, dass ein Substrat von EcsAB die Aktivität von RasP kompetitiv inhibiert.

Zusammenfassend ermöglichten die Ergebnisse dieser Arbeit ein neues Modell zu entwickeln. Der Abbau von RsiW durch RIP erfolgt in zwei proteolytischen Modulen. Jedes besteht aus einer Sequenz-spezifischen Peptidase (Site-1: PrsW, Site-2: RasP) die verkürztes RsiW dem weiteren Abbau durch unspezifische Proteasen (Site-1: Tsp-ähnliche, Site-2: Clp-Proteasen) zugänglich macht.

Chapter 1

Introduction and scope of this thesis

Janine Heinrich

1. Mechanisms of bacterial signaling

A fundamental mechanism of living cells is the transmission of signals from the environment to the cytoplasm to adapt to changing conditions. Alterations in physical parameters like the pH, osmolarity, temperature, deficiency in the availability of nutrients, and the occurrence of toxic compounds cause stress the cells have to counteract against. Also, signals that mediate cell-to-cell communication in developmental processes have to be transduced. Bacteria evolved universal signal transduction systems which regulate gene expression in response to such environmental changes. In most cases, regulation takes place at the level of transcriptional initiation. The extensive investigations of these systems have revealed general principles of differential gene expression (Jung and Krämer, 2010). Most of them have been elucidated with model organisms like the Gram-negative enterobacterium *Escherichia coli* and the Gram-positive soil bacterium *Bacillus subtilis*.

The simplest form of a bacterial signal transduction system consists of one single protein with a two-domain structure, a sensory input domain and a regulatory DNA-binding domain. Examples well known are repressors like LacI and activators like the CAP protein (Ulrich *et al.*, 2005). For these systems, the signaling molecule itself enters or is produced in the cytoplasm. In contrast, a variety of systems directly sense extracellular signals. In principle, transmembrane signaling is accomplished by a membrane spanning protein with an extracellular receptor-domain that transduces a signal to a cytoplasmic regulator.

1.1 Bacterial transmembrane signaling

Bacterial 'Two Component Systems' (TCSs) are the most prevalent group of trans-membrane signaling complexes (Stock *et al.*, 2000). TCSs are composed of modular proteins. A bitopic transmembrane sensor histidine kinase (HK) with an input and a transmitter domain is connected to a cytoplasmic response regulator (RR) with a receiver and a DNA-binding output domain. In an ATP dependent manner, the information is transduced through histidine-aspartate phosphorelays (Stock *et al.*, 2000; Mascher, 2006). As a result, the RR is able bind to the DNA by a helix-turn helix (HTH) motif, either to induce expression of genes which counteract the inducing signal, or to repress them.

In *E. coli*, more than 30 of such systems are predicted (Mizuno, 1997). For *B. subtilis*, 36 HKs and 34 RRs have been found (Fabret *et al.*, 1999). In addition, co-sensing components, e.g. ABC-transporters, are described to be involved in gene regulation via TCSs. Recently, a special family of intramembrane-sensing HKs which lack a distinct extracytoplasmic input domain have been discovered (Mascher, 2006).

While transmembrane signaling by TCSs is coordinated by a phosphotransfer mechanism, a variety of different systems depend on intramembrane proteolysis of a regulatory protein. This process, named regulated intramembrane proteolysis (RIP), had been discovered first for eukaryotic regulatory pathways. However, there is emerging evidence that also important prokaryotic systems involve this mechanism. First of all, stress responses coordinated by alternative sigma factors are regulated via RIP (Schöbel *et al.*, 2004; Ades, 2008). These mechanisms will be outlined in detail below. In addition, sporulation (Rudner *et al.*, 1999; Yu and Kroos, 2000), cell division (Bramkamp *et al.*, 2006), cell cycle regulation (Chen *et al.*, 2005), quorum sensing (Stevenson *et al.*, 2007), pheromone and toxin production (An *et al.*, 1999; Matson and DiRita, 2005), and biofilm formation (Qiu *et al.*, 2007; Heinrich *et al.*, 2008) depend on intramembrane proteolysis. Moreover, recent progress has established that RIP plays pivotal roles in pathogenic processes (Makinoshima and Glickman, 2006; Urban, 2009).

Transmembrane signaling has been well investigated for environmental changes that influence integrity of the cell envelope. For *E. coli*, the two TCSs CpxAR and BaeRS (Raivio and Silhavy, 2001; Ruiz and Silhavy, 2005) and the alternative sigma factor σ^E both are shown to counteract extracytoplasmic stress. In *B. subtilis* the activation of σ^W via RIP is the best investigated system, but there are TCSs [BceRS, LiaRS, YvcPQ, YxdJK (Jordan *et al.*, 2008)] described to be involved in envelope stress response as well.

1.2 Regulated intramembrane proteolysis in transmembrane signaling

About ten years ago, it became apparent that RIP is a control mechanism conserved from bacteria to humans (Brown et al., 2000). It is a two-step cleavage mechanism with the key step catalyzed by a family of peptidases, named intramembrane cleaving proteases (I-CLiPs). These are multispanning membrane proteins which cleave transmembrane domains of substrate proteins within the lipid environment of the membrane, to release a protein that functions in signaling (Weihofen and Martoglio, 2003).

This intramembrane cleavage event depends on a proteolytic processing of the substrate protein external to the membrane, also referred to as site-1 proteolysis.

1.2.1 Intramembrane cleaving proteases

I-CLiPs can be divided into four evolutionary unrelated families of highly hydrophobic multipass membrane proteins.

The mammalian Site-2 protease (S2P) was the first I-CliP discovered. It is involved in the sterol regulatory element binding protein (SREBP) activation pathway (Rawson *et al.*, 1997; Wolfe *et al.*, 1999), and contains a HExxH motif characteristic for metalloproteases (Rawlings and Barrett, 1995). Together with a second conserved LDG sequence motif, these amino acid residues coordinate a catalytic zinc atom. Orthologous proteins can be found from bacteria to higher organisms, and they were grouped to the S2P-family of I-CLiPs. Almost all members of the S2P-family with known substrates cleave type II membrane-spanning domains with the N-terminus in the cytoplasm and the C-terminus in the extra-cytoplasm. Such a restricted substrate orientation seems to be a general mechanism of substrate selection of intramembrane cleaving proteases.

The signal peptide peptidases (SPP) that constitute another family of I-CLiPs also seem to be restricted to substrates with type II topology, while members of the presenilin family (PS) cleave type I (N-out; C-in) transmembrane proteins. Both are proteases with a catalytically active aspartate residue. Although SPPs and PSs show only a low sequence homology, they have a high structural similarity and a conservation of some motifs, i.e. YD, GXGD and PAL in neighboring transmembrane helices (Weihofen *et al.*, 2002). For this reason they were grouped to one mechanistic family called GXGD-type of intramembrane proteases.

Another group of I-CLiPs is the rhomboid family of serine proteases. Rhomboids recognize substrates of type I topology and harbor a conserved GXSG motif that is characteristic for serine proteases of the chymotrypsin / trypsin / elastase family (Urban *et al.*, 2001; Gallio *et al.*, 2002; McQuibban *et al.*, 2003). Members of this family generally recognize their target directly without the need of a prior site-1 cleavage (Urban and Freeman, 2003).

1.2.2 The role of bacterial I-CLiPs in transmembrane signaling

In bacteria, only I-CLiPs of the S2P- and the rhomboid family have been discovered at present. S2Ps are involved in a variety of different transmembrane signaling pathways. One of the best investigated mechanisms of transmembrane signaling that makes use of S2Ps is the activation of alternative sigma factors of the extracytoplasmic function family (ECF). The sigma factor (σ) is a subunit of bacterial RNA-polymerase (RNAP) that is responsible for promoter recognition and initiation of transcription. ECF sigma factors are in many cases inactivated by a membrane spanning anti-sigma factor. These regulatory proteins sequester their corresponding sigma factor from interaction with core RNAP and prevent initiation of gene transcription. Upon an environmental signal, the anti-sigma factor is degraded through the mechanism of RIP under participation of an S2P (Fig. 1).

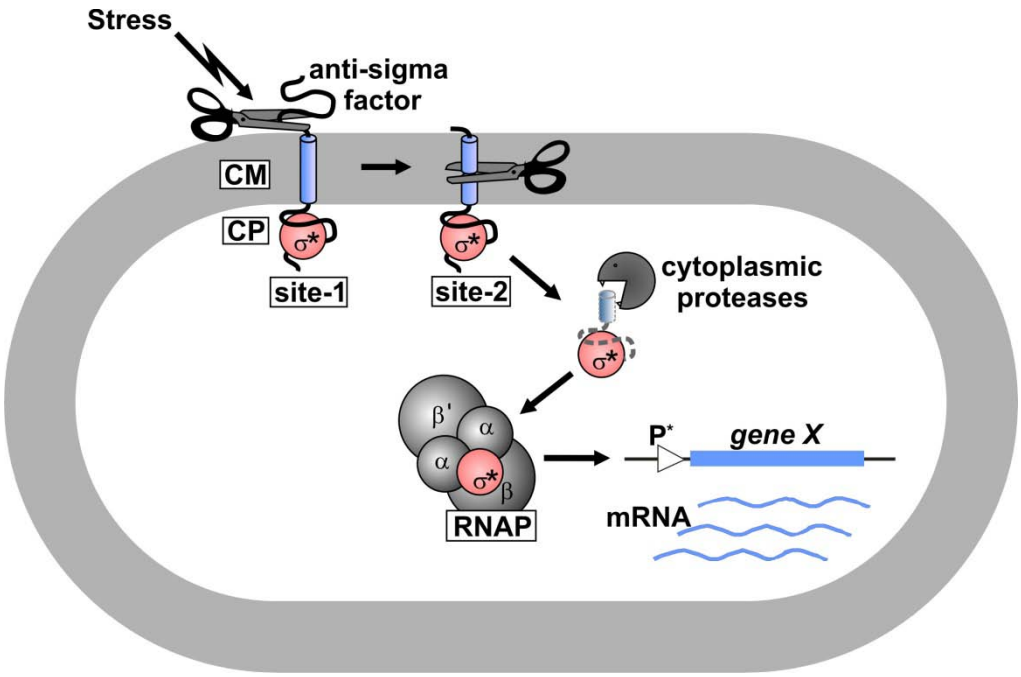

Fig.1 Model for the regulation of ECF sigma factor activity through transmembrane signaling that involves RIP. 'Site-1' and 'site-2' represent the proteases implicated in that process. CM: cytoplasmic membrane; CP: cytoplasm; RNAP: RNA polymerase. Further explanations see text. (Heinrich and Wiegert, 2009)

Other bacterial regulatory systems that involve I-CLiPs in a process of RIP have to be mentioned. In *B. subtilis*, the sporulation sigma factor σ^K is activated by cleaving off a transmembrane pre-sequence by the S2P SpoIVFB (Zhou and Kroos, 2005; Campo and Rudner, 2006). RasP (formerly YluC), another S2P of *B. subtilis*, is involved in timing cell-division by processing FtsL (Bramkamp *et al.*, 2006). In *Caulobacter crescentus*, the cycle of forming stalked and swarming cells is regulated by the S2P MmpA, which cleaves the polarity factor $PodJ_S$ (Chen *et al*., 2005). Finally, a peptide that mediates conjugation is released from the cell surface of *Enterococcus faecalis* by the S2P Eep (An et al., 1999).

In contrast, the only bacterial rhomboid protease with a defined substrate and function is *Providencia stuartii* AarA cleaving TatA. Thus, it activates TatA, which is a component of the TAT secretory pathway. It is believed that a so far unknown compound which mediates quorum sensing is then translocated through the membrane (Stevenson *et al*., 2007).

The crystal structures of the rhomboid GlpG of *E. coli* (Baker *et al.*, 2007) and *Haemophilus influenza* (Lemieux *et al.*, 2007), and of the S2P of the archaebacterial species *Methanocaldococcus jannaschii* [MjS2P (Feng *et al.*, 2007)] have revealed the active-site arrangement and gave answers to some of the main questions in intramembrane proteolysis.

The active sites are located within a folded, proteinaceous domain and sequestered from the surrounding lipids. GlpG holds a water-filled cavity which opens to the extracellular side that forms an aqueous environment surrounding the active serine residue. Additionally, GlpG contains helices within the membrane without crossing it, and a gating function was proposed in regulating access of substrates to the active site (Wang *et al.*, 2006; Wu *et al.*, 2006). In contrast, MjS2P has a polar channel that allows water entry from the cytoplasm to the catalytic zinc atom. An open and a closed conformation of MjS2P were crystallized, and there is evidence that two flexible transmembrane domains are responsible for these conformational changes, which is a mechanism for substrate gating (Feng *et al.*, 2007). However, these transmembrane domains are absent in a variety of other bacterial I-CLiPs, like *E. coli* RseP and *B. subtilis* RasP.

1.3 Activation of ECF sigma factors by RIP of a corresponding anti-sigma factor

While the housekeeping sigma factor is responsible for the basal expression of genes needed to keep the cell alive for general metabolic processes, alternative sigma factors are able to initiate transcription of specific sets of genes (regulons) in response to environmental signals. A subgroup of alternative bacterial sigma factors was first recognized as transcription factors involved in processes related to extracytoplasmic functions. Therefore, they were named ECF sigma factors (Lonetto *et al.*, 1994). The genome of *B. subtilis* encodes for at least 17 alternative sigma factors, seven of which belong to the ECF-family (Kunst *et al.*, 1997; Helmann and Moran JR, 2002). While only two were found in *E. coli* (σ^E and FecI), genomes of other bacteria encode up to 50 different ECF sigma factors (Helmann, 2002).

1.3.1 Activation of *E. coli* σ^E via RIP

On the molecular level, σ^E of *E. coli* is the best investigated ECF sigma factor. It controls the maintenance of the cell envelope integrity both under stress and normal growth conditions. The σ^E regulon includes 49 promotors, activating e.g. promoters that encode periplasmic chaperones (*dsbC*, *fkpA*, *skp*, and *surA*), enzymes for lipopolysaccharide biogenesis (*psd*, *lpxA*, *lpxD* and *lpxP*) and the promoter of the essential I-CLiP gene *rseP* (Rhodius *et al.*, 2006). σ^E is autoregulated and encoded in an operon that consists of the genes for the sigma factor itself (*rpoE*) and the cotranscribed genes *rseA*, *rseB* and *rseC* (regulator of sigma *E*) (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). RseA is a transmembrane anti-sigma factor with a type II topology (N-in, C-out), whereas RseB is a soluble periplasmic protein that interacts with a conserved region near the C-terminus of the poorly structured RseA periplasmic domain. RseC seems to exert no direct effect in modulating the σ^E-mediated stress response.

The σ^E regulon was initially discovered to control the periplasmic 'heat shock regulon' required for expression of one of the four promoters of *rpoH* (*rpoH3*), encoding the heat shock sigma factor σ^{32}, and the promoter of the periplasmic protease *degP* (*htrA*) gene, which were shown to be essential under growth conditions at 42°C (Erickson and Gross, 1989).

The specific inducing signal for inactivation of the anti-sigma factor RseA was shown to be the accumulation of non-native outer membrane porins (OMPs) like OmpC with a

C-terminal consensus sequence -YXF-COOH motif, which emerge upon a heat shock (Walsh *et al.*, 2003; Sohn *et al.*, 2007). In native OMPs the motif is inaccessible (Basle *et al.*, 2006). σ^E is also known to be induced by hyperosmotic shock (Bianchi and Baneyx, 1999) or alterations in the lipopolysaccharide (Tam and Missiakas, 2005).

To activate σ^E dependent transcription, σ^E has to be released from the anti-sigma factor to interact with the RNAP, but the affinity of σ^E to RseA is approximately 300-fold greater than to core-RNAP. In an off state, RseB binds to the periplasmic domain of RseA and enhances the inhibition of σ^E (Missiakas *et al.*, 1997; De la Penas *et al*., 1997).

In response to non-native OMPs that may appear in the periplasm upon stress (Flynn *et al.*, 2003), RseA is sequentially cleaved by the site-1 protease DegS (Ades *et al.*, 1999), the S2P-family I-CliP RseP (formerly named YaeL or EcfE) (Kanehara *et al.*, 2002; Alba *et al.*, 2002), and cytoplasmic proteases like ClpXP (Flynn *et al.*, 2004). DegS is a membrane-anchored protease with the catalytically active domain located in the periplasm. It forms a trimer with each active site cleaving the RseA anti-sigma factor about 30 residues from the C-terminal end of its transmembrane region. The truncated form of RseA is a direct substrate for RseP (Inaba *et al.*, 2008).

The activation of RseP as the key-player in RIP is tightly regulated to avoid signal independent activation of σ^E. First of all, this is accomplished by the regulation of DegS-activity itself. Second, RseB binding to the extracytoplasmic domain of RseA impedes DegS attack, and it is believed that RseB responds to different stress signals than DegS (Grigorova *et al.*, 2004). Third, RseP contains two overlapping PDZ domains facing the periplasm between the second and the third transmembrane helix. It was shown that one of the two periplasmic PDZ domains of RseP fully prevents premature intramembrane proteolysis of full-length RseA. In consequence of a deletion of this PDZ domain, RseA becomes a substrate for site-2 cleavage by RseP-ΔPDZ2 without an initiating site-1 cleavage step by DegS. Therefore, expression of σ^E is permanently activated in a deregulated manner. It was proposed that a Gln-rich region of the extracytoplasmic part of RseA directly interacts with the RseP-PDZ domains, preventing premature site-2 cleavage and ensuring the dependency on site-1 processing (Kanehara *et al.*, 2003).

Substrate requirements for RseP have been investigated to some detail. RseP is able to cleave any transmembrane domain without sequence similarities to RseA, provided that it contains helix-destabilizing amino acid residues (Akiyama *et al.*, 2004; Koide *et al.*, 2008).

Most recently it was shown that the C-terminal amino acid residue of RseA truncated by site-1 cleavage is essential to interact with one of the RseP's PDZ domains, thereby activating site-2 cleavage (Li *et al.*, 2009). Cleavage of site-1 processed RseA by RseP results in the release of the cytoplasmic part of RseA from the membrane, still interacting with σ^E. However, site-2 clipped RseA ends with the amino acid sequence -VAA at its C-terminus, which represents a recognition signal for the ClpXP AAA+ protease. Mediated by the SspB adaptor protein, ClpXP degrades RseA in an ATP-dependent manner. Therefore, the transmembrane domain of RseA contains a cryptic proteolytic tag that is uncovered after site-2 cleavage to ensure complete removal of the anti-sigma factor domain (Flynn *et al.*, 2004; Chaba *et al.*, 2007).

1.3.2 Activation of *B. subtilis* σ^W via RIP

B. subtilis is a ubiquitous bacterium of the soil that has become the Gram-positive counterpart of *E. coli* as a model prokaryote that can easily be accessed by molecular-genetic methods. Important developmental programs like sporulation and competence development have been investigated with this organism, and e.g. the concept of alternative sigma factors for differential gene regulation in bacteria has been discovered first in *B. subtilis* (Sonenshein *et al.*, 2002).

Besides the housekeeping sigma factor σ^A, the *B. subtilis* chromosome encodes for 17 alternative sigma factors, seven of which (σ^M, σ^V, σ^W, σ^X, σ^Y, σ^Z and YlaC) belong to the ECF-family. Their function and regulatory mechanisms are still not clearly understood (Helmann, 2002). Recent progress elucidated a possible role of σ^M, σ^W, and σ^X in regulatory networks activated after cell envelope stress (Mascher *et al.*, 2007). Respective regulons are induced by, and provide resistance to, antibiotics and other substances that affect the cell surface. Although extensive global transcriptional analyzes have been performed under overexpression conditions of the remaining four ECF sigma factors (Asai *et al.*, 2003; Zellmeier *et al.*, 2005), the biological function of respective regulons is unclear at present. This lack of information possibly is due to the fact that ECF sigma factors of *B. subtilis* are not essential under standard laboratory growth conditions (Asai *et al.*, 2008).

The *B. subtilis* σ^W-regulon and its induction are best understood. It consists of about 30 transcriptional units that were defined by different strategies.

First, the sequence of the autoregulated promoter of the gene encoding σ^W, *sigW*, was used in bioinformatic approaches to search for similar sequences in the 5' regions of *B. subtilis* genes (Huang *et al.*, 1999). Second, it was shown that alkaline shock specifically induces σ^W-controlled genes, and the regulon could be extended. However, there is obviously no involvement of σ^W in pH homeostasis (Wiegert *et al.*, 2001). Third, a method was developed by the group of John Helmann, where *in vitro* transcripts generated by σ^W-charged RNA-polymerase were used for DNA-array analysis [ROMA; Run-off Transcription / Macroarray Analysis; (Cao *et al.*, 2002a)].

About half of the σ^W-controlled gene products are membrane proteins of unknown function, some of which are related to transport and detoxification (Helmann, 2002). Recently it was proposed that these genes constitute an antibiosis regulon (Helmann, 2006). For example the *yqeZyqfAB* operon confers resistance to the lantibiotic sublancin, an antimicrobial peptide produced by *B. subtilis* strains itself. Likewise, *yfhLM* and *yknWXYZ* provide intrinsic immunity to the SdpC toxin that is involved in a cannibalism process upon entry of *B. subtilis* cells into sporulation (Butcher and Helmann, 2006). Moreover, the σ^W-controlled gene *fosB* is known to supply resistance to fosfomycin, an antibiotic produced by certain bacteria that disturbs peptidoglycan biosynthesis.

In addition to a slight activation at the early stationary growth phase, stress conditions like vancomycin treatment (Cao *et al.*, 2002b), addition of antimicrobial peptides to growing cultures (Pietiäinen *et al.*, 2005), phage infection and alkaline shock (Wiegert *et al.*, 2001), and the presence of excess NaCl (Petersohn *et al.*, 2001) induce the σ^W-regulon. In the absence of these inducing signals, σ^W is sequestered by the transmembrane anti-sigma factor RsiW [formerly named YbbM (Schöbel *et al.*, 2004)]. RsiW has a type II topology, with the cytoplasmic N-terminus responsible for sigma factor inhibition. Its gene is located immediately downstream of *sigW* in an autoregulated operon.

Comparable to *E. coli* RseA, it could be shown that RsiW-activity is controlled by RIP with the key step catalyzed by the S2P-family I-CLiP RasP [regulating anti-sigma factor protease, formerly known as YluC. (Schöbel *et al.*, 2004)]. To date, exclusively RsiW and FtsL are known substrates for RasP (Bramkamp *et al.*, 2006).

An initial site-1 cleavage step triggered by certain stresses like alkaline shock was also postulated for RIP of RsiW (Schöbel *et al.*, 2004), and the involvement of Clp peptidases in complete removal of cytoplasmic site-2 clipped RsiW was shown (Zellmeier *et al.*, 2006).

1.4 Scope of this thesis

Comparing the two known systems of induction of ECF sigma factor activity by RIP (*E. coli* σ^E and *B. subtilis* σ^W), there are striking similarities in the transmembrane signaling pathway. An extracytoplasmic signal triggers the inactivation of the cognate anti-sigma factor by a concerted proteolytic cascade in three cellular compartments, to transmit information and to elicit cellular responses. An initial cleavage event (site-1 cleavage) in the extracytoplasmic part of the anti-sigma factor prepares it to become a substrate of an I-CliP for site-2 cleavage. Finally, degradation of the residual anti-sigma domain within the cytosol by Clp peptidases liberates the ECF sigma factor to interact with the RNAP.

However, there are also apparent variations. A substantial difference to *E. coli* σ^E / RseA was discovered for the site-1 proteolysis step that renders RsiW a substrate for RasP. DegS homologs of *B. subtilis* could not be proven to participate in site-1 cleavage, and the postulated site-1 protease was unknown. Because both systems are induced by dissimilar stress signals, it can be assumed that molecular signals and the mechanism of site-1 cleavage are completely different.

Recent investigations on RIP in bacteria revealed a central role of this mechanism in processes related to pathogenesis (Makinoshima and Glickman, 2006). Therefore, the main issue of this thesis was to use *B. subtilis* and the σ^W / RsiW system as a Gram-positive model to analyze the mechanism of RIP in detail, and to establish themes and variations of this process.

First of all it was of major interest to identify and characterize the so far unknown site-1 protease. The basis of this work was a transposon-screen, by which several genes affecting RIP of RsiW were found. Chapter 2 describes the identification of the *ypdC* (*prsW)* gene as the site-1 protease (Heinrich and Wiegert, 2006). In chapter 3, site-1 cleavage of RsiW by PrsW is further characterized (Heinrich *et al.*, 2009). The involvement of other proteases downstream of site-1 is shown. Finally, the involvement of an ABC-transporter in site-2 proteolysis is presented in chapter 4 (Heinrich *et al*., 2008). Chapter 5 summarizes the relevance of RIP in transmembrane signaling in bacteria (Heinrich and Wiegert, 2009). Chapter 6 gives a short summary and discussion of the results of this thesis.

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Chapter 2

YpdC determines site-1 degradation in regulated intramembrane proteolysis of the RsiW anti-sigma factor of *Bacillus subtilis*

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SUMMARY

Genes of *Bacillus subtilis* controlled by the alternative ECF sigma factor σ^W constitute an antibiosis regulon. Its activity is modulated by RsiW, a transmembrane anti-sigma factor that sequesters and inactivates σ^W. Upon a stress signal, RsiW is degraded by a mechanism of regulated intramembrane proteolysis. To identify genes which influence RsiW degradation, a transposon screen with a reporter fusion of the green fluorescent protein to RsiW was performed. Among several gene loci identified, the *ypdC* (*prsW*) gene displayed a strong effect on RsiW stability. In a *ypdC* null mutant, induction of σ^W-controlled genes is abolished and site-1 proteolysis of RsiW is completely blocked. Transcriptional analysis revealed that *ypdC* is a monocistronic gene, and the defect of σ^W–induction of the null mutant was complemented by ectopically integrated *ypdC* under xylose control. Orthologs of YpdC can be found in a variety of different bacteria. Its membrane topology was analysed by alkaline phosphatase fusions, revealing that YpdC contains five transmembrane segments and two larger extracytoplasmic loops. In the first loop, two invariantly conserved glutamate residues can be found. In an *Escherichia coli* system, the cloned *ypdC* is the only determinant of efficient degradation of RsiW, however, YpdC does not display plain similarities to known proteases, suggesting that it either controls the activity of site-1 proteolysis of RsiW or represents a new type of protease.

Introduction

Alternative sigma-factors play an important role in differential gene expression of bacteria in response to environmental stresses or developmental changes. They are synthesized, activated, or stabilized by different mechanisms and replace the housekeeping sigma-factor in the RNA-polymerase complex to drive expression of a specific set of genes (Helmann and Moran JR, 2002). One subgroup of alternative sigma factors, displaying common sequence similarities, was named the extracytoplasmic function family (ECF), because many representatives control genes involved in cell envelope processes (Helmann, 2002; Lonetto *et al.*, 1994). There is increasing evidence that ECF sigma factors play an important role in cellular stress management with an impact on virulence of pathogenic bacteria (Bashyam and Hasnain, 2004; Raivio and Silhavy, 2001).

For *Escherichia coli* it has been shown that the activity of the ECF sigma factor σ^E is controlled by the transmembrane anti-sigma factor RseA. Upon severe heat stress or stress elicited by abnormal outer membrane proteins in the periplasm, RseA is degraded by a mechanism known as regulated intramembrane proteolysis (RIP) (Ades, 2004; Alba and Gross, 2004), where first the DegS protease is activated and cleaves RseA in its extracytoplasmic domain (site-1). Then, the site-1 product of RseA becomes a substrate of the intramembrane cleaving protease RseP that cuts in the transmembrane domain of site-1 clipped RseA (site-2). This cleavage event releases the sigma factor / anti-sigma factor complex from the membrane and uncovers a proteolytic tag at the C-terminus of site-2 clipped RseA that is recognized and degraded mainly by the cytoplasmic ClpXP protease in dependency on the SspB adaptor protein (Flynn *et al.*, 2004). In consequence, σ^E is liberated to interact with the RNA polymerase core enzyme.

In DNA array experiments, we noticed a specific induction of the *B. subtilis* ECF sigma factor σ^W when shifting the pH of the medium to 8.9 (Wiegert *et al.*, 2001). Further analysis showed that the σ^W anti-sigma factor RsiW is degraded in a RIP-like manner following alkaline stress, and RasP (YluC) could be identified as the site-2 intramembrane cleaving protease (Schöbel *et al.*, 2004). In addition, conserved alanine residues are found in the transmembrane segment of RsiW that constitute a cryptic

proteolytic tag, and active Clp peptidases are crucial for the induction of σ^W-controlled genes (Zellmeier *et al.*, submitted).

Despite the fact that RsiW and RseA display only minor sequence similarities, different signals elicit the stress response and different genes are controlled, the mechanism of σ^E and σ^W induction seems to be conserved. Therefore, it is conceivable that RIP of an anti-sigma factor is a general system for bacteria to control the activity of certain ECF sigma factors.

However, there seems to be an important difference in RIP of RseA and RsiW. The site-1 protease that triggers the first important step of the stress induced RsiW degradation cascade and that possibly senses the stress signal is unknown so far and one of the three DegS orthologs of *B. subtilis* is not involved (Schöbel *et al.*, 2004; our unpublishes results).

In the present study, we therefore designed a transposon screen using a reporter protein consisting of the green fluorescent protein (GFP) fused to the N-terminus of RsiW. Among several transposon insertions that stabilise the GFP-RsiW reporter, two mutants are of major interest. First, insertions in the *ecsAB* genes encoding an ABC transporter of unknown function impair degradation of RsiW and induction of σ^W. Second, knockout of the *ypdC* gene abolishes the site-1 degradation step of RsiW. The site-1 degradation of RsiW can be reconstituted by the cloned *ypdC* gene in a heterologous *E. coli* system, suggesting that YpdC either catalyses the site-1 degradation step or controls the activity of the site-1 protease.

Results

Design of a transposon screen to identify genes involved in degradation of RsiW

In a previous work we analysed the involvement of Clp proteases in the complete degradation of site-2 clipped RsiW using a translational fusion of the green fluorescent protein (GFP) to the N-terminal end of the RsiW anti-sigma factor (Zellmeier *et al.*, submitted) (Fig. 1A). We could observe that colonies of a *B. subtilis* strain that constitutively overproduce the RsiW-GFP fusion display a clearly reduced fluorescence when compared to respective strains with a deletion of the genes encoding the site-2 protease RasP or the peptidase ClpP (Fig. 1B). Therefore, the decreased fluorescence results from degradation of the GFP-RsiW fusion protein by intramembrane

proteolysis of the RsiW part, uncovering an SsrA-like proteolytic tag at the C-terminal end of the truncated fusion protein that is recognized mainly by ClpXP, which completely degrades the fusion protein.

In order to identify the site-1 protease or associated factors in intramembrane proteolysis of RsiW, we made use of the GFP-RsiW fusion as a reporter in a mutagenic screen with the mini-Tn10 transposon encoded on plasmid pIC333. Transposon mutagenesis was performed according to a published procedure (Servant *et al.*, 2005; Steinmetz and Richter, 1994b) and about 50,000 single colonies were screened for enhanced fluorescence (an example is shown in Fig. 1C). To test transposon mutants with enhanced fluorescence of the GFP-RsiW fusion for σ^W activity, chromosomal DNA of the about 50 clones that were obtained was isolated and transformed to *B. subtilis* TW51, which encodes a transcriptional fusion of the strong σ^W-controlled *yuaF* promoter to *lacZ*. In a wild type background, colonies of TW51 are blue when grown on X-Gal plates for 36 h at 37 °C, because of the induction of σ^W in the stationary growth phase (Huang *et al.*, 1998), whereas under conditions of stabilisation of RsiW (e.g., knockout of *rasP*) they remain white (Schöbel *et al.*, 2004). Transformants were selected for spectinomycin resistance. Chromosomal DNA of single colonies displaying a white phenotype on X-Gal plates was isolated and *Hind*III-digested for plasmid rescue.

Identification of genes affecting stability of RsiW

To ensure maximal variability of plasmids rescued, restriction analysis was performed and only plasmids exhibiting different restriction patterns were subjected to DNA sequencing. Genes identified are listed in Table 1. In summary, six gene loci were hit that influence fluorescence of the GFP-RsiW reporter and induction of the σ^W-controlled P_{yuaF}-*lacZ* transcriptional fusion.

The *clpX* gene encodes the ATP-binding subunit of the ClpXP protease that we could show to be involved in the complete degradation of site-2 clipped RsiW (Zellmeier *et al.*, submitted) and, therefore, shows the feasibility of the transposon screen to isolate genes involved in degradation of RsiW. Note that the *rasP* gene could not be found,

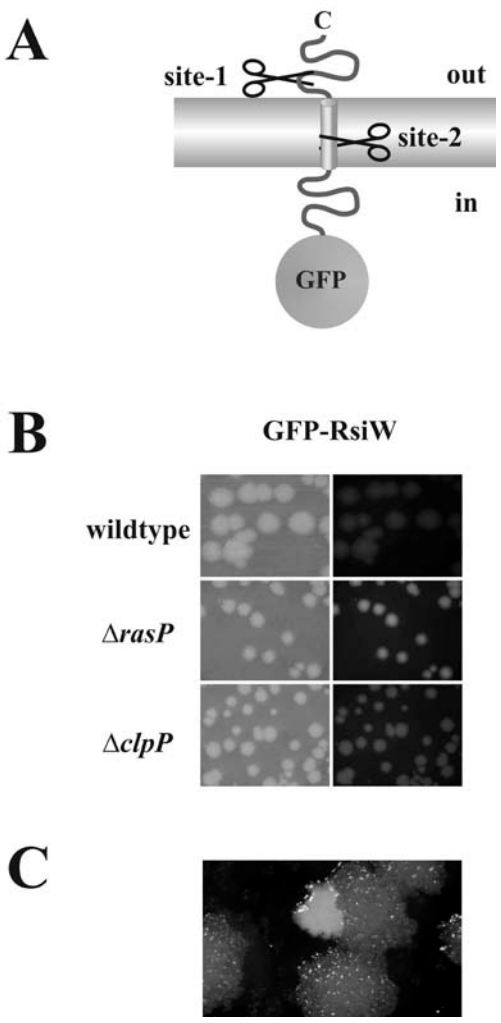


Fig. 1. Transposon screen to identify genes involved in degradation of RsiW. A. Schematic representation of a fusion of GFP to the N-terminus of the RsiW anti-sigma factor as a reporter for RIP. Site-1 degradation is performed by a protease unknown so far, site-2 proteolysis is catalysed by RasP (YluC). **B.** GFP fluorescence of *B. subtilis* colonies encoding the reporter fusion depicted in (A) in *rasP* and *clpP* minus background in comparison with the wild type (strain TW705). Photos on the left were taken under normal white light; on the right, under blue light (480 nm). The deletion strains show enhanced fluorescence due to the block in RsiW degradation. **C.** Example of a single colony that displays significantly higher GFP fluorescence than the majority of transposon mutants.

because it is placed in an operon with the essential genes *proS* (encoding prolyl-tRNA synthetase) and *polC* (encoding DNA polymerase III, alpha chain) downstream of it, which stresses limitations of a transposon screen using pIC333.

The genes *ecsA* and *ecsB* encode the ATP-binding protein subunit and the permease subunit of an ABC transporter with unknown function that is known to influence protein secretion, competence, sporulation and biofilm formation (Branda *et al.*, 2004; Leskela *et al.*, 1996; Leskela *et al.*, 1999; Pummi *et al.*, 2002). *ecsB* was hit twice at different positions. The gene *srfAA* is part of a huge operon that encodes an enzyme complex responsible for non-ribosomal synthesis of surfactin. In addition, the *comS* gene, involved in competence development, is encoded in that operon (Nakano *et al.*, 1991). *yorR* is an SP-β prophage gene of unknown function. *scoC* (also denoted as *hpr* or *catA*) belongs to the group of transition state regulators and was first described in a study of *B. subtilis* mutants overproducing alkaline and neutral proteases (Perego and Hoch, 1988). It has been shown that *scoC* plays a direct role in the initiation of sporulation by acting as a repressor of the two major

signaling peptide transport systems, *opp* and *app* (Koide *et al.*, 1999). *yorR*, *srfAA* and *scoC* transposon mutants display an intermediate phenotype with enhanced but not strong fluorescence and reduced but not zero β-galactosidase activity. The *ypdC* gene was hit at three different positions, where the last has occurred at the very end of the gene. It encodes a protein of unknown function with several putative transmembrane regions. The *ypdC* and *ecs* transposon mutants display the strongest phenotypes of enhanced GFP fluorescence and of absence of β-galactosidase activity of the P_{yuaF}-*lacZ* reporter fusion (Table 1), and the influence of the other genes on RsiW stability seems to be more indirect. In the present study we, therefore, present the detailed analysis of *ypdC*, whereas the influence of *ecsAB* on degradation of RsiW will be addressed elsewhere.

Deletion of ypdC *abolishes induction of* σ^W*-controlled genes*

First, a clean deletion / insertion mutant was constructed through replacement of *ypdC* by a phleomycin resistance gene.

The Δ*ypdC::bleo* construct was combined with the P_{yuaF}-*lacZ* reporter fusion and β-galactosidase activities of cultures with and without alkaline shock were determined in comparison to the wild type background strain. In the wild type background, β-galactosidase activity is induced by a factor of about 15 one hour after NaOH addition, whereas the *ypdC* minus strain displays no activity at all (Fig. 2A, columns 1 and 2).

In an independent experiment, the *B. subtilis* wild type strain 1012 was analysed in comparison to the isogenic *ypdC* minus strain and to an isogenic *sigW* knockout strain in Northern blots probed for signals of *ypdC* and *pbpE* (Fig. 2B). The *ypdC* transcript was detected at about 0.7 kb and was not induced upon NaOH addition to the medium, revealing that *ypdC* is a monocistronic gene not responding to alkali stress. The *ypdC* transcript was not detectable in the knockout strain.

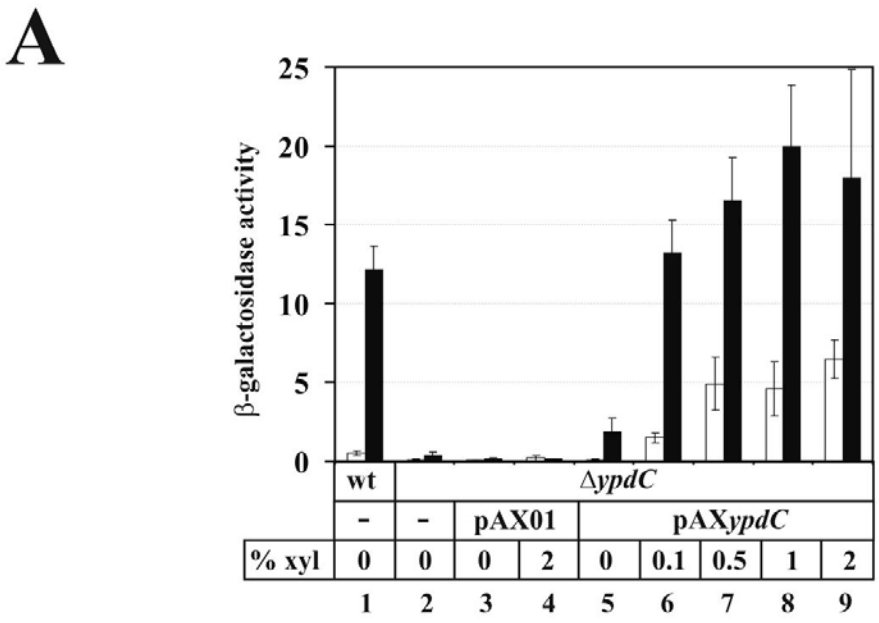
The σ^W-controlled *pbpE* gene was strongly induced by an alkaline shock in the wild type background, but neither in the *sigW* nor in the *ypdC* minus strains, confirming that the presence of *ypdC* is crucial for induction of σ^W-controlled genes.

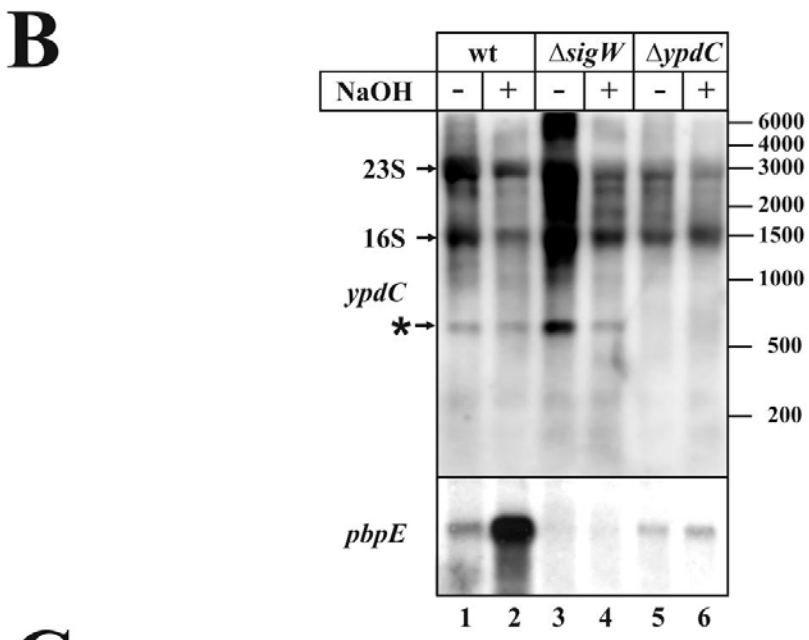
The *ypdC* gene was complemented ectopically under control of a xylose regulatable promoter. Alkali induction of the *lacZ* reporter fusion was restored in a xylose-dependent manner (Fig. 2A, columns 5-9).

TABLE 1. Identification of genes which affect fluorescence of GFP-RsiW and induction of a σ^W-controlled *lacZ* reporter

Mini-Tn10 insertion site[a]	Gene[b] and/or features	Presumed function	Fluorescence GFP-RsiW[c]	LacZ phenotype[d]
2884472	*clpX* (672)	ATP-dependent Clp protease ATP-) Binding subunit	++	-
1076988	*ecsA* (244)	ABC transporter (ATP-binding protein)	+++	-
1077774	*ecsB* (294)	ABC-2 type transport system permease protein	+++	-
1078451	*ecsB* (971)	"	+++	-
1072807	*scoC* (169)	transcriptional regulator for peptide transport and sporulation initiation (MarR family)	++	-/+
379703	*srfAA* (3179)	surfactin synthetase subunit 1	+	-/+
380611	*srfAA* (4087)	"	+	-/+
2173083	*yorR* (72)	SP-β protein, unknown	+	-/+
2399625	*ypdC* (437)	unknown; similar to unknown proteins	+++	-
2399582	*ypdC* (480)	"	+++	-
2399408	*ypdC* (654)	"	+++	-/+

[a] Position in the *B. subtilis* genome.
[b] Position of the Mini-Tn10 insertion site relative to the first nucleotide of the gene is given in parentheses.
[c] The intensity of GFP-RsiW fluorescence of colonies of each strain was monitored after growth on LB plates overnight and is indicated as follows: +++ (very strong, same intensity as *rasP* knockout) , ++ (strong) , + (less strong).
[d] Expression of the σ^W-controlled *yuaF* promoter in each strain was measured by growth on LB Xgal plates. Colony color was observed after 36 h of growth at 37°C and is indicated as follows: -/+ (light blue),- (white).


A
β-galactosidase activity
25
20
15
10
5
0
wt
ΔypdC
-
-
pAX01
pAXypdC
% xyl
0
0
0
2
0
0.1
0.5
1
2
1 2 3 4 5 6 7 8 9


B
wt
ΔsigW
ΔypdC
NaOH
- + - + - +
23S
16S
ypdC
*
pbpE
6000
4000
3000
2000
1500
1000
500
200
1 2 3 4 5 6

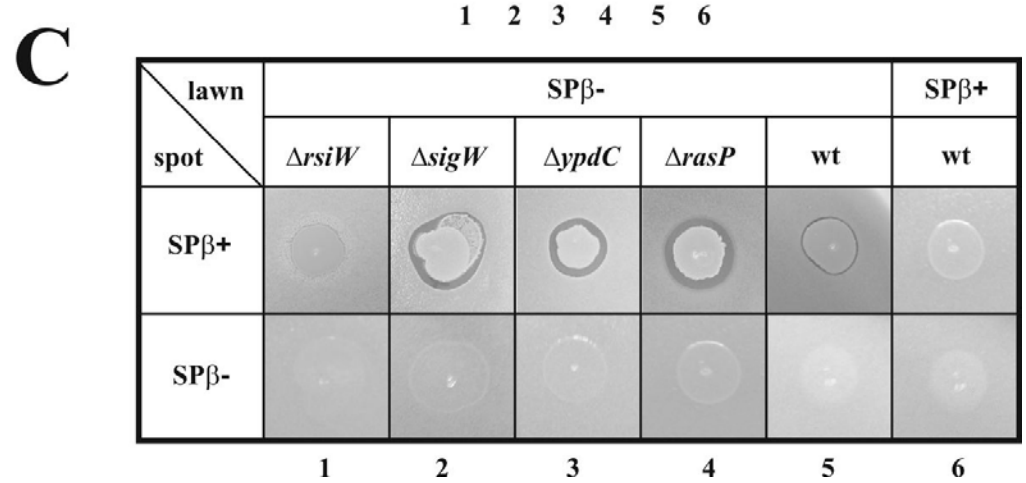

C
lawn
spot
SPβ-
SPβ+
ΔrsiW
ΔsigW
ΔypdC
ΔrasP
wt
wt
SPβ+
SPβ-
1 2 3 4 5 6

Fig. 2. Influence of *ypdC* on the expression of σ^W-controlled genes.
A. β-Galactosidase activities of *B. subtilis* reporter strains encoding a transcriptional fusion of the σ^W-controlled *yuaF* promoter to *lacZ*. NaOH was added to a final concentration of 24 mM at an OD_{578} of 0.7. Samples were taken 60 min after NaOH addition (solid bars) and at the same time point for unshocked cells (open bars). (1) TW30; wild type background. (2) JAH01; *ypdC::bleo*. (3, 4) JAH02; *ypdC::bleo, lacA::*pAX01. (5 – 9) JAH03; *ypdC::bleo, lacA::*pAX*ypdC*. Different concentrations of xylose were added to the cultures to induce expression of *ypdC* in strain JAH03 as indicated.
B. Northern blot of total RNA of *B. subtilis* 1012 wild type, 1012 *sigW::spec*, and 1012 *ypdC::bleo* cultured with and without alkaline treatment. Samples were taken 10 min after NaOH addition and at the same time point for the unshocked cells. Each lane was loaded with a sample of 2 µg of total RNA. Blots were developed with riboprobes against *ypdC* and the σ^W-controlled *pbpE* gene. The *ypdC* transcript is marked with a star. The position of 16S and 23S rRNA cross reacting with the *ypdC* riboprobe is also indicated. Lane 3 was overloaded due to improper dissolution of the RNA.
C. Spot-on-lawn assay to test for σ^W-dependent intrinsic immunity against the lantibiotic sublancin. *B. subtilis* 1A100 (SPβ-, lacks genes for sublancin production and resistance) and 1012 (SPβ+, sublancin producer) with different genetic backgrounds (wt: 1012 wild type, Δ*sigW*: 1012 *sigW::spec*, Δ*rsiW:* 1012 *rsiW::spec*, Δ*ypdC:* 1012 *ypdC::bleo,* Δ*rasP:* 1012 *rasP::tet*) were poured on LB agar as a lawn with low cell density. Wild type strains *B. subtilis* 1A100 and 1012 with a high cell-density were spotted on this lawn.

Most interestingly, β-galactosidase activities were significantly enhanced in cultures not alkali-stressed when the xylose concentration exceeded 0.1 %, suggesting that overproduction of YpdC activates σ^W under non-stress conditions. An isogenic strain without the cloned *ypdC* gene as a control displayed no alkali-induced β-galactosidase activity in presence and absence of xylose (Fig. 2A, columns 3 and 4). Alkali induction of σ^W is most probably artificial and there is no involvement of σ^W in pH homeostasis. Most recently, it was shown that σ^W is induced by antimicrobial peptides (Pietiäinen *et al.*, 2005) and that one of the true functions of the σ^W antibiosis regulon is the defence of antimicrobial peptides produced by other bacilli (Butcher and Helmann, 2006). To analyse whether functional YpdC is of importance to provide intrinsic immunity to the lantibiotic sublancin, we performed spot on lawn assays (Fig. 2C) as described previously (Butcher and Helmann, 2006). The sublancin synthesis and resistance genes are encoded on the SPβ prophage region, therefore a sublancin producer is resistant to the lantibiotic (Fig. 2C, upper part, 6). In contrast, intrinsic resistance of an SPβ-strain depends on σ^W-induction, as can be seen for the *sigW* null mutant (large halo) in comparison to the *rsiW* minus strain (faint halo) (Fig. 2C, uppper part 1 and 2). The *ypdC* knockout strain displays the same sensitivity as the *sigW* and the *rasP* knockout strains, visible by larger halos around the sublancin producing bacterial spot (Fig. 2C, upper part, 2, 3, and 4), corroborating that the YpdC function is of crucial importance for the biological function of σ^W.

YpdC affects site-1 proteolysis of RsiW

To investigate stabilisation of RsiW in the *ypdC* minus strain, localisation of the GFP-RsiW reporter fusion was analysed by confocal laser scanning microscopy and Western blotting of cell fractions. Confocal microscopy shows localisation of the reporter protein in the membrane, similar to the *rasP* minus strain. Therefore, site-1 or site-2 proteolysis of RsiW is impaired in the *ypdC* mutant, as a block in cytoplasmic degradation of site-2 clipped RsiW shows an even distribution of fluorescence in the cytoplasm, exemplified by a *clpP* knockout (Fig. 3A). Western blots of cell fractions showed that, compared to the wild type, an increased amount of GFP-RsiW can be detected in the membrane of the *ypdC* minus strain (Fig. 3B, compare lanes 3 and 6), whereas the

rasP knockout mainly accumulates site-1 clipped RsiW as described earlier (Schöbel *et al.*, 2004). A truncated form of the GFP reporter fusion lacking the extracytoplasmic part of RsiW (GFP-RsiWΔ1) is not stabilised in the *ypdC* minus strain, in contrast to the r*asP* knockout (Fig. 3B, lanes 15 and 18). RsiWΔ1 is a constitutive substrate for RasP, hence YpdC either directly catalyses site-1 proteolysis or is crucial for the sensing of the stress signal that finally triggers site-1 proteolysis, in the absence of which the RsiW anti-sigma factor can not be degraded. To ascertain that the stabilising effect of the *ypdC* minus strain is not restricted to the artificial GFP-RsiW fusion protein, a wild type and an *ypdC* minus strain with authentic chromosomally encoded RsiW was analysed by Western blotting (Fig. 3C). It is evident that also wild type RsiW is not degraded after alkaline shock in the *ypdC* minus background and that induction of the σ^W-controlled *pbpE* gene, encoding penicillin binding protein Pbp4*, is impaired (Fig. 3C, compare lanes 2 and 6). Furthermore, the site-1 degradation product that is prominent in the *rasP* knockout strain also without shock is not detectable in the *ypdC* and a *ypdC* / *rasP* double knockout strain (Fig. 3C, lanes 5-8).

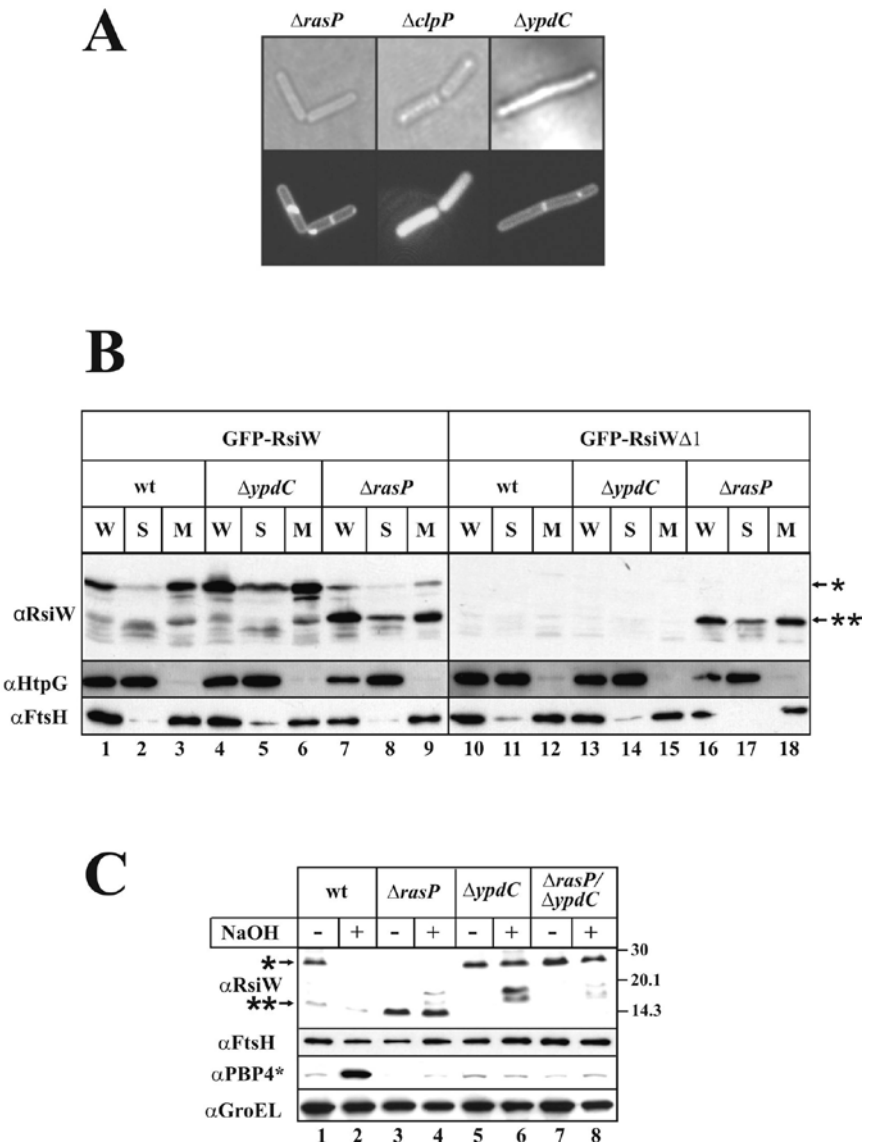


Fig. 3. YpdC influences site-1 proteolysis of the RsiW anti-sigma factor
A. Confocal laser scanning- and light-microscopy (lower and upper panels, respectively) of *B. subtilis* TW705 cells expressing the GFP-RsiW reporter fusion in *rasP*, *clpP*, and *ypdC* null mutants. **B.** Western blots of *B. subtilis* strains expressing reporter proteins of GFP fused to the amino terminus of RsiW (TW705) and RsiWΔ1 (TW706; extracytoplasmic amino acid residues 108-208 of RsiW missing) in wild type, *rasP*, and *ypdC* knockout background. Samples of cultures in the late exponential growth phase were withdrawn and cells were disrupted by sonication. To localize fusion proteins, whole cell extracts (W) were further fractionated to the membrane- (M) and soluble-protein fraction (S) by ultracentrifugation. Blots were developed with polyclonal antibodies against RsiW and, as a loading control, with polyclonal antibodies against FtsH and HtpG. GFP-RsiW is marked with a star, truncated GFP-RsiW with two stars.
C. Western blot analysis of *B. subtilis* strain 1012 wild type, 1012 *rasP::tet*, 1012 *ypdC::bleo*, and 1012 *rasp::tet ypdC::bleo*. Cells were alkali- shocked as described in the legend of Fig. 2, and samples were withdrawn 10 min after the shock. Blots of membrane fractions were treated with antibodies against RsiW and FtsH (loading control), blots of soluble fractions were developed with antibodies against Pbp4* and GroEL (loading control).

YpdC is the only determinant of site-1 proteolysis of RsiW in an E. coli *system*

During overproduction of RsiW as an N-terminally His-tagged protein for purification and antibody production, it became evident that the protein is rather stable in the heterologous host *E. coli* (Schöbel et al., 2004; compare Fig. 4A, lanes 1 and 2). To test whether the cloned *ypdC* gene influences stability of the anti-sigma factor in *E. coli*, a two plasmid system was designed with the gene encoding His_6-RsiW constitutively expressed from a pBR322-derived plasmid and the *ypdC* gene under IPTG control on compatible plasmid pHSG575. Western blot experiments clearly showed that induction of *ypdC* upon IPTG addition results in accumulation of a truncated form of RsiW in *E. coli*. The truncated form migrates at a similar size compared to the site-1 clipped form produced in a *B. subtilis rasP* mutant expressing His6-RsiW (Fig. 4A, lanes 4 and 5). Pulse-chase experiments were performed with the *E. coli* system. Instead of immunoprecipitation, NiNTA was used to enrich His_6-RsiW for SDS-PAGE and fluorography.

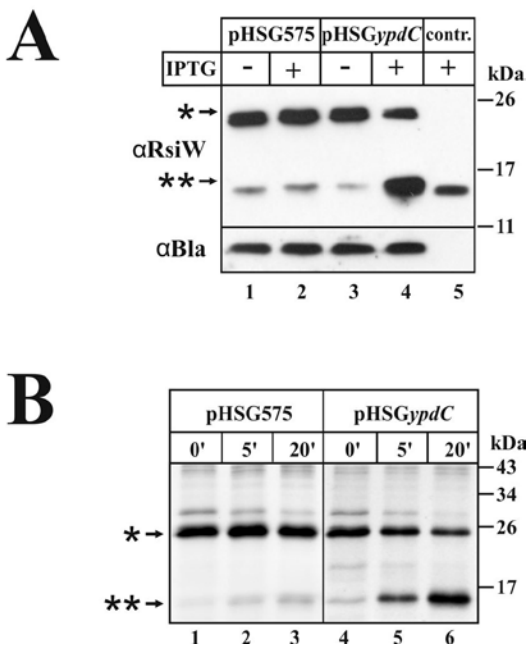


Fig. 4. YpdC causes degradation of RsiW in *E. coli*
A. *E. coli* XL1blue / pMA01 constitutively expressing His_6-tagged RsiW containing either the empty vector pHSG575 or the plasmid pHSG*ypdC* with the cloned *ypdC* gene under IPTG control were grown at 37 °C to stationary growth phase in the presence or absence of 1 mM IPTG. Samples were withdrawn and subjected to SDS-PAGE and Western blotting. Antibodies against RsiW and the *E. coli* β-lactamase (Bla) were used. As a control, a sample of the membrane fraction of *B. subtilis* JAH05, expressing His_6-RsiW in the *rasP* minus background, was loaded to the same gel. His_6-RsiW is marked with a star, truncated His_6-RsiW with two stars.
B. *E. coli* strains described above were grown in minimal medium in the presence of 1 mM IPTG and analysed in a pulse chase experiment with chase times as indicated. For details, see the Material and Methods section. His_6-RsiW is marked with a star, truncated His_6-RsiW with two stars.

It became obvious that only under conditions of *ypdC* expression His_6-RsiW was significantly converted to a C-terminally truncated form (Fig. 4B) that remains membrane bound (data not shown).

Characterisation of YpdC

The above results suggest YpdC being the site-1 protease in RIP of RsiW. In data base searches using the BLAST algorithm, genes with homology to *ypdC* can be found in a variety of different Gram-positive and-negative bacteria, as well as in some archeae. Patterns of highly conserved amino acid residues can be found, which are most likely crucial for YpdC function. YpdC is a member of the COG2339 cluster of orthologous groups with 14 representatives (for an alignment see http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=COG2339) and more than 200 proteins related to COG2339 are listed. However, for all these genes no function has been assigned so far and we were not able to detect similarities to known proteases. Assuming YpdC represents a new type of protease, conserved amino acid residues of the active site should be placed in the extracytoplasm. Topology prediction of YpdC using different programs did not give clear results. Depending on the program, one to six transmembrane regions are proposed. We therefore analysed YpdC topology by fusing different proportions of it to *E. coli* alkaline phosphatase that displays activity only in the oxidizing extracytoplasmic environment. We were not able to clone fusions in a system that we previously used for *rsiW* (Schöbel *et al.*, 2004), most probably due to the toxic effect of *ypdC* when expressed in *E. coli*. With a pMUTIN based system allowing direct fusion of *phoA* to *ypdC* in the *B. subtilis* chromosome under control of the authentical *ypdC* promoter, low but reproducible PhoA activities were measured with fusions after amino acid residues at position 90 and 160, whereas at position 25, 125, and 218 activities did not exceed background (Fig. 5). These fusions favour a topology proposed by the SOUSI algorithm (Hirokawa *et al.*, 1998) with two larger extracytoplasmic loops (Fig. 5). To further investigate YpdC membrane topology, we used a random PhoA fusion system in *E. coli*. Five active (at position 57, 66, 75, 104, and 175) and two inactive (at position 26 and 128) PhoA fusions were obtained, the position of which fully corroborate the topology depicted in Fig. 5.

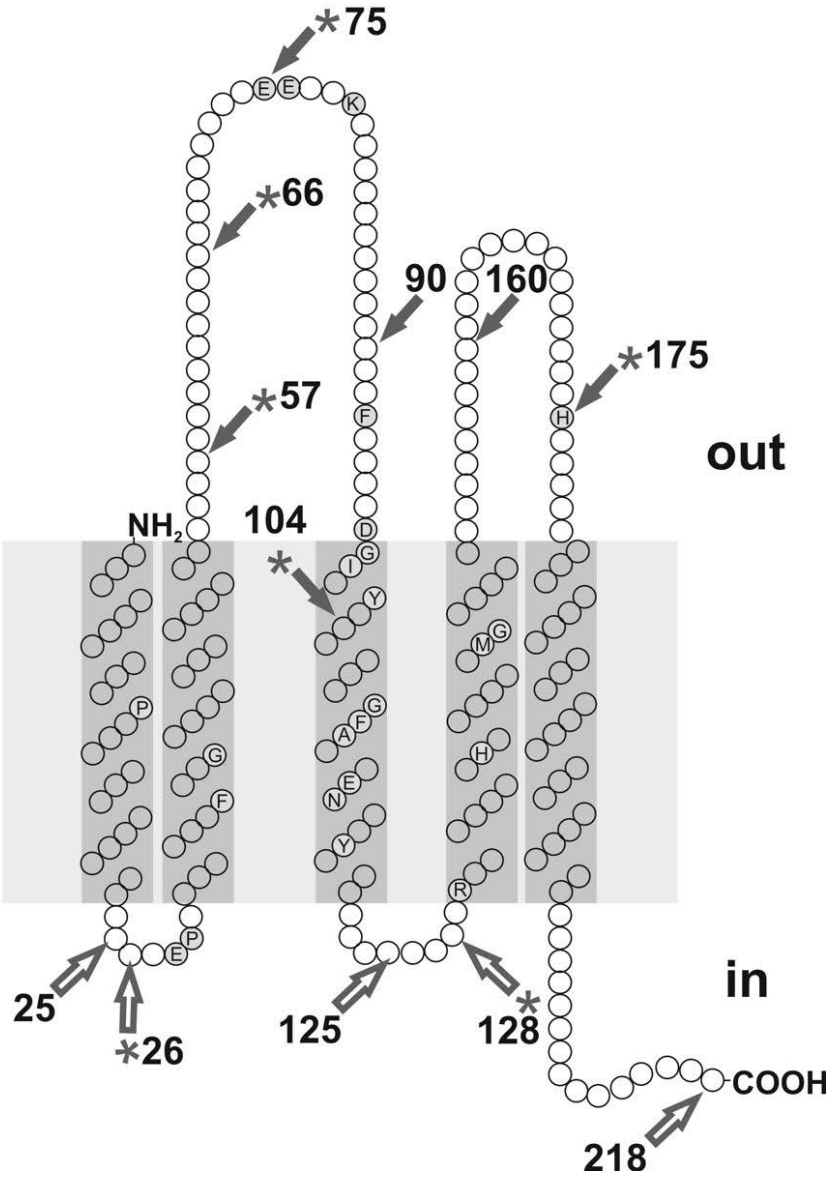


Fig. 5. Schematic representation of the YpdC topology.
Transmembrane domains predicted by the SOUSI program (Hirokawa *et al.*, 1998) are depicted. Fusion joints to the *E. coli* alkaline phosphatase that were constructed in a *B. subtilis* system (arrows) and fusions randomly generated by exonuclease III digestion of the *ypdC* 3' end in an *E. coli* system (arrows with a star) are indicated. Solid arrow: high phosphatase activity, open arrow: low phosphatase activity. Conserved residues in YpdC are denoted. Alkaline phosphatase activities for the *B. subtilis* system were: 90: 80 mU; 160: 70 mU; 25, 125, 218: 0 mU (background activity of 1012 wild type). For the *E. coli* system activities were: 26: 9 U; 57: 63 U; 66: 255 U; 75: 112 U; 104: 82 U; 128: 5 U; 175: 42U.

According to this model, two highly conserved glutamate residues (E75, E76) can be found in a large extracytoplasmic loop, as well as a lysine (K79) and phenylalanine (F93) residue. In a second extracytoplasmic loop, a highly conserved histidine (H175) residue is present. Future experiments have to show whether these residues are of importance for site-1 proteolysis of RsiW.

Discussion

The σ^W regulon controls expression of about 60 genes, most of unknown function and encoding membrane proteins. Bioinformatic as well as experimental analyses proposed that it constitutes an antibiosis regulon that both produces and defends antimicrobial compounds (Cao *et al.*, 2001; Cao *et al.*, 2002b; Cao *et al.*, 2002a; Huang *et al.*, 1999; Turner and Helmann, 2000).

Alkaline stress, phage SPP1 infection (Wiegert *et al.*, 2001), the absence of the FtsH protease (Zellmeier *et al.*, 2003), salt shock (Petersohn *et al.*, 2001), as well as antibiotics like vancomycin (Cao *et al.*, 2002b) and antimicrobial peptides active on the cell envelope (Pietiäinen *et al.*, 2005) have been described to induce the σ^W regulon. The true biological function remained elusive as there was no inducing compound or stress condition known against which σ^W-controlled genes confer resistance. However, most recently, it was shown that the σ^W regulon plays an important role in providing intrinsic resistance against peptide antibiotics produced by competing *Bacillus* strains, and five σ^W-dependent operons that supply resistance to at least four different antimicrobial compounds were identified (Butcher and Helmann, 2006).

The molecular stress signal that elicits the first degradation step of RsiW and the postulated site-1 protease are unknown so far, but it is conceivable that antimicrobial peptides itself trigger activation of the site-1 proteolytic step, in the same manner as non-native outer membrane proteins activate the DegS protease in stress-induced degradation of *E. coli* RseA (Kanehara *et al.*, 2003; Walsh *et al.*, 2003).

In our transposon screen to identify genes involved in RsiW degradation we found the *ypdC* gene having a strong influence on RsiW stability. Further analysis clearly showed that YpdC is a membrane protein involved in site-1 degradation of RsiW. In the absence of *ypdC*, the full length RsiW is stabilised in the membrane and also not degraded

upon challenge to alkali shock. In the *ypdC* knockout, σ^W-controlled genes are not induced and the intrinsic resistance of *B. subtilis* cells to antimicrobial peptides like sublancin is abolished. However, it is unclear whether YpdC constitutes the site-1 protease itself or is an associated factor, possibly a sensor, that is crucial for activating the site-1 proteolytic step *in trans*. The main argument against YpdC being a protease is the fact that standard BLAST searches did not reveal plain homology to known proteases, and we could only find limited similarities to a rhomboid like protease of *Anopheles gambiae* (gi:58376061) by blasting the MEROPS peptidase database (http://merops.sanger.ac.uk).

The eukaryotic S1P protein is the paradigm of site-1 proteases. It is a subtilisin-like serine protease bound to the membrane of the endoplasmatic reticulum (ER) via a transmembrane segment, the active site facing the ER lumen. It cleaves the Sterol Regulatory Element-Binding Protein (SREBP) which is a membrane-bound transcription factor that controls the synthesis and uptake of cholesterol and fatty acids in animal cells (Brown and Goldstein, 1999). For bacteria, two examples of proteases active in a site-1 proteolytic step on one substrate are described so far. First, DegS is a membrane-anchored serine protease cleaving the RseA anti-sigma factor facing the periplasm of *E. coli*. Its activity is modulated by its own PDZ domain (Walsh *et al.*, 2003). Second, PerP, an aspartic protease that is also amino-terminally anchored to the membrane of *Caulobacter crecentus* degrades $PodJ_L$, a factor responsible for recruiting proteins involved in polar organelle biosynthesis. PerP itself is transcriptionally regulated (Chen *et al.*, 2006). For *B. subtilis*, a RIP mechanism processes and activates pro-σ^K, involving the intramembrane cleaving protease SpoIVFB. Here, proteolytic steps comparable to a site-1 mechanism are crucial to activate site-2 cleavage, i.e. proteins that are in a complex with SpoIVFB (SpoIVFA and BofA) and inhibit its activity are degraded. First, the SpoIVB protease cleaves SpoIVFA at its C-terminus, which then makes BofA susceptible to degradation by CtpB (Zhou and Kroos, 2005). Taken together, the data for bacterial proteases involved in site-1 cleavage events show that, despite the fact that related Zn-dependent intramembrane cleaving proteases catalyse the site-2 degradation, there seems to be a greater variability for site-1 proteases.

Our experimental data can be interpreted in favour of a site-1 proteolytic activity of YdpC. Efficient site-1 cleavage of RsiW can be reconstituted in an *E. coli* system when the *ypdC* gene is expressed upon IPTG addition. This cleavage is independent of a stress signal like alkaline pH of the medium (data not shown). These data either suggest that YpdC sensitizes RsiW to cleavage by an intrinsic *E. coli* protease, or that YpdC itself is catalysing site-1 proteolysis. *E. coli* does not encode a YpdC homolog and it is unlikely that such a sophisticated mechanism of proteolytic cleavage of RsiW, true orthologs of which are not encoded by the phylogenetically distant *E. coli*, can be perfomed by another intrinsic protease. However, it can not be excluded that YpdC function is indirect, and in fact slow degradation of RsiW to a site-1 cleaved form is visible in pulse chase experiments with the *E. coli* system (Fig. 4B, lanes 1-3).

As it could be observed earlier, site-1 proteolysis is deregulated in the absence of RasP, because the full length RsiW is hardly detectable in membrane fractions of a *rasP* knockout strain and the site-1 proteolysis product accumulates even in the absence of a stress signal (Schöbel *et al.,* 2004), suggesting that RasP is an inhibitor of site-1 proteolysis. This uncontrolled degradation of RsiW in the absence of RasP is abrogated in a *rasP / ypdC* double knockout (Fig. 3C), again favouring YpdC being the site-1 protease. It is an intriguing question, whether an interaction of RasP to YpdC and / or RsiW abolishes site-1 cleavage of RsiW under non-stress conditions. Further experiments, most desirable *in vitro*, have to show whether YpdC acts directly in site-1 proteolysis of RsiW. It also has to be shown; whether the conserved residues in the extracytoplasmic loops of YpdC are indeed invariant residues of a catalytic center, keeping in mind that the site-1 proteolytic process takes place in the extracytoplasmic part of RsiW close to the transmembrane domain. Given the fact that orthologs of YpdC can be found in a great variety of bacteria, including pathogens like *Staphylococcus aureus*, it will be a great challenge to further investigate the role of YpdC proteins in bacteria.

After we had submitted the present manuscript a paper by Ellermeier and Losick was published, where they identified *ypdC* coincidentally by a totally different approach being involved in site-1 proteolysis of RsiW (Ellermeier and Losick, 2006). In summary, they isolated *B. subtilis* clones suppressing the toxic effect of the

SdpC toxin that partly mediates killing in the cannibalism process in entering sporulation. Among others, suppressors were mapped in the *ypdC* gene, and they could show that suppression was due to constitutive activation of the σ^W regulon, which is known to confer resistance to the SdpC toxin in cells lacking the SdpI immunity protein (Butcher and Helmann, 2006). In contrast to the suppressor mutation in *ypdC* (E95K) that constitutively activates σ^W, they show, in accordance to our results, that a knockout of *ypdC* and also mutation of the conserved glutamate residues at position 75 and 76 to alanine abolishes site-1 proteolysis of RsiW.

On the basis of the HHpred algorithm they find similarities of YpdC to the COG1266 group with the yeast RCE1 protein as a CAAX prenyl endopeptidase as protease. Hence they propose YpdC to be a novel site-1 protease and rename it PrsW (protease responsible for activating σ^W). However, as the only direct evidence for PrsW being a protease is reconstitution of RsiW site-1 proteolysis in *E. coli* in our present work and by the work of Ellermeier and Losick, future experiments have to address proteolytic activity of PrsW directly.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Bacterial strains as well as plasmids used in this study are listed in Table 2. *E. coli* and *B. subtilis* strains were grown aerobically at 37 °C in Luria Broth (LB) or in mineral salts medium (Tanaka *et al.*, 1967) with 0.4% glycerol as a carbon source for pulse chase experiments. When necessary, LB was supplemented with ampicillin (100 µg/ml), phleomycin (1µg/ml), neomycin (10 µg/ml), spectinomycin (100 µg/ml), chloramphenicol (10 µg/ml), tetracycline (10 µg/ml) or erythromycin (1 µg/ml or 100 µg/ml).

If required, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), X-P (5-bromo-4-chloro-3-indolyl-Phosphat) together with 1 mM K-phosphate, 1mM IPTG (isopropyl- β-D-thiogalacto-pyranoside), NaOH, or xylose in varying concentrations were added to solid or liquid media.

DNA manipulation and sequencing

DNA manipulation was performed according to standard procedures (Sambrook and Russell, 2005). For PCR reactions, high-fidelity Deep $Vent_R$ DNA Polymerase (New England Biolabs, Inc.)

was used, for colony-PCR the Taq-DNA Polymerase. Standard DNA sequencing was carried out by a company (Seqlab, Göttingen, Germany) with appropriate sequencing primers.

Transposon mutagenesis, detection of GFP fluorescence and confocal laser scanning microscopy

To isolate new genes involved in proteolysis of RsiW, transposon mutagenesis was performed with plasmid pIC333 as previously described (Steinmetz and Richter, 1994b). As a reporter, *B. subtilis* TW705 was used for screening for high fluorescent mutants on LB-plates containing a spectinomycin concentration of 100 µg/ml. Chromosomal DNA of positive clones was isolated and transformed to strain TW51 with selection on X-Gal plates containing neomycin (10 µg/ml). Transformants were screened for chloramphenicol sensitivity and a white colony phenotype. Again, chromosomal DNA of positive clones was isolated, retransformed to TW705 and checked for enhanced fluorescence For plasmid rescue, 10 µg of the chromosomal DNA was digested with *Hind*III, ligated and transformed to *E. coli* DH10B with selection for spectinomycin resistance. Plasmids were analysed by restriction analysis and the transposon integration sites were identified by DNA-sequencing using primer: 5´-CCA ATACGCAAACCGCCTCTC-3´.

To localise GFP fusion proteins *in vivo*, either the Leica (Heidelberg, Germany) MZFLIII microscope or the TCS/SP confocal scanning laser microscope equipped with an argon-ion laser for excitation at 488 nm were used. Detection occurred at 510 nm, and data were collected with fourfold averaging. Preparation of samples was performed according to a published procedure (Wehrl *et al.*, 2000).

Construction of a B. subtilis ypdC *minus strain*

The *ypdC* gene was inactivated by a deletion and insertion of a phleomycin resistance gene. To delete *ypdC*, the chromosomal 5´ flanking region was PCR amplified using primers 5´-GGCCATCTGCAGAGATGATTG CAAACATCAGCAAC-3´ and 5´ GGC CATGAGCTCGATTCTGCCGGAATT TGAAGCTCT-3´ (1) and chromosomal DNA of *B.subtilis* 1012 as a template. The PCR product was cloned into pBlueCATII (Versteeg *et al.*, 1999) between *Pst*I and *Sac*I sites.

TABLE 2: Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80dlacZ ΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*) 7697 *gal*U *gal*K λ⁻ *rps*L *nup*G	(Grant *et al.*, 1990)
XL1-Blue	F'::Tn10 *proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96*(Nal^r) *thi hsdR17* ($r_K^-m_K^+$) *glnV44 relA1 lac*	Stratagene
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	(Saito *et al.*, 1979)
1A100	*trpC2* , sensitive to SPβ (also: *CU1065*)	(Zahler *et al.*, 1977)
TW30	*1012 amyE::*P_{yuaF} *– lacZ, lacA::spec* (Neo^R, Spec^R)	(Schöbel *et al.*, 2004)
TW51	*1012 amyE::*P_{yuaF} *– lacZ* (Neo^R)	(Zellmeier *et al.*,2003)
JAH01	TW30 *ypdC::bleo* (Neo^R, Bleo^R)	This work
JAH02	TW30 *ypdC::bleo, lacA::pAX01* (Neo^R, Bleo^R, Em^R)	This work
JAH03	TW30 *ypdC::bleo, lacA::pAXypdC* (Neo^R, Bleo^R, Em^R)	This work
JAH04	*1012 rasP::tet, ypdC::bleo* (Bleo^R, Tet^R)	This work
JAH05	TW30 *rsiW::spec, rasP::tet, lacA*::pAL-His$_6$-*rsiW* (Spec^R, Tet^R, Em^R, Neo^R)	This work
1012 *rasP::tet*	*1012 yluC::tet* (Tet^R)	(Schöbel *et al.*, 2004)
1012 *ypdC::bleo*	*1012 ypdC::bleo* (Bleo^R)	This work
1012 *rsiW::spec*	1012 with *spec*- insertion in *rsiW* (Spec^R)	(Schöbel *et al*., 2004)
1012 *sigW::spec*	1012 with *spec*- insertion in *sigW-rsiW* (Spec^R)	(Schöbel *et al*., 2004)
TW705	*1012 amyE::gfp rsiW* (Cm^R)	(Zellmeier, submitted)
TW706	*1012 amyE::gfp rsiW Δ1* (Cm^R)	(Zellmeier, submitted)
Plasmids		
	pIC333 mini- Tn10 transposon delivery vector (Spec^R, Em^R)	(Steinmetz and Richter, 1994b)
pBlueCATII	cat-cassette between flanking cloning sites (Amp^R, Cat^R)	(Versteeg *et al.*, 1999)
pJH02	*ypdC* up- and down-stream regions flanking cat- of pBluCATII	This work
pJH03	pJH03, *cat* exchanged for *bleo*-resistance	This work
pAL01	Plasmid with IPTG regulatable promoter for integration into the *B. subtilis lacA* locus	(Schöbel *et al.*, 2004)
pJH05	pAL01 with cloned His$_6$*rsiW*	This work
pMA01	pBR322 standard vector with cloned His$_6$*rsiW*	This work
pAX01	Plasmid with xylose regulatable promoter for integration into the *B. subtilis lacA* locus	(Härtl *et al.*, 2001)
pAX-*ypdC*	Plasmid for integration of inducible *ypdC* in the *lacA*-locus	This work
pHSG575	pSC101 ori and IPTG regulatable promoter	(Takeshita et al.,1987)
pHSG-*ypdC*	pHSG575 with *ypdC* under IPTG control	This work
pMUTIN*phoA*	pMUTIN derived plasmid with cloned *phoA*, similar to pMUTIN-GFP$^+$ (Kaltwasser *et al.*, 2002)	(Schumann, unpublished)
pMUTIN-*ypdC-phoA*	pMUTIN*phoA* with cloned fragments of 6x His-tagged- *ypdC* (pMutin *phoA-ypdC*-Y25,-Y90, -V125,-A160 and-V218)	This work
pQE30	expression vector for overproduction of proteins in *E. coli* (Amp^R)	Qiagen
pQE30-*ypdC*	pQE30 with cloned *ypdC* gene	This work
pQE30-*phoA-ypdC*	pQE30-*ypdC* with cloned *phoA* downstream of *ypdC*	This work

The resulting plasmid was cut with *Hind*III and *Sal*I and ligated to the 3´ flanking region of *ypdC* that was PCR amplified using primers 5´-GGCCATAAGCTTAAGCCGCAC AGCAACCGTGC-3´ and 5´-GGCCAT GTCGACGAGCTGTTCCATGACATA TTCACG-3´ (2) and digested with with *Hind*III and *Sal*I, yielding plasmid pJH02.

PCR- amplification of the phleomycin-resistance gene using primers 5´-GGCCATGAATTCACATATGAGCGA ATTGAATTTATAATA-3´ and 5´-GGC CATAAGCTTTGAACAGATTAATAA TAGATTTTAGCT-3´ with plasmid pER::Pm (Steinmetz and Richter, 1994a) as a template, and replacement of the cat-cassette of pJH02 via restriction with *EcoR*I / *Hind*III and ligation of the isolated vector fragment with the digested PCR- product of *bleo* resulted in plasmid pJH03. After ligation, all constructed plasmids were transformed to *E. coli* DH10B and checked via colony-PCR and restriction analysis. The *B. subtilis ypdC* minus strain was constructed via PCR-amplification of *ypdC::bleo* with the flanking regions using primers (1), (2) and pJH03 as a template, and the product was transformed into *B. subtilis* TW30. Chromosomal DNA of transformants resistant to phleomycin and neomycin was analysed by Southern blot analysis with a DIG- labelled DNA probe against *ypdC*, yielding strain JAH01. For construction of either *ypdC::bleo* or double- mutant strains (e.g. *B. subtilis* 1012 *ypdC::bleo*) for different experiments, chromosomal DNA of JAH01 *rasP::tet* was transformed to respective strains.

Complementation of ypdC

Plasmid pAX01 was used for construction of pAX-*ypdC*. To amplify *ypdC* via PCR with chromosomal DNA of *B.subtilis* 1012 as a template, primers 5´-GGCCATGGATCCATGTTTGCAA TCATCTCTGCAGG-3´ (3) and 5´-GGCCATGGATCCGATATCCTATAC TTGCATCATATTAACGGA-3´ (4) were used. The PCR product was restricted with *BamH*I and ligated to *BamH*I digested and dephosphorylated pAX01. The orientation of the integration was confirmed by restriction analysis of pAX01-*ypdC* with *EcoR*V and sequencing with the primer 5´-TTTGAAGCTTGAATTAGATATTTA AAA-3´, yielding plasmid pAX01-*ypdC*. For complementation experiments, different strains were constructed, starting from strain *B. subtilis* TW30, encoding a P_{yuaF}-*lacZ* reporter fusion and the spectinomycin resistance gene in

lacA (Schöbel *et al.*, 2004). In the first step, chromosomal DNA of JAH01 was transformed to *B. subtilis* TW30. Phleomycin-resistant cells of the resulting strain TW30*ypdC* were transformed with either plasmid pAX-*ypdC* or pAX01 as a negative control. Transformants were plated on LB containing erythromycin with a concentration of 1 µg/ml and screened for phleomycin- (1 µg/ml), erythromycin- (100 µg/ml), and neomycin- (10 µg/ml) resistance and spectinomycin-sensitivity, yielding strains JAH02 and JAH03.

Spot- on lawn Assay

For competition experiments, *B. subtilis* 1A100 (Spβ-) which is sensitive to sublancin and *B. subtilis* 1012 (Spβ+; produces sublancin) were used with varying genetic backgrounds. The *sigW* and the *rsiW* genes were deleted as controls. The assay was performed in accordance with a recently described protocol (Butcher and Helmann, 2006). Strains were grown to an OD_{578} ~ 0.6 and 2 µl of each were spotted on 1.5% LB agar plates with a bacterial lawn.

NaOH- shock experiments and preparation of B. subtilis *cell fractions*

NaOH- shock experiments were performed as previously described (Schöbel *et al.*, 2004). Cells were harvested by centrifugation, washed and suspended in 1 ml of cold disruption buffer (50 mM Tris/HCl, 100 mM NaCl, pH 7.5) containing the Complete protease inhibtor cocktail (Roche). Samples were adjusted to the same OD_{578} by dilution with cold disruption buffer. 1 ml of cell suspensions were sonicated (Cell Disrupter B15, Branson) on ice. 100 µl were removed (whole cell fraction, W) and the remaining 900 µl were centrifuged at 5000 x g for 15 min at 4 °C to remove cell debris. Then, 800 µl of the supernatant was ultracentrifuged at 45000 x g for 1 h at 4 °C. The supernatant (soluble fraction, S) was removed and the resulting membrane pellet (membrane fraction, M) was washed with 500 µl of disruption buffer, ultracentrifuged again (45000 x g, 0.5 h, 4 °C), dissolved in 100 µl of Laemmli buffer and heated for 5 min at 95 °C. The protein content of the W and S fractions was estimated according to Bradford, and 10 µg of total protein was loaded on each lane for SDS-PAGE and Western blotting. A volume equivalent to the S fraction was loaded for the M

fraction, i.e. 1/8 of the volume of the S fraction containing 10 µg soluble protein.

Enzymatic assays, Northern blot, and Western blot analysis

Activities of β-galactosidase and alkaline phosphatase were measured as described elsewhere (Schöbel *et al.*, 2004). Northern blot analyses were performed according to a published procedure (Homuth *et al.*, 1997) with riboprobes against *ypdC* and the σ^W-dependent gene *pbpE* as an internal control for activation of the σ^W regulon. Western blots were performed as described previously (Homuth *et al.*, 1996) using a semi-dry blotting procedure (Biorad, Trans-Blot-SD). Blots were developed with polyclonal antibodies against either GFP (Clontech) or RsiW, FtsH, HtpG, Pbp4*, Bla and GroEL.

Pulse-chase experiments

Pulse-chase experiments were carried out as previously published (Blaudeck *et al.*, 2001). Instead of immunoprecipitation, His_6-tagged RsiW was enriched using 100 µl of a NiNTA suspension according to a standard purification protocol (Qiagen, Hilden, Germany).

Analysis of YpdC *function in an* E.coli *system*

For testing a possible site-1 proteolytic function of YpdC in *E.coli* a two-plasmid system was established including construction of pMA01 and pHSG-*ypdC*. Plasmid pMA01 which encodes the N-terminally His_6-tagged RsiW under control of a constitutive promoter was cloned by amplifying His_6*rsiW* from Plasmid pQE30*rsiW* (Schöbel *et al.*, 2004) with primers 5'-GGCCATGAGCTCAAAGAGGAGAA ATTAACTATGAGAG-3' and 5'-GGCCATGCATGCGAGCAAAGCGG CTAACGCGCT-3' (5), restriction of the product with *Ecl*136II / *Sph*I and ligation to the *Eco*RV / *Sph*I restricted vector fragment of plasmid pBR322. The *ypdC* gene was cloned to plasmid pHSG575 (Takeshita *et al*., 1987), which contains the pSC101 origin of replication and allows IPTG regulatable expression. *ypdC* was PCR amplified using primers (3) and (4) and chromosomal DNA of *B. subtilis* 1012 as a template, restricted with *BamH*I and ligated to the *BamH*I digested and dephosphorylated pHSG575. The right orientation was checked by restriction analysis, yielding pHSG-*ypdC*. As a *B. subtilis* control encoding wild type *ypdC*, His_6-tagged *rsiW* was cloned to plasmid pAL01

(Schöbel *et al.*, 2004) by PCR amplifying His_6*rsiW* from Plasmid pQE30*rsiW* with primers 5'-GGCCAT AGATCTATGAGAGGATCGCATCAC CATCACCATCACGGA-3' and (5), restriction of the product with *Bgl*II / *Sph*I and ligation to pAL01 restricted with *Bam*HI / *Sph*I, resulting in plasmid pJAH05. The plasmid was transformed to strain TW30 and combined with the *rsiW::spec* and *rasP::tet* mutant as described previously (Schöbel et al., 2004), resulting in strain JAH05.

PhoA Assays for determination of YpdC membrane topology

To determine the membrane topology of YpdC, five pMutin derived integration vectors for generation of different YpdC-PhoA fusion proteins were constructed. To that purpose, pMutin*phoA* was used, which is a pMUTIN derived plasmid that enables to translationally fuse the 3'-end of gene fragments to *phoA* in analogy to a method described earlier (Kaltwasser et al., 2002). Constructs with a fusion of PhoA to amino acid residues Y25, Y90, V125, A160 and V218 of YpdC were generated. First, all *ypdC* fragments were amplified via a PCR- reaction with one of the following 3´ primers: PhoAypdC-Y25 5´-GGCCATCGGCCGATACT GATCTTTTAAATAAAAATAACTTA-3´; PhoAypdC-Y90 5´-GGCCAT CGG CCGGTAAACACTGATCATCAGTAT AAAC-3´; PhoAypdC-V125 5´-GGC CATCGGCCGCACGCCGTGGCCAAT TAAATAAA-3´; PhoAypdC-A160 5´-GGCCATCGG CCGCGCCTTATCAGCGGAAAAACG -3´; PhoAypdC-V218 5´-GGCCATCGG CCGTACTTGCATCATATTAACGGA ACG-3´ plus the 5´oligo (3) and *B. subtilis* 1012 chromosomal DNA as a template. The resulting PCR products were *Xma*III, *Kpn*I digested and cloned into pMutin*phoA* yielding pMutin*phoA-ypdC* -Y25, -Y90, -V125, -A160 and -V218. Respective plasmids were transformed to *B. subtilis* 1012. Transformants selected on plates containing erythromycin (1µg/ml) were screened for resistance of 100 µg/ml erythromycin. The exact chromosomal integration site was verified via PCR with primers (1) and 5´-GGCCATGTCGACTTATTTCAGCCC CAGAGCGGCTTT-3´ (6) and *Eco*RI restriction analysis of the PCR products.

To clarify the membrane-topology of YpdC with randomly generated PhoA fusions, an assay with an ExoIII / SI Deletion Kit (Fermentas) was performed. This experiment is based on directed exonuclease III degradation of a linearized plasmid- DNA in 3´->5´

direction that is stopped after different time points. Single-stranded regions are removed by S1 Nuclease followed by recircularization and transformation to *E. coli* XL1-Blue. Plasmid pQE30 (Qiagen, Hilden, Germany) was used for construction of pQE30-*ypdC* and subsequently pQE30-*ypdC-phoA*. In the first step, *ypdC* was amplified by PCR and digested as described for pAX-*ypdC*, followed by transformation of the ligated product to *E.coli* XL1-Blue. Verification of the resulting plasmid was done by restriction analysis of pQE30-*ypdC* with *Pst*I and sequencing with primer 5´-GAGCGGATAACAATTTCACACAG-3´. In the next step, the *phoA*- gene was amplified via PCR using Primer (6) and 5´-GGCCATGATATCGAATCCGAG CTCACGTGTTAACCGGGCTGCTC-3´and cloned between *EcoR*V and *Sal*I sites of pQE30-*ypdC*. The ligated product pQE30-*ypdC-phoA* was transformed to *E. coli* XL1-Blue and checked by restriction analysis. To generate translational *phoA* fusions, pQE30-*ypdC-phoA* was digested with *EcoR*V, producing a blunt end as substrate for the ExoIII reaction, and *Sac*I, resulting in a degradation-resistant 3´overhang. The unidirectional nuclease reaction was performed in 1x Exo III Buffer containing 80 mM NaCl at 25 °C causing the Exonuclease III to delete ~ 30 bases per minute of the 3´end of *ypdC*. Samples were taken every minute for 25 time-points, treated with S1 nuclease and T4-DNA ligase and transformed to *E. coli* XL1-Blue plated on LB containing ampicillin (100 µg/ml), X-P and potassium phosphate (1 mM). Colonies were screened for a blue colour on X-phosphate plates as a result of extracytoplasmic PhoA-activity. Fusion joints of truncated *ypdC* were identified by sequencing of selected plasmids with primer 5´-GTCTGATCACCCGTTAA ACG-3´. PhoA activities were measured as described earlier (Schöbel et al., 2004), with the exception that *E. coli* strains were induced at an OD578=0.8 with 100 µM of IPTG for 1 h. Background activities of *B. subtilis* 1012 or *E. coli* XL1-Blue respectively were subtracted from values estimated for the samples.

Computer analysis

To determine the loci of mini-Tn10 insertion, the sequence of chromosomal DNA flanking the mini-Tn10 was blasted against the *B. subtilis* genome using either the SubtiList website at http://www.pasteur.fr/Bio/SubtiList.html or the NCBI blast search tool at http://www.ncbi.nlm.nih.gov/BLAST/,

the BSORF *B. subtilis* Genome Database at http://bacillus.genome.jp/ or the Micado website at http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl using default settings. Protein localization and transmembrane domains were predicted using public programs TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), PSORT (http://psort.nibb.ac.jp/), SOUSI (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html), UniProt (http://www.expasy.uniprot.org/), TMap (http://bioinfo.limbo.ifm.liu.se/ tmap/), and DAS (http://www.sbc.su.se/~miklos/DAS/). The MEROPS peptidase database is available under http://merops.sanger.ac.uk/.

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Chapter 3

Two proteolytic modules are involved in regulated intramembrane proteolysis of *Bacillus subtilis* RsiW

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SUMMARY

Stress-induced degradation of the *Bacillus subtilis* anti-sigma factor RsiW results in the induction of genes controlled by the ECF sigma factor σ^W. RsiW is cleaved by the mechanism of regulated intramembrane proteolysis at site-1 and-2 by PrsW and RasP, respectively, and is then further degraded by cytoplasmic Clp peptidases. In a reconstituted *Escherichia coli* system, PrsW removes 40 amino acids from RsiW by cleaving between Ala168 and Ser169 of the extracytoplasmic domain, thereby generating RsiW-S1. Further trimming of RsiW-S1's C-terminus by the periplasmic tail-specific protease Tsp is crucial for subsequent RasP-catalyzed clipping. In *B. subtilis*, mutation of RsiW at Ala168 severely impairs site-1 processing. RsiW-S1 is undetectable in wild type *B. subtilis* and knockout strains lacking various extracytoplasmic proteases. While it can be stabilized by C-terminal tagging, even this fusion protein is still attacked. Thus, several peptidases seem to be involved in trimming of RsiW downstream of PrsW and upstream of RasP in *B. subtilis*. Overall, the RsiW degradation pathway can be subdivided into two modules each consisting of a site-specific peptidase which prepares RsiW for further degradation by downstream proteases.

Introduction

Bacterial ECF sigma factors play an important role in controlling genes of extracytoplasmic function that are involved in processes related to transport, cell envelope integrity and antibiosis, but also to certain stresses like oxidative conditions and heat. In many cases, a transmembrane anti-sigma factor sequesters the sigma factor from interaction with RNA polymerase (Helmann, 2002). For several examples it has been shown that, in response to an environmental signal, the sigma factor is released through regulated intramembrane proteolysis (RIP) of the transmembrane anti-sigma factor. This mechanism seems to represent a common regulatory mechanism in bacteria, with *E. coli* σ^E / RseA best understood (Ades, 2004; Alba and Gross, 2004; Ehrmann and Clausen, 2004).

σ^W is one of the seven ECF sigma factors encoded by the Gram positive model bacterium *Bacillus subtilis*. It is autoregulated and controls about 30 promoters. The σ^W regulon is induced by a variety of signals, like vancomycin treatment (Cao *et al.*, 2002), antimicrobial peptides (Pietiäinen *et al.*, 2005; Butcher and Helmann, 2006), phage infection, and strongly by an alkaline shock (Wiegert *et al.*, 2001). It appears to confer intrinsic immunity against different antimicrobial agents and resistance to some antibiotics, but it is not involved in maintenance of pH-homeostasis following alkaline shock. Therefore, σ^W seems to constitute an antibiosis regulon acting against cell envelope stress (Helmann, 2006; Butcher *et al.*, 2008). We could show that the mechanism of activation of σ^W involves stress induced RIP of the transmembrane anti-sigma factor RsiW, in a manner similar to *Escherichia coli* RseA, under participation of the S2P-family intramembrane protease RasP (formerly YluC) (Schöbel *et al.*, 2004). Site-2 clipped RsiW (RsiW-S2) also presents a proteolytic tag at its C-terminus that is recognized mainly by ClpXP (Zellmeier *et al.*, 2006).

A substantial difference to *E. coli* σ^E / RseA was discovered for the site-1 cleavage step that renders RsiW a substrate for RasP. A participation of DegS homologs of *B. subtilis* could not be proven, and two different experimental approaches identified the *prsW* gene (formerly *ypdC*) as the determinant of site-1 proteolysis (Heinrich and Wiegert, 2006; Ellermeier

and Losick, 2006). It was proposed that PrsW (protease responsible for activating σ^W) represents a novel site-1 protease (Ellermeier and Losick, 2006). However, *in vitro* assays to directly demonstrate protease activity of PrsW were not possible so far, mainly because purification of the protein failed. Also, the molecular signal(s) that activates the site-1 cleavage step and other regulatory factors that might be involved in degradation of RsiW remain elusive at present.

PrsW does not display obvious similarities to known protease families, and in the database of Clusters of Orthologous Groups of proteins (COGs) it belongs to COG2339, where multispanning membrane proteins of unknown function found in Gram-positive and Gram-negative bacteria as well as some Archaea are grouped. Ellermeier and Losick (2006) discovered similarities of PrsW to COG1266, a group that shares three conserved sequence motifs with the eukaryotic type II CAAX prenyl endopeptidase family, with the yeast RCE1 protease (Ras and a-factor converting enzyme) as a paradigm. COG1266 and the type II CAAX prenyl endopeptidase family had been grouped earlier to a superfamily of probable membrane embedded metalloproteases, in the following abbreviated as MEM-superfamily, that were suspected to be potentially involved in protein and/or peptide modification and secretion (Pei and Grishin, 2001). It can be assumed that PrsW belongs to this large MEM-superfamily, and it represents the first prokaryotic member with a defined substrate and function.

As the anti-sigma factor RsiW seems to be the direct substrate for PrsW, it is an ideal model for proteins of the MEM-superfamily to investigate the catalytic mechanism and the activation of proteolysis and to perform structural and functional analyzes. PrsW most probably is a zinc-dependent protease, and site-directed mutagenesis of the conserved twin glutamate residues that are proposed to bind zinc and to be catalytically active abolish site-1 cleavage of RsiW (Ellermeier and Losick, 2006; our own unpublished results). Two models for PrsW membrane topology have been proposed that substantially differ in the location of these glutamate residues. One model suggests that they are located within transmembrane regions and that site-1 cleavage of RsiW occurs within or close to the transmembrane helix of RsiW, supported by the molecular mass of site-

1 cleaved RsiW as determined by SDS-PAGE (Ellermeier and Losick, 2006).

Here we present evidence that PrsW cleaves RsiW more distal to the transmembrane helix between amino acid residues Ala168 and Ser169 to generate a site-1 processed form truncated for 40 C-terminal amino acid residues (RsiW-S1). Furthermore, our results suggest that most of the residual extracytoplasmic part of RsiW-S1 has to be removed from the C-terminal end by other protease(s) to produce RsiW-S1Δ, which is the substrate for RasP. Therefore, RIP of RsiW seems to involve two proteolytic modules, each consisting of one site-specific processing protease (site-1: PrsW; site-2: RasP) that renders RsiW susceptible to more unspecific degrading proteases (site-1: unknown peptidase(s); site-2: Clp peptidases).

Results

Truncated and point mutated RsiW is degraded to a site-1 proteolysis product in the absence of PrsW.

In a previous transposon screen with a GFP-RsiW fusion protein as a reporter we had identified the *prsW* gene as determinant of site-1 cleavage of RsiW in *B. subtilis* (Heinrich and Wiegert, 2006). Because our database searches did not reveal PrsW being a potential protease, we wondered whether it could be an associated factor that somehow influences or regulates site-1 cleavage of RsiW by a protease that had not been hit in our transposon screen. In order to identify such a protease, we performed an additional random mutagenesis screen with strain JAH01. Here, a transcriptional fusion of the σ^W controlled *yuaF* promoter to *lacZ* serves as a reporter for σ^W induction, and the *prsW* gene is knocked out. Due to the absence of site-1 cleavage and stabilisation of RsiW, colonies of JAH01 grown in presence of IPTG remain white, in contrast to the isogenic *prsW* wild type strain, where stationary phase induction of σ^W results in blue colonies on LB plus X-Gal. All of the mutants obtained that re-established the blue colony phenotype proved to be point mutations in *rsiW*, located in the region encoding the extracytoplasmic part of RsiW (Fig. 1). As one of the mutations resulted in a C-terminal truncated form of RsiW, we constructed a series of *rsiW* alleles deleted for the 5, 10, 15, and 20 C-terminal amino acid residues encoding region. Mutation Δ5 exhibited a wild

type phenotype, whereas Δ10, Δ15, and Δ20 resulted in blue colonies (data not shown). Together with evidence for PrsW being a site-1 protease (Ellermeier and Losick, 2006), there are two explanations for the observed effects. Either, the point mutations or deletion of ten or more C-terminal amino acid residues bypasses the need for site-1 cleavage and make RsiW a direct substrate for RasP, or they result in an unstable extracytoplasmic domain of RsiW that is degraded in an unspecific and PrsW independent manner. We performed Western blot analyses of different strains expressing the mutated *rsiW* alleles.

All of the five point mutations and C-terminal RsiW deletions Δ10, Δ15, and Δ20 showed the band of the designated site-1 cleavage product of RsiW at approximately 14 kDa in the *rasP* minus background, whereas for the wild type and *prsW* minus strains no RsiW protein could be detected (Fig. 2A, exemplified for the Δ20 form of RsiW). These data strongly suggest that C-terminal truncations and point mutations produce an unstable extracytoplasmic domain that is degraded in an unspecific manner to a position where transmembrane location of RsiW abolishes further proteolysis.

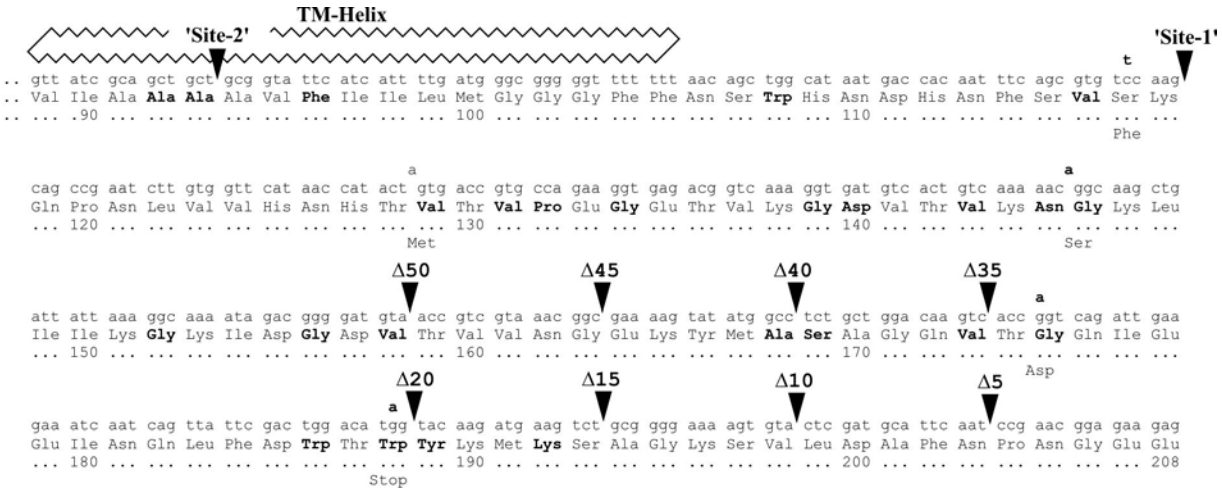

Fig. 1. Amino acid sequence of the transmembrane- and extracytoplasmic part of RsiW. The putative site-2 processing site of RasP in the transmembrane helix of RsiW and the approximate site-1 cleavage form of RsiW detectable in *B. subtilis rasP* minus cells are depicted with arrows. Single point mutations found in the random mutagenesis screen resulting in PrsW independent degradation of RsiW (S117F, V129M, G146S, G175D, W188stop), and truncated forms constructed (Δ50, Δ45, Δ40, Δ35, Δ20, Δ15, Δ10, and Δ5) are indicated.

As a consequence, PrsW catalysed site-1 cleavage does not necessarily take place at around amino acid residue 118, and the so far designated site-1 form of RsiW at 14 kDa might be a secondary product. This hypothesis could also explain why there is a small but marked difference in the molecular mass of site-1 cleaved RsiW detectable in *B. subtilis* membranes compared to the form that arises in the reconstituted *E. coli* system (Fig. 2B). These observations prompted us to have a closer look on site-1 proteolysis of RsiW and to identify the exact cleavage position of PrsW in RsiW.

The tail specific protease is involved in site-1 proteolysis in a reconstituted E. coli *system.*

B. subtilis rsiW expressed in *E. coli* results in a stable RsiW protein. Upon expression of *prsW*, the full length form of RsiW is converted to a 14 kDa proteolysis product, independently of an external shock like alkaline stress (Heinrich and Wiegert, 2006; Ellermeier and Losick, 2006). To analyse RIP of RsiW in more detail, we designed a reconstituted system in *E. coli*, with RsiW expressed from a constitutive promoter as an N-terminally His6-tagged protein, and the gene for *prsW* under arabinose control (Fig 3A, lines 1 – 8). With this system it can be seen that RsiW is processed to the 14 kDa form upon arabinose addition to the medium (Fig. 3A, lanes 1 and 2). When a mutated allele of *prsW* with the designated active-site glutamate residues at position 75 and 76 changed to alanine is expressed (*prsW**), RsiW remains stable (Fig. 3A, lanes 3 and 4). To test our hypothesis that further proteases besides the PrsW site-1 protease are involved in the formation of the 14 kDa RsiW proteolysis product, several single and multiple *E. coli* knockout strains in periplasmic proteases ((HM120 (*degP*, *ompT*, *tsp*), KS474 (*degP*), HM101 (*tsp*), CAG 33315 (*degS*)) were analyzed in the reconstituted system. A different size of RsiW upon induction of *prsW* was found for strains with a knockout for *prc* (*tsp*). This gene encodes a periplasmic endoprotease of *E. coli* that recruits a peptide or protein substrate via the nonpolar C-terminus of the substrate (Beebe *et al.*, 2000). It is involved in processing of PBP 3 (*prc*; processing involving the C-terminal cleavage, (Hara *et al.*, 1991)) and in degradation of proteins with destabilizing C-termini (*tsp*; tail specific protease, (Silber *et al.*, 1992)).

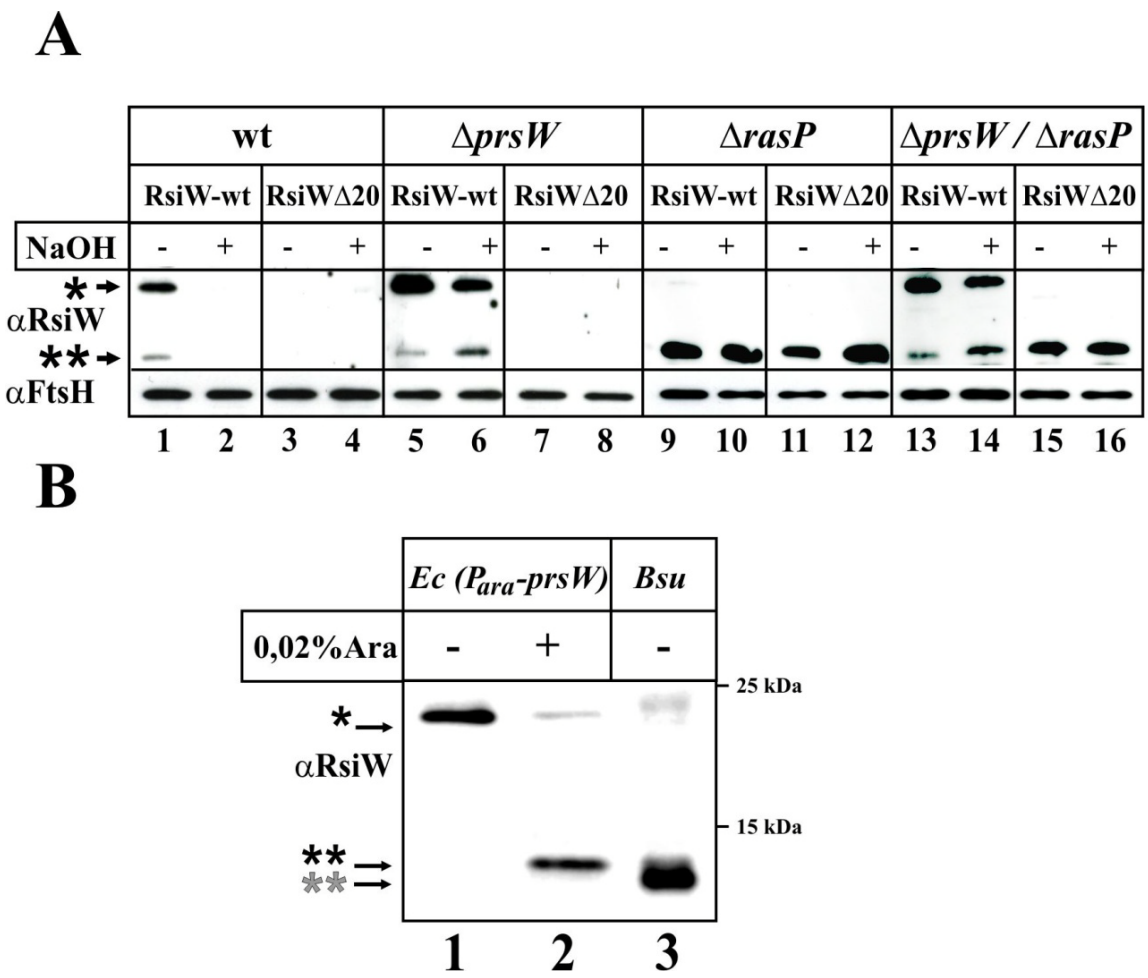

Fig. 2. Truncated RsiW is degraded to a site-1 proteolysis product.
A. Western blots of *B. subtilis* strains TW32-FLAG (wild type RsiW; lanes 1, 2, 5, 6, 9, 10, 13, 14) and JAH100 (RiWΔ20; lanes 3, 4, 7, 8, 11, 12, 15, 16) in wild type, Δ*prsW* (JAH101), Δ*rasP* (JAH102), and Δ*rasP* / Δ*prsW* (JAH103) double- knockout background. NaOH was added to a final concentration of 24 mM at an OD_{578} of 0.7. Samples were taken 10 min after NaOH addition and at the same time point for unshocked cells. The cells were disrupted by sonication, and whole cell extracts were further fractionated to the membrane- and soluble-protein fraction by ultracentrifugation. For SDS-PAGE, an equivalent of 10 µg of soluble protein was used for each membrane fraction. Blots were developed with polyclonal antibodies against RsiW and, as a loading control, with polyclonal antibodies against FtsH. Full length 3xFLAG-tagged RsiW is marked with a star, site-1 truncated 3xFLAG-tagged RsiW with two stars. **B.** *E. coli* DH10B (lanes 1, 2) containing both, plasmid pMA01 to constitutively express 6His-tagged RsiW and plasmid pJAH17 with the *prsW* gene under arabinose control, were grown at 30 °C to an OD of 0.5. The culture was divided; to one culture arabinose was added to a final concentration of 0.02 %, followed by further incubation for 3h. Samples were withdrawn and subjected to SDS-PAGE and Western blotting. Antibodies against RsiW were used. As a control, a sample of the membrane fraction of *B. subtilis* JAH05 (lane 3) expressing 6His-RsiW in the *rasP* minus background was loaded to the same gel. 6His-RsiW is marked with a star, site-1 truncated 6His-RsiW with two stars.

In the *prc* minus strain, RsiW is converted to an approximate 19 kDa product (Fig. 3A, lane 6), in contrast to the 14 kDa form detectable in the wild type background (Fig. 3A, lane 2). These data suggest that the 19 kDa form of RsiW is the actual PrsW site-1 cleavage product (RsiW-S1), which is further degraded by Tsp to the 14 kDa form (RsiW-S1Δ). In pulse chase experiments with the Δ*prc* strain, conversion of RsiW to RsiW-S1 is visible over chase time, whereas in the *prc* wild type background only RsiW-S1Δ is produced (Fig. 3B, lanes 1 – 8). Also in the *prc* minus strain, a slight conversion to RsiW-S1Δ takes place (Fig. 3B, lanes 5 – 8). This form is not completely stable as the amount decreases after prolonged chase time (Fig. 3B lanes 4 and 8), which might be the reason why RsiW-S1Δ is not visible in the Δ*prc* strain in static Western blots experiments.

To test whether *prc* homologs are involved in further degradation of site-1 cleaved RsiW in *B. subtilis*, strains with a single knockout of *ctpA* (QBP159) and *ctpB* (QBP161) and a combined double knockout (QBP170; Pan et al., 2003) were analyzed in Western blot experiments. However, there was no difference in alkaline shock induced degradation of RsiW in comparison to the isogenic wild type strain. The same was true for the WB800 strain (Murashima *et al.*, 2002) deleted for 8 extracytoplasmic proteases (data not shown).

Mapping of the PrsW site-1 cleavage site in RsiW.

At present, PrsW is the only prokaryotic member of the MEM-superfamily with known function. The reconstituted *E. coli* system with the *prc* minus strain enabled us for the first time to map the exact processing site of such a MEM protein. For a rough estimation, we first expressed different truncated forms of His6-tagged RsiW at a molecular mass around 19 kDa that we used as molecular weight markers in SDS-Page. It becomes evident that PrsW-cleaved RsiW in the *prc* minus strain migrates at the position of RsiW truncated for the last 40 C-terminal amino acid residues (RsiWΔ40) (Fig. 4A). To refine our estimation, we used a technique that involves covalent modification of engineered cysteine residues at the processing site with methoxypolyethyleneglycol 5000 maleimide (malPEG), which adds a molecular mass of ~5 kDa to the polypeptide and retards its SDS-PAGE mobility (Akiyama *et al.*, 2004).

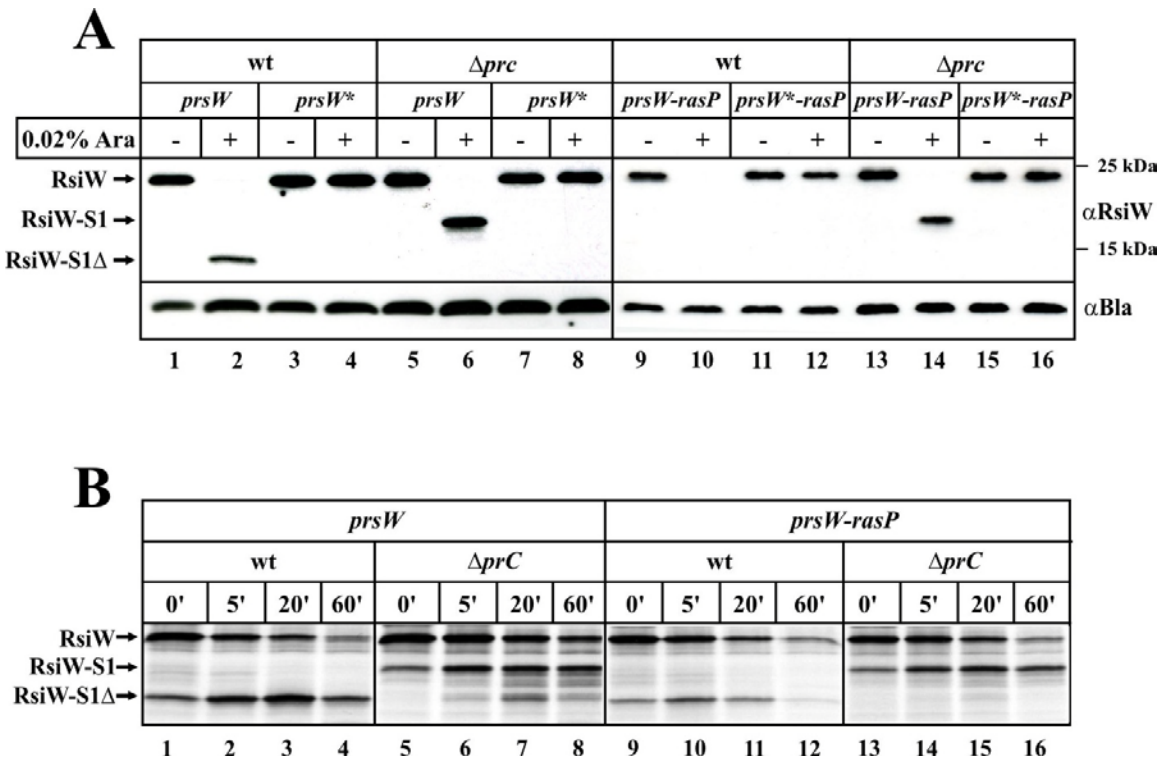

Fig. 3. The tail specific protease Tsp encoded by the *prc* gene is involved in formation of truncated RsiW in the reconstituted *E. coli* system.
A. Western blot experiments of *E. coli* strains constitutively expressing 6His-tagged RsiW from plasmid pMA01 and additionally containing either plasmid pJAH17 (*prsW*) and pJAH19 (*prsW-rasP*), or pJAH18 (*prsW**, mutation of active site residues) and pJAH20 (*prsW***-rasP*) were grown as described in the legend to Fig.2 B in an *E. coli prc* wild type (HM105; lanes 1 – 4, 9 – 12) or Δ*prc* (HM101; lanes 5 – 8, 9 – 12) background. Cells were harvested by centrifugation 3 h after induction with 0.02% arabinose, and for the same time point of the uninduced cells. Antibodies against RsiW and the *E. coli* β-lactamase (Bla) as a loading control were used. The former designated site-1 product is labelled as RsiW-S1Δ and the PrsW dependent truncated form as RsiW-S1. **B.** Pulse chase experiment of *E. coli* HM105 (*prc* wild type; lanes 1 – 4, 9 – 12) and HM101 (Δ*prc*; 5 – 8, 13 – 16) strains harbouring pMA01 and pJAH17 (*prsW*) or pJH19 (*prsW-rasP*). Cells were grown at 30°C in minimal medium in the presence of 0.02% arabinose and analysed in a pulse chase experiment with chase times as indicated. For details, see the Experimental Procedures section.

Four point mutations at amino acid position 167 to 170 of RsiW were constructed (M167C, A168C, S169C, A170C) and first examined for PrsW processing in the reconstituted *E. coli* system in the Δ*prc* background. All of the site-directed mutant forms of RsiW were site-1 cleaved comparable to wild type RsiW, exemplified with RsiW-M167C in Fig. 4B (lanes 1 and 2). The isolated site-1 clipped RsiW proteins were then treated with malPEG and subjected to SDS-Page afterwards. Wild type site-1 cleaved RsiW that contains

three cysteine residues in the cytoplasmic part of the anti-sigma factor is shifted to a single band at a molecular mass of approximately 30 kDa (Fig. 4B, lane 3). RsiW-S169C and A170C are shifted in the same manner, whereas RsiW-M167C and RsiW-A168C show a second super shifted band at about 35 kDa (Fig. 4B, lanes 4 – 7). Although we were not able to find experimental conditions to allow a complete super shift, these data strongly suggest that the site-1 cleaved forms of RsiW-M167C and RsiW-A168C have retained the engineered cysteine residue, which is equivalent with a PrsW processing site between Ala168 and Ser169. These residues are highly conserved in six RsiW proteins of different bacterial species that can be found in databases (Fig. 1). To examine whether there is site specificity in RsiW for PrsW processing, we mutated respective amino acid residues.

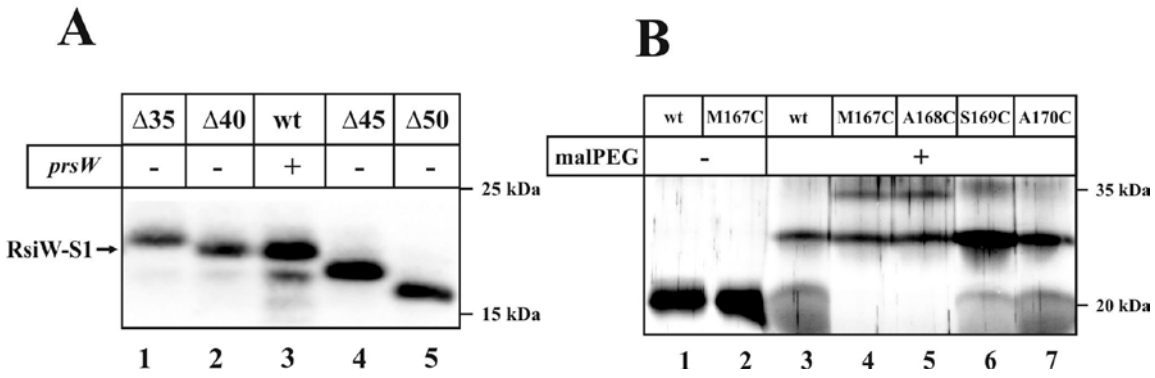

Fig. 4. Mapping of the PrsW cleavage site in RsiW.
A. Western-blot of *E. coli* HM101 constitutively expressing 6His-tagged RsiW from plasmid pMA01 and additionally containing plasmid pJAH17 (lane 3) was grown as described in the legend to Fig.2 B. As a molecular weight marker, different C-terminal truncated forms of 6His-tagged RsiW (Δ35, Δ40, Δ45, Δ50) were expressed likewise from plasmids pJH35-38 together with the empty vector pBAD33 (lanes 1, 2, 4, 5). Samples were withdrawn and subjected to SDS-PAGE and Western blotting. Specific antibodies against RsiW were used. The PrsW-processed form of 6His-RsiW is labelled as RsiW-S1.
B. MalPEG- shift assay of PrsW-processed RsiW proteins with engineered cysteine residues M167C, A168C, S169C, and A170C. *E. coli* HM101 strains constitutively expressing cysteine engineered (lanes 2, 4 – 7) and wild type 6His-RsiW (lanes 1 and 3) containing plasmid pJAH17 were grown as described in the legend to Fig.2 B. PrsW-processed 6His-RsiW was enriched via NiNTA chromatography and further purified via preparative SDS-PAGE and electroelution. MalPEG modification was performed where indicated (lanes 3 – 7). Samples were subjected to SDS-PAGE and silver staining. For details, see the experimental procedures section.

We chose an exchange of alanine 168 and serine 169 to glutamine in RsiW (RsiW-A168Q and RsiW-S169Q), as the polar and relatively large side chain of glutamine should interfere with substrate binding and peptide bond cleavage in an active site, like it has been shown e.g. for the processing of signal peptides (Shen *et al.*, 1991). The mutant alleles were ectopically expressed under IPTG control as 3xFLAG-tagged proteins with the wild type *rsiW* gene knocked out by deletion and insertion of a spectinomycin resistance gene. Anti-sigma factor activity and stress induced RIP in *B. subtilis* was monitored at the hand of a σ^W-controlled *lacZ* reporter fusion and Western blotting as described earlier (Schöbel *et al.*, 2004). Both mutant *rsiW* alleles retained anti-sigma factor activity, as addition of IPTG significantly reduced β-galactosidase activity of the σ^W controlled *lacZ* reporter (Table 1). However, alkaline stress induced β-galactosidase activity was only detectable for wild type RsiW and RsiW-S169Q. Membrane and cytoplasmic fractions of alkaline shocked cells were analysed by Western blotting. Antibodies against PBP4* served as a marker for σ^W induction, and FtsH and HtpG antibodies as loading controls for membrane and cytoplasmic proteins respectively. In addition to the wild type strain background, minus mutations of *prsW*, *rasP*, and *prsW / rasP* double knockouts were analysed in parallel (Fig. 5). For the RsiW wild type control it is evident that alkaline stress results in the degradation of RsiW and induction of the σ^W controlled *pbpE* gene encoding PBP4* (Fig. 5, lane 9). In the *prsW* minus and the *prsW / rasP* minus background RsiW is stabilized, whereas in the *rasP* minus background RsiW-S1Δ accumulates in the membrane (Fig. 5, lanes9 – 12). In accordance to the β-galactosidase activity tests (Table 1), RsiW-S169Q displays wild type behaviour, with the exception of a faint 14 kDa RsiW-S1Δ band in the *prsW / rasP* minus background (Fig. 5, lanes 2, 4, 6, and 8). In contrast, RsiW-A168Q is not degraded following alkaline shock, and mainly the full length form is found in *rasP* minus background (Fig. 5 lanes 1 and 5). These data show that site-1 proteolysis of RsiW is significantly impaired when the conserved amino acid residue A168 in the PrsW processing site is altered. This points to sequence specificity of PrsW activity with respect to the alanine residue at position 168.

TABLE 1:
β-Galactosidase activities of TW32-FLAG, JAH104 and JAH108 expressing wild type or mutated *rsiW* under IPTG-regulatable control, and a transcriptional fusion of the σ^W-controlled *yuaF* promoter to *lacZ*. Cells were cultured in the absence and presence of 1 mM of IPTG, one culture with IPTG was alkaline shocked at an OD_{578} = 0.7 by the addition of NaOH to a final concentration of 24 mM, resulting in pH 8.9. Samples were taken 1 h after NaOH addition for shocked and unshocked cells. Activities were determined in three independent experiments and mean values of units / OD are given. Standard deviations were in the range of 1 to 20 %.

strain	**β-galactosidase activity**		
	-IPTG	+IPTG	+IPTG +NaOH
***rsiW* wt**	9.3	0.3	7.0
***rsiW* A168Q**	8.4	0.1	0.1
***rsiW* S169Q**	10.5	0.8	6.8

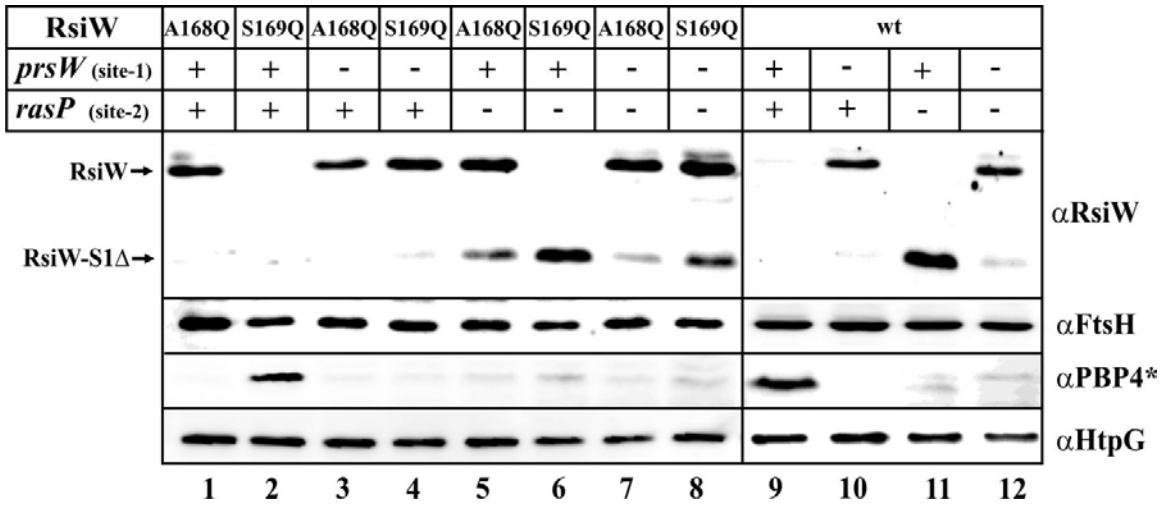

Fig. 5. PrsW catalysed cleavage of RsiW is dependent on Ala168.
Western blot experiment of *B. subtilis* strains TW32-FLAG (lanes 9 – 12), JAH104 (3xFLAG-RsiW-A168Q; lanes 1, 3, 5, 7), JAH108 (3xFLAG-RsiW-S169Q; lanes 2, 4, 6, 8) in the wild type, *prsW* and, or *rasP* minus background to analyse intramembrane proteolysis of RsiW. The wild-type *rsiW* gene of respective strains is deleted and the different *rsiW* alleles are ectopically expressed under IPTG control. Cultures were alkaline- shocked as described in the legend to Fig. 2 A. Blots of membrane fractions were treated with antibodies against RsiW and FtsH (loading control), blots of soluble fractions were developed with antibodies against σ^W controlled Pbp4* and HtpG as a loading control.

Further degradation of PrsW processed RsiW is crucial for RasP catalyzed site-2 cleavage.

A major question that arises from above experiments is, whether further extracytoplasmic degradation of RsiW-S1 to RsiW-S1Δ is a prerequisite for the site-2 cleavage step catalysed by RasP. To address this issue, the *rasP* gene was placed downstream of *prsW* in an artificial operon under control of the arabinose inducible promoter. Western blot and pulse chase experiments were performed with the reconstituted *E. coli* system as described above. In the *prc* wild type background, RsiW is completely removed from the cells upon arabinose addition (Fig. 3A, lane 10). Pulse chase experiments show that the co-expression of *rasp* results in degradation of RsiW-S1Δ (Fig. 3B, lanes 9 – 12), showing that RasP is active in the reconstituted system. In contrast, RsiW-S1 that arises in the *prc* minus strain is detectable in Western blots (Fig. 3A, lane 14) and stabilized in the pulse chase experiment in the presence of *rasP* (Fig. 3B, lanes 13 – 16).

These data suggest that RasP is not able to efficiently recognize RsiW-S1. In order to become a substrate for the site-2 protease RasP, the C-terminal end of RsiW-S1 has to be further degraded, in a figurative sense to be shaved off, to form RsiW-S1Δ.

In *B. subtilis*, neither RsiW-S1 nor an engineered RsiWΔ40 form could be detected in knockouts of *prc* homologs and the multiple protease knockout strain WB800 (see above). Therefore, to analyse site-1 cleavage and the requirement for further truncation for subsequent site-2 proteolysis of RsiW in *B. subtilis*, we made use of translational fusions of the green fluorescent protein GFP to the N-terminus and the Alkaline Phosphatase PhoA to the C-terminus of different RsiW forms (Fig. 6A). GFP directly serves as a reporter for RsiW stability (Zellmeier *et al.*, 2006), and PhoA mimics a stably folded extracytoplasmic domain. In addition, a mutant, unstable form of PhoA (PhoA*) was used with the stabilizing cysteine residues involved in disulfide bond formation altered to serine (Kihara *et al.*, 1999). Respective fusion proteins were expressed in *B. subtilis* wild type, *rasP* and *prsW* knockout background, and membrane fractions of samples with and without alkaline shock were analysed in Western blots using RsiW antibodies. GFP-RsiW$_{1-208}$-PhoA with full length RsiW displayed the normal RsiW behaviour. In comparison to other signals, relatively small amounts of the 97,4 kDa fusion protein can be detected in addition to a GFP-RsiW-S1Δ product migrating at about 35 kDa (Fig. 6B, lane 1). Both disappear after alkaline shock (Fig. 6B, lane 2). The amount of the fusion protein strongly increases in the *prsW* minus background (Fig. 6B, lanes 9, 10), and in the *rasP* background a strong signal for GFP-RsiW-S1Δ is detectable, irrespectively of an alkaline shock (Fig. 6B, lanes 17, 18). This is equivalent with GFP-RsiW$_{1-208}$-PhoA being attacked by PrsW and undergoing RIP.

Constant degradation of overexpressed RsiW, probably due to titration of a stabilizing factor, has been observed earlier (Heinrich and Wiegert, 2006). GFP-RsiW$_{1-112}$-PhoA with almost the entire extracytoplasmic domain of RsiW missing shows a strong signal and is not removed from the membranes after alkaline shock (Fig. 6B, lanes 5 and 6). This form also remains more stable in the *rasP* minus background when compared to GFP-RsiW$_{1-208}$-PhoA (Fig. 6B, lane 21). The same experiments were performed with fusions to the

unstable PhoA*. For GFP-RsiW$_{1-208}$-PhoA* it is evident that the unstable PhoA* proportion is completely absent, with only GFP-RsiW and GFP-RsiW-S1Δ present (Fig. 6B, lane 3). With construct GFP-RsiW$_{1-112}$-PhoA*, degradation of PhoA* results in GFP-RsiW$_{1-112}$ becoming a substrate for RasP, as only in the *rasP* knockout background a signal can be detected (Fig. 6B, lanes 7, 8, 15, 16, 23, and 24).

In summary, these results show that the exchange of the extracytoplasmic domain of RsiW by PhoA prevents site-2 cleavage, unless PhoA is destabilized and degraded.

Like RsiWΔ20 (Fig. 2A), truncated RsiW that mimics the PrsW processed form identified in the reconstituted *E. coli* system (RsiWΔ40) is completely removed from *B. subtilis* membranes unless *rasP* is knocked out (data not shown). To estimate whether the free C-terminus ending with an alanine residue is absolutely required for degradation of RsiWΔ40, fusion protein GFP-RsiW$_{1-168}$-PhoA was constructed (Fig. 6A).

Fusion of PhoA to the C-terminal alanine residue masks RsiW from complete degradation in *B. subtilis*, as 93 kDa GFP-RsiW$_{1-168}$-PhoA is detectable in Western blots in wild type background (Fig. 6B, lanes 25, 26). In the *rasP* minus background, large amounts of GFP-RsiW-S1Δ become visible, whereas the amount of full length GFP-RsiW$_{1-168}$-PhoA remains the same (Fig. 6B, lane 29). These data show that, like GFP-RsiW$_{1-112}$-PhoA, GFP-RsiW$_{1-168}$-PhoA is not directly cleaved by RasP. Furthermore, masking the C-terminal end of PrsW processed RsiW is not sufficient to protect it against proteolytic attack. In conclusion, in *B. subtilis* the RsiW-S1 protein is attacked also by extracytoplasmic proteases other than Tsp-like ones, because there is no absolute dependence on a C-terminal degradation signal. For GFP-RsiW$_{1-168}$-PhoA it is not clear whether PhoA itself or already the about 60 extracytoplasmic C-terminal amino acid residues of RsiW$_{1-168}$ prevent site-2 cleavage. Experiments with the reconstituted *E. coli* system are in support of the latter.

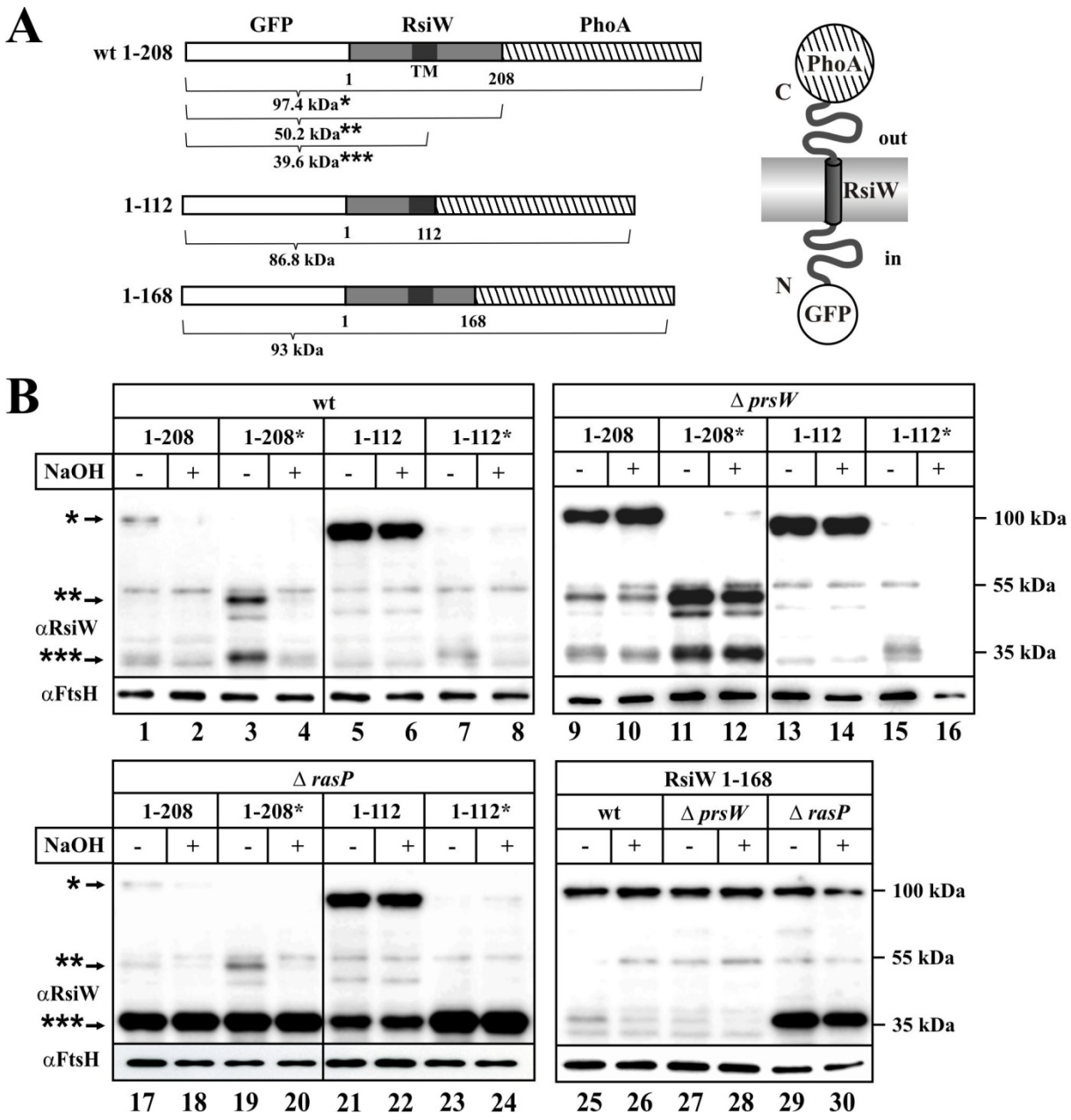

Fig. 6. Truncated RsiW is protected against RasP catalyzed site-2 cleavage by C-terminal PhoA fusions.

A. Schematic representation of translational fusions of GFP to the N-terminus, and PhoA to the C-terminus of wild type and truncated forms of RsiW as a reporter for regulated intramembrane proteolysis. The molecular masses of fusions proteins and different degradation products are depicted. **B.** Western blot of *B. subtilis* 1012 *rsiW::spec* reporter strains encoding the different translational fusions shown in A. A star indicates fusions to unstable PhoA with cysteine residues involved in disulfide-bond formation mutated to serine. 1-208: GFP-RsiW$_{1-208}$-PhoA; 1-112: GFP-RsiW$_{1-112}$-PhoA; 1-168: GFP-RsiW$_{1-168}$-PhoA. 50 µM IPTG was added to induce expression of the reporter-constructs. Cells were alkaline-shocked as described in the legend to Fig. 2 A. Blots were developed with polyclonal antibodies against RsiW and, as a loading control, with polyclonal antibodies against FtsH. GFP-RsiW$_{1-208}$-PhoA is marked with a star, GFP-RsiW that arises when the unstable PhoA* is degraded is marked with two stars, and site-1 cleaved and further truncated GFP-RsiW (GFP-RsiW-S1Δ) with three stars.

Discussion

Bacterial proteases involved in RIP are promising targets for the design of novel antibiotics, because several investigations revealed their crucial function in pathogenic processes (Urban, 2009). For example, an S2P protease has been connected to the regulation of cell envelope composition and *in vivo* growth and persistence of *Mycobacterium tuberculosis* in its host (Makinoshima and Glickman, 2005), and it seems likely that there is a link to the control of one or more of the ten ECF sigma factors *M. tuberculosis* encodes. Therefore, our aim is to use the σ^W / RsiW system of *B. subtilis* as a Gram-positive model to analyse variations in transmembrane signalling via RIP.

In vitro experiments have revealed that the *E. coli* site-1 protease involved in processing of the σ^E anti-sigma factor RseA, DegS, cleaves RseA about 30 residues from the end of its transmembrane region (Walsh *et al.*, 2003). The site-1 product becomes a direct substrate for the S2P site-2 protease RseP (Inaba *et al.*, 2008). It was shown that the two periplasmic PDZ domains of RseP prevent premature intramembrane proteolysis of full length RseA through interaction of the RseP-PDZ domains with a so far not clearly defined ligand. Removal of this ligand would induce a conformational change in RseP to allow entry of full length RseA. There are experimental data that this negatively regulating ligand is equivalent to a Gln-rich region of the extracytoplasmic RseA domain (Kanehara *et al.*, 2003; Bohn *et al.*, 2004; Inaba *et al.*, 2008). Furthermore, the periplasmic RseB protein and the site-1 protease DegS negatively regulate RseP activity, with RseB in a manner dependent on the PDZ domain of RseP (Grigorova *et al.*, 2004). Very recently it was shown that the C-terminal valine residue of RseA exposed after site-1 cleavage may facilitate site-2 processing through direct interaction with one PDZ domain of RseP (Li *et al.*, 2009). σ^W is the sole alternative ECF sigma factor of a Gram-positive bacterium for which it has been proven at present that it is regulated via RIP of its membrane spanning anti-sigma factor. The process very much resembles RIP of the *E. coli* σ^E anti-sigma factor, but there seem to be important differences in the mechanism mainly for the site-1 cleavage step that is catalysed by the MEM-superfamily member PrsW.

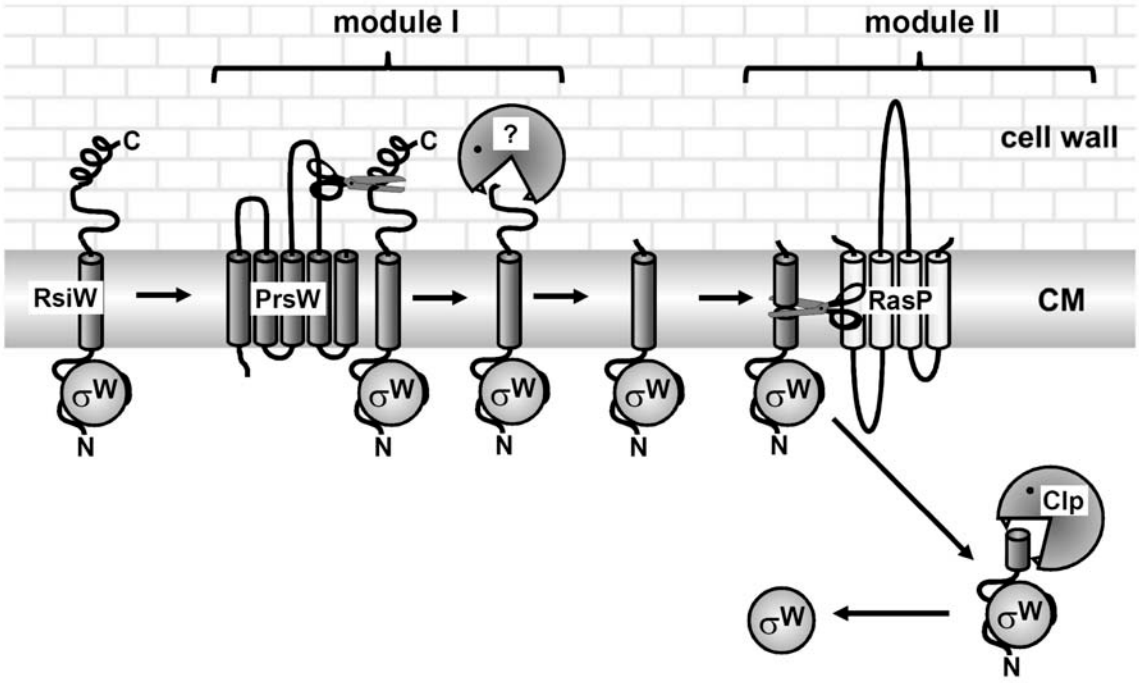

Fig. 7. Model for regulated intramembrane proteolysis of RsiW.
Two proteolytic modules are involved. In module I, PrsW cuts between Ala168 and Ser179 of RsiW to remove 40 extracytoplasmic C-terminal amino acid residues. This site-1 cleavage destabilizes the remaining extracytoplasmic domain of RsiW, rendering it a substrate for degradative extracytoplasmic bulk proteases like Tsp. Only then, RsiW that is shaved for nearly the entire extracytoplasmic part becomes a substrate for proteases of module II, which are first the processing site-2 protease RasP that uncovers a cryptic proteolytic tag, which is recognized by degradative cytoplasmic proteases like ClpXP.

Our present investigations were hampered by the fact that isolation of the active multispanning transmembrane proteases PrsW and RasP for *in vitro* experiments was not possible. However, reconstitution of PrsW as well as RasP catalysed proteolysis in *E. coli* has successfully been used earlier (Ellermeier and Losick, 2006; Bramkamp *et al.*, 2006; Heinrich and Wiegert, 2006). Using such a system, we could map the obvious PrsW processing site between amino acid residues Ala168 and Ser169, and we could show that this truncation of RsiW makes it susceptible to other general proteases like Tsp, to further degrade the extracytoplasmic part, both in *E. coli* and in *B. subtilis*.

The process of removal of bulk amino acids residues in the extracytoplasmic part seems to be crucial for RsiW to become a substrate for the site-2 protease RasP, as the direct PrsW site-1 product RsiW-S1 was not attacked by RasP in the reconstituted *E. coli* system.

Moreover, a fusion protein of the stable PhoA to the C-terminus of the designated site-1 cleavage product RsiWΔ40 is not entirely degraded in wild type background (Fig. 6, lanes 25, 26), further indicating that this form is unstable but not a direct substrate for RasP, and that the free C-terminus is crucial for efficient degradation by so far undefined proteases. Secondary structure prediction indicates an α-helix in the extracytoplasmic region of RsiW that is cleaved off by PrsW, and it will be the aim of future work to analyze, whether this helix interacts with another RseB-like component to shield RsiW from PrsW attack.

Tail-specific proteases, also designated as carboxyl-terminal processing peptidases (Ctp), have been reported earlier to be implicated in RIP. First of all, CtpB of *B. subtilis* is able to trigger activation of the sporulation sigma factor σ^K indirectly by cleaving the SpoIVFA protein that is part of a signalling complex together with the intramembrane cleaving protease SpoIVFB. SpoIVFA cleavage activates SpoIVFB to process membrane bound pro-σ^K to σ^K that is released to the mother-cell cytoplasm (Campo and Rudner, 2006). Second, Tsp is implicated in the Gram-negative pathogen *Pseudomonas aeruginosa* AlgU / MucA sigma factor / anti-sigma factor system. This system is homologous to *E. coli* σ^E / RseA and regulates genes involved in alginate production (Wood and Ohman, 2006). It became evident that MucA is controlled via RIP by the same components as RseA, i.e. AlgW (DegS) for site-1 cleavage, MucP (RseP) for site-2 proteolysis, and MucB (RseB). In addition, a small secretory protein (MucE) that might represent an outer membrane protein was identified, that, upon overexpression, induced mucoidy of *P. aeruginosa*. Like *E. coli* OmpC its C-terminal amino acid residues are believed to activate AlgW through interaction with its PDZ domain, although there are some variations in the recognition motif (Qiu *et al.*, 2007). Tsp is believed to affect alginate production in mucoid strains expressing truncated forms of MucA (e.g. *mucA22*), and inactivation or overexpression of *prc* did not affect alginate production in strains with wild type MucA (Reiling *et al.*, 2005; Qiu *et al.*, 2007). This could be explained by the fact that the truncated MucA form lacking 51 amino acid residues of the 90 amino acid residues extracytoplasmic domain can neither be attacked by AlgW nor directly by MucP,

and further truncation by Tsp is crucial to become a substrate for MucP. Because RIP of wild type MucA is dependent on AlgW but not Tsp, it has to be assumed that MucA is cleaved by the DegS homolog AlgW, like *E. coli* RseA, more proximal to the transmembrane region of the anti-sigma factor. This differs to the action of PrsW that obviously cleaves more distal. In contrast, truncation studies on *E. coli* RseA showed that RseP is able to process RseA165 and RseA140, lacking 51 and 75 C-terminal residues of the 98 residue extracytoplasmic part respectively, without prior action of DegS. As additional deletion of *rseP* stabilized Rse165 and RseA140, there seems to be no participation of Tsp or other proteases (Kanehara *et al.*, 2003).

Like RseP, RasP belongs to the diverse phylogenetic group I of S2P proteases that contains a PDZ domain (Kinch *et al.*, 2006). However, sequence alignment shows that RasP is not a direct homolog of RseP, as there is a high variation in interjacent amino acid residues between the three conserved transmembrane spanning segments. Notably, our data suggest that site-1 truncated RsiW depends on RasP for site-2 proteolysis and is not efficiently cleaved by *E. coli* RseP. This is in accordance with our observation that in contrast to the *rasP* gene, *rseP* does not complement a *B. subtilis rasP* knockout with respect to σ^W induction (our unpublished data). The recently published crystal structure of the *Methanocaldococcus jannaschii* S2P revealed that the three conserved transmembrane regions of S2P proteins constitute a polar channel that allows water entry to the embedded catalytic zinc atom (Feng *et al.*, 2007). Furthermore, two different forms of the molecule were crystallized; a closed conformation and an open conformation that would allow lateral substrate entry. A mechanism of substrate gating by transmembrane segments was proposed, however, S2P regions assigned for gating are not conserved and are absent in *E. coli* RseP as well as *B. subtilis* RasP. Our data can be interpreted in a way that the substrate channel of RasP is covered from the extracytoplasmic face by a structure that is able to accommodate only a limited number of residues at the C-terminal extracytoplasmic part of its substrate protein, but that site-2 cleavage is a passive event that takes place as soon as the major part of the extracytoplasmic domain of RsiW is shaved off. This is in agreement with the mechanism of RIP in eukaryotes. The site-1 proteolysis sites

of the three well investigated substrate proteins SREBP (sterol regulatory element binding protein), Notch, and APP (β-amyloid precursor protein) differ to a great extent, but the cleavage shortens the extracytosolic segment to less than 30 amino acids, which is a prerequisite for the secondary intramembrane proteolysis step. It is believed that bulk domains or distinct residues flanking the transmembrane region of uncleaved substrate proteins affect interaction with the site-2 protease (Brown *et al.*, 2000).

Two questions remain unanswered. First, it is unclear which protease(s) attack PrsW processed RsiW (RsiW-S1) in *B. subtilis*. PhoA fusions to the C-terminal end of RsiWΔ40 and the fact that single point mutations in the extracytoplasmic domain (Fig. 1) destabilize RsiW to become degraded in a PrsW independent manner point to the additional participation of proteases not related to Tsp. This hypothesis is supported by the fact that a double knockout of the *B. subtilis* Tsp homologs CtpA and CtpB is not impaired in σ^W induction. Also for the *E. coli prc* knockout strain HM101, Tsp independent truncation of PrsW processed RsiW could be detected in pulse chase experiments upon prolonged chase times (Fig. 3B). Therefore, it is most likely that C-terminal truncation of RsiW by PrsW or single point mutations massively destabilize the extracytoplasmic part of RsiW that will then be attacked by numerous extracytoplasmic bulk proteases that are produced by *B. subtilis*.

The second question is how site-1 proteolysis of RsiW is regulated that only after a stress signal PrsW becomes active. Two observations may give an answer. First, PrsW in the reconstituted *E. coli* system is constitutively active without a stress signal, suggesting that a negatively regulating element is missing. Second, RsiW becomes a constitutive substrate for site-1 proteolysis when overexpressed in *B. subtilis*, suggesting that a negatively regulating element is titrated by excess amounts of RsiW. Therefore, it seems that it is the extracytoplasmic domain of RsiW that interacts with a factor that prevents site-1 proteolysis, and it has to be assumed that this interaction is abrogated upon a stress signal. So far we were not able to identify this negatively regulating element, but experimental data show that it is not the RasP PDZ domain (data not shown).

In summary, we propose a model for RIP of RsiW that includes two proteolytic modules, each consisting of a processing

protease and one or more degrading proteases (Fig. 7). In this model, the only stress-related protease is PrsW, and it is an astonishing example for the concerted engagement of different proteases just to degrade one regulatory protein. Furthermore, we were for the first time able to characterize the cleavage site of a bacterial protease of the MEM-superfamily, which is not in the vicinity of the transmembrane part of the substrate. As cleavage is blocked by only one point mutation in the processing site, our results can contribute to the finding of specific inhibitors of MEM proteases.

Experimental Procedures

Bacterial strains and growth conditions.

Bacterial strains as well as plasmids used in this study are listed in Table 2. *E. coli* and *B. subtilis* strains were grown aerobically at 30°C or 37°C in Luria Broth (LB) or in mineral salts medium (Tanaka *et al.*, 1967) with 0.4% glycerol as a carbon source for pulse chase experiments. When necessary, LB was supplemented with ampicillin (100 µg/ml), phleomycin (1 µg/ml), neomycin (10 µg/ml), spectinomycin (100 µg/ml), chloramphenicol (10 µg/ml), tetracycline (10 µg/ml) or erythromycin (1 µg/ml or 100 µg/ml). If required, final concentrations of 50 µM or 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), NaOH (24 mM) or arabinose (0.02%) were added to liquid media.

ß-Galactosidase assays, Western blot and Pulse Chase experiments.

β-Galactosidase activities were measured as described elsewhere (Wiegert *et al.*, 2001). Western blots were performed as described previously (Homuth *et al.*, 1996) using a semi-dry or a tank blotting procedure (Biorad). For western blotting experiments polyclonal antibodies against β-lactamase of *E. coli* or *B. subtilis* RsiW, FtsH, Pbp4* at a dilution of 1:5000 or HtpG (1:10000) were used. Pulse-chase experiments were performed as described (Heinrich and Wiegert, 2006).

Plasmid construction

Deletions of 5, 10, 15, and 20 amino acid residues at the C-terminal end of RsiW were constructed using 5´oligo GGCCATGGATCCATGAGCTGTCCT GAACAAATTGTG (1) and the corresponding 3´ oligonucleotides GGCCATGCATGCTTAATTGAATGC ATCGAGTACACTTTTC, GGCCAT GCATGCTTATACACTTTTCCCCGC

AGACTTC, GGCCATGCATGCTTAA GACTTCATCTTGTACCATGTCCA or GGCCATGCATGCTTACCATGTCCA GTCGAATAACTGAT. The PCR products were inserted into the pAL-FLAG*rsiW* vector fragment via BamHI and PaeI. Resulting plasmids (pJAH-Δ5, pJAH-Δ10, pJAH-Δ15, pJAH-Δ20) were transformed to TW30. Transformants were selected on LB plus erythromycin (1µg/ml) and then screened for double crossover for erythromycin (100µg/ml) resistance and spectinomycin sensitivity.

Finally, wild type *rsiW* was knocked out by transformation of chromosomal DNA of *B. subtilis* 1012 *rsiW::spec*, yielding strain JAH97 (*rsiW*Δ5), JAH98 (*rsiW*Δ10, JAH99 (*rsiW*Δ15), and JAH100 (*rsiW*Δ20). Strain JAH100 was further combined with 1012 *prsW::bleo* (JAH101), 1012 *rasP::tet* (JAH102), or both (JAH103).

Intramembrane proteolysis of RsiW was analyzed in a reconstituted *E. coli* system essentially as described earlier (Heinrich and Wiegert, 2006). Plasmid pMA01 encodes the N-terminally 6His-tagged RsiW under control of a constitutive promoter. The genes for *prsW*, or *prsW-rasP* in tandem, were placed under arabinose control using plasmid pBAD33, which is compatible to pMA01.

Chromosomal DNA of *B. subtilis* 1012 was used as a template to amplify *prsW* using primers GGCCATGAGCTCGGA AAGAGGTTGCCTGATGTTTGC (2) and GGCCATGGATCCGTCGAC*TTA* AAGATCTTCTTCGCTAATAAGTTT TTGTTCTACTTGCATCATATTAACG GAACG (3). The 3' oligonucleotide (3) adds a c-myc tag to the C-terminal end of PrsW. The PCR product was restricted with SacI / SalI and ligated to pBAD33 cut with the same enzymes, resulting in plasmid pJAH17. The mutant allele *prsW* E75,76A was cloned likewise to yield plasmid pJAH18. Mutations were introduced using a PCR megaprimer method as described earlier (Schöbel *et al.*, 2004) with primer TTTTATCTTCAGGGTTTTTGGCGGC ATCATTAAAATGGTTTATACT (4). A first PCR reaction with the mutagenesis primer (4) together with (3) was used to amplify a 490 bp fragment. The resulting PCR fragment served as a megaprimer in a second PCR together with primer (2). The *rasP* gene with its ribosome binding site was PCR amplified with primers GGCCAT AGATCTTTAAGTATAAGAAGGAG ATATACATATGTTC and GGCCAT GTCGACATCCAATTGAGATCCTCA TTTCTTCGCACC and was placed downstream of *prsW* in pJAH17 and pJAH18 via BglII and SalI restriction

sites; to gain pJAH19 and pJAH20. All constructs were checked by DNA sequencing.

In order to map the processing site of PrsW in the *E. coli* Δ*prC* background, different C-terminal truncated forms of RsiW were constructed essentially as described for plasmid pMA01 (Heinrich and Wiegert, 2006) using 5'-primer GGCCATGGATCCATGAGCTGTCCTGAACAAATTGTG with one of the following 3'-primers GGCCATGCATGC*TTA*GACTTGTCCAGCAGAGGCCATAT (RsiWΔ35), GGCCATGCATGC*TTA*GGCCATATACTTTTCGCCGTTTA (RsiWΔ40), GGCCATGCATGC*TTA*GCCGTTTACGACGGTTACATCC (RsiWΔ45), and GGCCATGCATGC*TTA*TACATCCCCGTCTATTTTGCCTT (RsiWΔ50). PCR fragments were restricted with BamHI and PaeI and ligated to pBR322 cut with the same enzymes, resulting in pJAH35, pJAH36, pJAH37, and pJAH38.

For the exact determination of the PrsW cleavage site, different mutant alleles of *rsiW* (M167C, A168C, S169C and A170C) were constructed. The 5' oligonucleotide GGCCATGCATGCGGTACCGAATTCGAGCAAAGCGGCTAACGCGCT was used with one of the following 3' oligonucleotides GGCCATACCGGTGACTTGTCCAGCAGAGGCGCAATACTT,GGCCATACCGGTGACTTGTCCAGCAGAGCACATATAC, GGCCATACCGGTGACTTGTCCAGCACAGGCCATAT or GGCCATACCGGTGACTTGTCCACAAGAGGCCATATACTT in a PCR with chromosomal DNA of *B. subtilis* 1012 as a template. To gain pJAH40 (M167C), pJAH41 (A168C), pJAH42 (S169C) and pJAH43 (A170C), the PCR products were digested with AgeI and PaeI and ligated to the vector fragment of pMA01 cut with the same enzymes. Constructs were checked by restriction analysis and DNA sequencing.

As residues Ala168 and Ser169 in the PrsW processing site of RsiW are highly conserved, mutant alleles of *rsiW* were constructed with the megaprimer method as described earlier (see above) using primer GGCCATGCATGCGAGCAAAGCGGCTAACGCGCT (5) and GTCGTAAACGGCGAAAAGTATATGCAGTCTGCTGGACAAGTCACCGG or TAAACGGCGAAAAGTATATGGCCCAGGCTGGACAAGTCACCGGTCA in the first PRC, and the megaprimer and oligo number (1) in a second one. The following steps are the same as described for plasmids pJAH97-100 resulting in pJAH104 (RsiW-A168Q) and pJAH108 (RsiW-S169Q).

Plasmids were transformed to *B. subtilis* TW30 as described, yielding *B. subtilis* strains JAH104 and JAH108.

Strains *B. subtilis* TW32-FLAG as well as JAH100, JAH104 and JAH108 were combined with *prsW::bleo* and / or *rasp::tet* and analyzed by Western blotting and β-galactosidase tests.

To further analyze RsiW degradation in *B. subtilis*, we made use of translational fusions as a reporter, where the green fluorescent protein GFP was fused to the N-terminus and the Alkaline Phosphatase PhoA of *E. coli* to the C-terminus of RsiW. We made use of plasmid pHT01 that stably replicates in *B. subtilis* (Nguyen *et al.*, 2005). First, *gfp*$^+$ was PCR amplified with oligonucleotides GGCCATAGATCT ATGAGCGCTAGCAAAGGAGAA and GGCCATAGATCTGTTCAACTAGCA GACCATTATCAA and GGCCATCCC GGGCTGCAGGAGCTCTTAGGATCC TTTGTAGAGCTCATCCATGCCATG using plasmid pQE*gfp*$^+$ (Zellmeier *et al.*, 2006) as a template. The PCR product was restricted with BamHI / SmaI and ligated to pHT01 cut with the same enzymes. Next, the intrinsic BamHI site of gfp$^+$ was destroyed via silent mutagenesis, resulting in plasmid pHT*gfp*. In the next step, different forms of RsiW-PhoA were fused to GFP.

As a template, the $RsiW_{1-208}$PhoA and $RsiW_{1-112}$PhoA encoding regions on plasmids pQE30*rsiW phoA*-2 and pQE30*rsiW*-3 *phoA* were used (Schöbel *et al.*, 2004). In addition, mutated *phoA** with the four wild type cysteine residues changed to serine (Kihara *et al.*, 1999) was amplified with oligonucleotides GGCCATGATATCACGTGTTAACCG GGCTGCTCAGGGCGAT and GGC CATAAGCTTGGATCCTTATTTCAG CCCCAGAGCGGCTTT and pSTD422 (Y. Akiyama, unpublished) as a template. Wild type *phoA* of pQE30*rsiW phoA*-2 and pQE30*rsiW*-3 *phoA* were replaced by mutated *phoA** via Ecl136II and HindIII restriction sites. In addition, plasmid pQE30*rsiW*168-*phoA* was constructed as described for pQE30*rsiW-phoA*-2 using 3´oligonucleotide GGCCATAAGCTT GAGCTCGGCCATATACTTTTCGCC GTTTAC. For amplifying all constructs, oligonucleotides GGCCATGGATCCA TGAGCTGTCCTGAACAAATTG and GGCCATCCCGGGTTATTTCAGCCC CAGAGCGGC were used.

TABLE 2: BACTERIAL STRAINS AND PLASMIDS USED IN THIS STUDY

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80dlacZ ΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara,leu*) 7697 *gal*U *gal*K λ⁻ *rps*L *nup*G	(Grant *et al.*, 1990)
XL1-Blue	F'::Tn10 *proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/recA1 endA1 gyrA96*(Nal^r) *thi hsdR17* ($r_K^-m_K^+$) *glnV44 relA1 lac*	*S*tratagene
KS272	F- Δ*lacX*74 *galE galK thi rpsL*(*strA*) Δ*PhoA*(PvuII)[25]	(Meerman and Georgiou, 1994)
HM101	KS272 Δ*tsp3*::Ω*Kan*^{r} *eda51*::Tn10(Tet^r)	(Meerman and Georgiou, 1994)
HM105	KS272 *eda51*::Tn10(Tet^r)	(Meerman and Georgiou, 1994)
HM120	KS272 *degP*41(Δ*PstI*):: Ω*Kan*^{r}Δ*tsp3*::Ω*Kan*^{r} *eda51*::Tn10(Tet^r) Δ*ompT*	(Meerman and Georgiou, 1994)
KS474	KS272 *degP*41(Δ*PstI*):: Ω*Kan*^{r}	(Meerman and Georgiou, 1994)
CAG 33315	MC1061 Δ*degS* [Φλ*rpoH*P3::*lacZ*]	(Ades *et al.*, 1999)
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	(Saito *et al.*, 1979)
WB800	Δ*trpC* Δ*npr* Δ*aprE* Δ*eprE* Δ*vpr* Δ*bpf* Δ*mpr::ble* Δ*nprB::bsr* Δ*wprA::hyg*	(Murashima *et al.*, 2002)
QPB159	Δ*ctpA*::*tet*	(Pan et al., 2003)
QPB161	Δ*ctpB*::*tet*	(Pan et al., 2003)
QPB170	Δ*ctpA*::*cat* Δ*ctpB*::*tet*	(Pan et al., 2003)
1012 *prsW::bleo*	1012 *ypdC::bleo* (Bleo^R)	(Heinrich and Wiegert, 2006)
1012 *rasP::tet*	*tet* insertion in *rasP* (Tet^R)	(Schöbel *et al.*, 2004)
1012 *rsiW::spec*	*spec* insertion in *rsiW* (Spec^R)	(Schöbel *et al.*, 2004)
TW30	1012 *amyE::pyuaF-lacZ lacA*::*spec* (Spec^R, Neo^R)	(Heinrich and Wiegert, 2006)
JAH05	TW30 *rsiW::spec rasP::tet lacA*::pAL-6His*rsiW*	(Heinrich and Wiegert, 2006)
TW32-FLAG	TW30 *rsiW::spec lacA*::pAL-FLAG*rsiW;* in this work combined with *prsW::bleo* (JAH80) *rasP::tet* (Schöbel *et al.*,2004), and *prsW::bleo / rasP::tet* (JAH81)	(Schöbel *et al.*, 2004)
JAH100	TW30 *rsiW::spec lacA*::pAL-FLAG*rsiW* Δ20 combined with *prsW::bleo* (JAH101) *rasP::tet* (JAH102) or both (JAH103)	this work
JAH104-107	TW30 *rsiW::spec lacA*::pAL-FLAG*rsiW*-A168Q (JAH104), combined with *prsW::bleo* (JAH105) *rasP::tet* (JAH106) or both (JAH107)	this work

JAH108-111	TW30 *rsiW::spec lacA*::pAL-FLAG *rsiW*-S169Q (JAH108), combined with *prsW::bleo* (JAH109) *rasP::tet* (JAH110) or both (JAH111)	this work

Strain or plasmid	Relevant characteristics	Source or reference
Plasmids		
pMA01	pBR322 standard vector encoding 6His-RsiW	(Heinrich and Wiegert, 2006)
pJAH17	standard cloning vector pBAD33 with *prsW* controlled by arabinose regulatable promoter	this work
pJAH18	pBAD33 encoding mutated *prsW* E75,76A	this work
pJAH19 / 20	pJAH17 / 18 with *prsW* fused to *rasP*	this work
pJAH35-38	pMA01 encoding C-terminally truncated *rsiW* Δ35 (35), Δ40 (36), Δ45 (37), and Δ50 (38)	this work
pJAH40-43	pMA01 encoding His_6*rsiW* alleles M167C (40), A168C (41), S169C (42) and A170C (43)	this work
pJAH97-100	pAL-FLAG *rsiW* encoding RsiW truncated for 5 (97), 10 (98), 15 (99) and 20 (100) C-terminal amino acid residues	(Schöbel *et al.*, 2004) this work
pJAH104 / 108	pAL-FLAG *rsiW* A168Q (104) and S169Q (108)	this work
pHT-GFP	Plasmid for translational GFP^+ fusions	this work
pKH01-05	Encode translational fusions GFP-$RsiW_{1-208}$-PhoA(01), GFP-$RsiW_{1-208}$-PhoA* (02), GFP-$RsiW_{1-112}$-PhoA (03), GFP-$RsiW_{1-112}$-PhoA* (04), GFP-$RsiW_{1-168}$-PhoA (05)	this work

The PCR fragments as well as pHT-GFP were restricted with BamHI / SmaI and ligated to gain pKH01 (encoding GFP-RsiW$_{1-208}$-PhoA), pKH02 (encoding GFP-RsiW$_{1-208}$-PhoA*), pKH03 (encoding GFP-RsiW$_{1-112}$-PhoA), pKH04 (encoding GFP-RsiW$_{1-112}$-PhoA*), and pKH05 (encoding GFP-RsiW$_{1-168}$-PhoA). All plasmids were transformed to *B. subtilis* 1012 *rsiW::spec*, selected for chloramphenicol resistance and further combined with *prsW::bleo* and / or *rasP::tet*.

Nitrosoguanidin mutagenesis.

In order to identify mutants of TW30 *prsW::bleo* exhibiting functional intramembrane proteolysis of RsiW in a PrsW independent manner, random mutagenesis was performed with 1 % N-methyl-N-nitro-N-nitroso-guanidine according to standard protocols (Cutting and Vander Horn, 1990). Colonies were screened for a blue phenotype on LB plates with phleomycin and X-Gal. In order to test whether the observed phenotypes were due to a mutation in *rsiW*, clones were supplied with a wild type copy of *rsiW* via transformation with pAL-FLAG *rsiW* (Schöbel *et al.*, 2004). Under inducing conditions, all of the clones remained white on LB plates with X-Gal after induction of the ectopically integrated *rsiW*. PCR products amplified with chromosomal DNA of the mutants as a template and oligos (1) and (5) were cloned to pAL-FLAG via BamHI / PaeI as described for pJAH97-100 and *rsiW* alleles were DNA-sequenced with oligonucleotide (1). All of the obtained *rsiW* alleles that encoded RsiW degraded in a PrsW independent manner displayed single-point mutations in the extracytoplasmic domain of RsiW (Fig. 1).

Determination of the PrsW cleavage site by malPEG modification of engineered cyteins.

To identify the exact processing site of PrsW in RsiW, *E. coli* HM101 harboring pJAH17 together with pMA01, pJAH40, pJAH41, pJAH42 or pJAH43 and were grown in 3 l of LB. Expression of *prsW* was induced by the addition of 0,02 % of arabinose to the medium at an OD of 0.5 with further growth at 30 °C for 3 h. Cells were harvested by centrifugation and disrupted by sonification, and cell-free extracts were treated with malPEG according to a procedure described earlier (Akiyama *et al.*, 2004). It was not possible to detect signals for malPEG modified RsiW in Western blot experiments using specific RsiW

antibodies, probably due to low blotting efficiency of polyethylenglycol modified RsiW. Therefore 6His-RsiW was enriched using 2 ml of a NiNTA suspension according to a standard purification protocol (Qiagen, Hilden, Germany). Suitable elution fractions that still contained large amounts of contaminating proteins were submitted to preparative SDS-PAGE (Protean 2, Bio-Rad), and site-1 clipped RsiW was electro-eluted over night (Model 422 Electro-Eluter, Bio-Rad) in protein elution buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) according to the manufacturer's protocol.

The elution volume for each sample was approximately 600 µl. Samples were dialyzed against 100 mM Tris-HCL (pH 8.1), 1% SDS, and concentrated to a volume of 100 µl using Microcon Centrifugal Filter Devices (3000 MWMCO). Electro-eluted proteins were checked by Western blotting using a 5 µl sample volume and specific antibodies against RsiW. MalPEG modification was performed with 20 µl of each sample in a final reaction volume of 50 µl. First, a pre-incubation for 5 minutes at room temperature followed by 95 °C for 5 minutes in 100 mM Tris-HCL (pH 8.1), 1% SDS, 1 mM TCEP, 0.5 mM DTT was performed. Then, 2.5 mM malPEG was added and the mixtures were further incubated for 20 minutes at room temperature. Due to low blotting efficiency of malPEG-modified RsiW, 20 µl of each sample was analyzed by SDS-PAGE followed by silver-staining of the gels.

NaOH- shock experiments and preparation of B. subtilis cell fractions.

NaOH- shock experiments were performed as previously described (Schöbel *et al.*, 2004). Cells were harvested by centrifugation, washed and suspended in 1 ml of cold disruption buffer (50 mM Tris/HCl, 100 mM NaCl, pH 7.5) containing the Complete protease inhibitor cocktail (Roche). 1 ml of cell suspensions were sonicated (Cell Disrupter B15, Branson) on ice, followed by centrifugation at 5000 x g for 15 min at 4 °C to remove cell debris. Then, 800 µl of the supernatant was ultracentrifuged at 45000 x g for 1 h at 4 °C. The supernatant (cytoplasmic fraction) was removed and the resulting membrane pellet was washed with 500 µl of disruption buffer and ultracentrifuged again (45000 x g, 0.5 h, 4 °C). The protein content of the cytoplasmic fraction was estimated according to Bradford and the membrane

fractions were dissolved in an equivalent volume of Laemmli-buffer to a final concentration of 10 µg/µl. 10 µg of total protein was used for SDS-PAGE and Western blotting.

DNA manipulation, sequencing and computer analysis

DNA manipulation was performed according to standard procedures (Sambrook and Russell, 2005). For PCR reactions, the Deep $Vent_R$ DNA Polymerase (New England Biolabs, Inc.) was used. Standard DNA sequencing was carried out by a company (Seqlab, Göttingen, Germany) with appropriate sequencing primers.

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Chapter 4

The *Bacillus subtilis* ABC transporter EcsAB influences intramembrane proteolysis through RasP

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SUMMARY

The *Bacillus subtilis* σ^W regulon is induced by different stresses that most probably effect integrity of the cell envelope. The activity of the ECF sigma factor σ^W is modulated by the transmembrane anti-sigma factor RsiW that undergoes stress induced degradation in a process known as regulated intramembrane proteolysis, finally resulting in the release of σ^W and the transcription of σ^W–controlled genes. Mutations in the *ecsA* gene, encoding an ATP binding cassette of an ABC transporter of unknown function, block Site-2 proteolysis of RsiW by the intramembrane cleaving protease RasP (YluC). In addition, degradation of the cell division protein FtsL which represents a second RasP substrate is blocked in an *ecsA* minus strain. The defect of σ^W induction of an *ecsA* knockout strain could be partly suppressed by overproducing RasP. A *B. subtilis rasP* knockout strain displays the same pleiotropic phenotype as an *ecsA* knockout, which is a defect in processing α-amylase, in competence development, and in formation of multicellular structures known as biofilms.

Introduction

Genes of *Bacillus subtilis* controlled by the alternative ECF sigma factor σ^W constitute an antibiosis regulon (Helmann, 2002; Helmann 2006) responding to a variety of envelope stresses such as certain antibiotics (Cao *et al.*, 2002) and antimicrobial peptides (Pietiäinen *et al.*, 2005; Butcher & Helmann, 2006), but also by an alkaline shock and phage infection (Wiegert *et al.*, 2001). Its activity is modulated by RsiW, a transmembrane anti-sigma factor that sequesters and inactivates σ^W. Upon a stress signal, RsiW is degraded in a mechanism of regulated intramembrane proteolysis (RIP). In a concerted action at least three proteases in three different compartments of the cell degrade the RsiW anti-sigma factor in a sequential manner, finally resulting in the release of σ^W and the transcription of σ^W-controlled genes. The first protease that has been identified to be involved in that process is RasP (regulating anti-sigma factor protease; formerly YluC). RasP belongs to the group of Zn-dependent intramembrane cleaving proteases (iClips) and cleaves RsiW in its transmembrane domain after the extracytoplasmic part of the anti-sigma factor has been removed by a Site-1 protease (Schöbel *et al.*, 2004). The Site-2 degradation step catalysed by RasP uncovers a cryptic proteolytic tag that ensures further degradation of the RsiW remnant by cytoplasmic proteases, mainly ClpXP (Zellmeier *et al.*, 2006). These two steps are related to RIP of the σ^E anti-sigma factor RseA of *Escherichia coli* (Ades, 2004; Alba & Gross, 2004). RasP is an ortholog of *E. coli* RseP, and the concept of a cryptic proteolytic tag recognized by ClpXP has been described first for that system (Akiyama *et al.*, 2004; Flynn *et al.*, 2004). However, there is a marked difference in the Site-1 proteolytic step, because no dependency on *B. subtilis* Deg (Htr) proteases was found and the inducing stress is apparently different. The probable Site-1 protease of RsiW was identified by two different approaches. First, *B. subtilis* clones suppressing the toxic effect of SdpC, a protein which is involved in a cannibalism process of killing sibling cells upon entry the sporulation programme, were mapped in a gene now renamed as *prsW* (protease responsible for activating σ^W, formerly *ypdC*). (Ellermeier & Losick, 2006). It was shown that suppression was due to constitutive activation of the σ^W regulon,

which is known to confer resistance to the SdpC toxin in cells lacking the SdpI immunity protein (Butcher & Helmann, 2006). Second, a transposon screen with a reporter consisting of the green fluorescent protein (GFP) fused to the aminoterminus of RsiW was performed, and several transposon insertions in the *prsW* gene were identified to stabilize GFP-RsiW and to prevent Site-1 cleavage of the anti-sigma factor (Heinrich & Wiegert, 2006). In addition to *prsW*, transposon insertions in a second gene locus, *ecsAB*, showed marked stabilisation of the fluorescent reporter and prevented induction of σ^W. Here, we show that mutations in the *ecsA* gene block Site-2 proteolysis of RsiW by the intramembrane cleaving protease RasP.

Results

Mutations in B. subtilis ecsAB prevent induction of σ^W-controlled genes.

In a previous transposon screen we had made use of a GFP-RsiW reporter in order to identify genes involved in RIP of the anti-sigma factor (Heinrich & Wiegert, 2006). Different transposon insertions in *ecsA* or *ecsB* causing stabilisation of the GFP-RsiW reporter and abolishing induction of a transcriptional fusion of *lacZ* to the strong σ^W-controlled *yuaF* promoter were obtained. These genes encode the ATP binding cassette and the transmembrane part of an ABC transporter that has been characterized earlier (Leskela *et al.*, 1996) and orthologs of which can be found in a great variety of bacterial species. However, the function of EcsAB is still unknown. To analyse the obvious influence of the EcsAB ABC-transporter on degradation of RsiW, a *B. subtilis ecsA* knockout mutant was constructed by replacing major part of *ecsA* by a spectinomycine resistance gene (*ecsA::spec*). When compared to the wild-type, the *ecsA* knockout strain does not induce the *yuaF-lacZ* reporter following alkaline shock, a stress condition that strongly activates the σ^W regulon. (Fig. 1A, lanes 1, 2 compared to 7, 8). Northern blot analysis with riboprobes against the σ^W-controlled genes *yuaG* and *pbpE* revealed that the *ecsA::spec* strain is completely unable to induce the σ^W regulon, comparable to *rasP* or *sigW* knockout strains (Fig. 1B). In contrast, alkali induction of the σ^W independent *yhaU* gene remained unaffected.

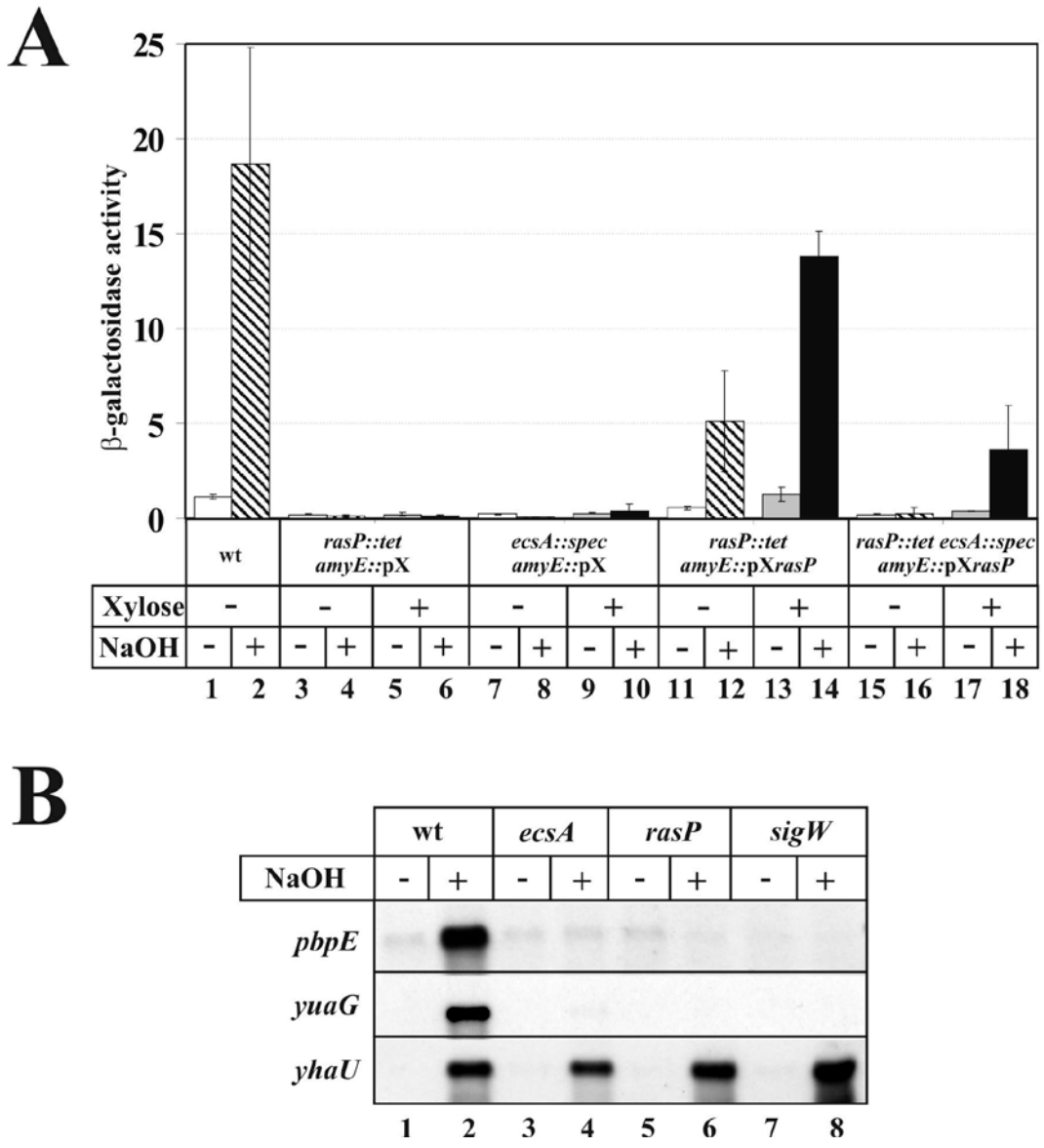

Fig. 1. *B. subtilis ecsA* mutant strains do not induce σ^W-controlled genes.
A. β-Galactosidase activities of *B. subtilis* strains containing a reporter of the strong σ^W-controlled *yuaF* promoter fused to *lacZ*. Xylose to a final concentration of 2 % was added to the cultures where indicated. NaOH shock experiments to induce σ^W were performed as described in the Materials and Methods section. Strains were BFS233 (1, 2), JAH12 *rasP::tet* (3- 6), JAH12 *ecsA::spec* (7 – 10), TW601 (11 – 14), and JAH14 (15 – 18). **B.** Northern-blot analysis of *B. subtilis* strain 1012 (1, 2) and isogenic knockouts of *ecsA* (3, 4), *rasP* (5, 6) and *sigW* (7, 8). An alkaline shock was performed at an OD_{578} of 0.7 by the addition of NaOH to a final concentration of 24 mM (pH 8.9) where indicated. Samples were drawn 10 min after NaOH addition and at the same time point for unshocked cells. 2 µg of total RNA was loaded to each lane. Riboprobes against σ^W-controlled *pbpE* and *yuaG*, and alkaline inducible *yhaU* were used.

To exclude that the failure to induce σ^W in the *ecsA::spec strain* is due to a polar effect on downstream genes, *ecsA* was ectopically expressed as an aminoterminally 3xFLAG epitope tagged protein under IPTG control. Alkali induction of the *yuaF* reporter fusion could be measured in the absence of IPTG already (Fig. 2A lanes 1 and 2), whereas full induction was restored in the presence of IPTG (Fig. 2A lanes 3 and 4).

To examine whether the effect of EcsA on σ^W induction was due to the absence of its catalytical activity or the absence of EcsA protein, an *ecsA* allele mutated in the catalytic Walker-B ATPase domain (E160Q) was expressed. Whereas both, 3xFLAG-EcsA and 3xFLAG-EcsA-E160Q were detectable in Western blots after IPTG addition (Fig. 2B), the E160Q mutant was unable to induce the *yuaF* reporter (Fig. 2A lanes 5- 8).

Spot-on-lawn assays according to a published procedure (Butcher & Helmann, 2006) showed that a sublancin producing strain spotted onto a lawn of a sublancin negative *ecsA::spec* strain generates a larger halo than on a lawn of a respective *ecsA* wild-type strain, indicating that σ^W-mediated sublancin immunity is absent in *ecsA* minus. In the same way, vancomycine treatment, an antibiotic that targets cell wall biosynthesis and induces the *B. subtilis* σ^W regulon (Cao *et al.*, 2002), did not induce the σ^W-controlled *pbpE* and *yuaG* genes in an *ecsA* minus strain, in contrast to the *ecsA* wild-type (data not shown). These experiments clearly demonstrate that EcsAB ABC-transporter activity is crucial for induction of σ^W, and that the failure for induction is not limited to specific σ^W-inducing stress signals.

B. subtilis ecsA minus accumulates Site-1 clipped RsiW.

The general failure of the *ecsA* mutant strains to induce σ^W, and the strong fluorescence of the GFP-RsiW reporter of transposon insertions, suggest that stress induced intramembrane proteolysis of RsiW is impaired in the absence of EcsAB activity. Therefore, the *ecsA::spec* mutation was introduced to strains expressing the GFP-RsiW and a GFP-RsiWΔ1 reporter, respectively. GFP-RsiWΔ1 is a constitutive substrate for RasP and not dependent on Site-1 proteolysis for degradation (Zellmeier *et al.*, 2006). Colonies of both strains are highly fluorescent, whereas in the *ecsA* wild-type background only weak fluorescence is visible (data not shown).

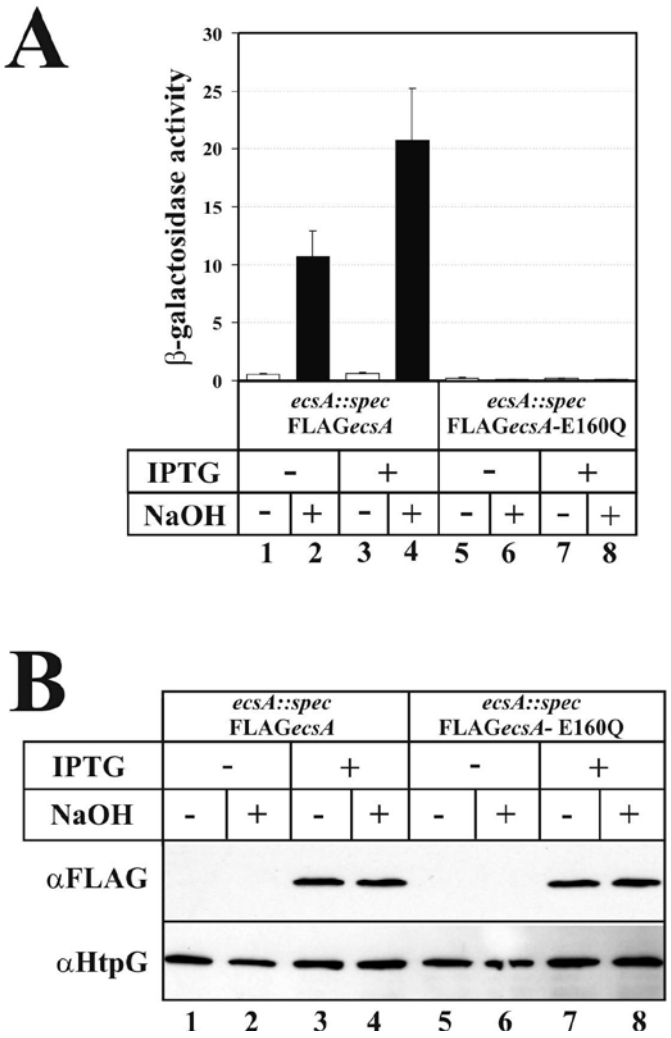

Fig. 2. Complementation of the *ecsA::spec* deletion.
NaOH shock experiments to induce σ^W were performed as described in the Materials and Methods section. IPTG to a final concentration of 1 mM was added to the cultures where indicated. Strains were *B. subtilis* JAH67 (1012 *ecsA::spec lacA::pAL-FLAGecsA amyE::*P_{yuaF}*-lacZ*) and JAH68 (1012 *ecsA::spec lacA::pAL-FLAGecsA-E160Q amyE::*P_{yuaF}*-lacZ*) **A.** β-Galactosidase activities. **B.** Western blots of whole cell extracts probed with antibodies against the FLAG epitope tag and, as a loading control, HtpG.

In Western blots, mainly the Site-1 clipped truncated form of GFP-RsiW is detectable in membrane fractions (Fig. 3A, compare lanes 3 and 9). GFP-RsiWΔ1, that is absent in the wild-type due to proteolysis by RasP and further cytoplasmic proteases (Zellmeier *et al.*, 2006), is stabilised in the *ecsA::spec* background (Fig. 3A, lanes 6, 12). Membrane fractions prepared from *B. subtilis* 1012 *ecsA::spec* cells cultured without and with a 10 min alkaline shock give strong signals for Site-1 clipped RsiW in Western blots and very weak signals for full length RsiW, both with and without shock, and just like the *rasP::tet* strain (Fig. 3B, lanes 3-6). For the wild-type control, mainly the full length RsiW is detectable only for non-shocked cells (Fig. 3B, lanes 1, 2). In conclusion, RasP catalysed intramembrane Site-2 proteolysis of RsiW does not take place in the absence of EcsAB activity, concomitantly with a deregulated Site-1 proteolysis that takes place without an external stress.

The intramembrane cleaving protease RasP is inactive in a B. subtilis ecsA minus strain.

To investigate whether the inability of RasP to attack Site-1 clipped RsiW or RsiWΔ1 in the absence of EcsAB is substrate specific for RsiW, degradation of a second RasP substrate was analysed. Recently it has been demonstrated that FtsL, a transmembrane protein of the *B. subtilis* cell division machinery, is attacked by RasP (Bramkamp *et al.*, 2006). Therefore, in analogy to the GFP-RsiW reporter, a GFP-FtsL fusion was constructed and expressed in different genetic backgrounds. Stability of the GFP-FtsL reporter protein was monitored by the fluorescence of whole cells grown on LB agar plates. Fluorescence in the wild-type and *prsW* knockout background is low, indicating that GFP-FtsL is unstable and degraded in a PrsW independent manner (Fig. 3C). The *rasP* minus strain as well as the *ecsA* minus strain are highly fluorescent, indicating that GFP-FtsL is proteolysed in a RasP dependent manner, and that this degradation is dependent on EcsAB activity. Interestingly, GFP-FtsL is also stabilised in a *clpP* minus background, meaning that the alanine residues in its transmembrane domain constitute a cryptic proteolytic tag as described for RsiW (Zellmeier *et al.*, 2006).

Taken together, the failure to degrade both RsiW and FtsL suggest that, in the absence of EcsAB activity, RasP is not functional.

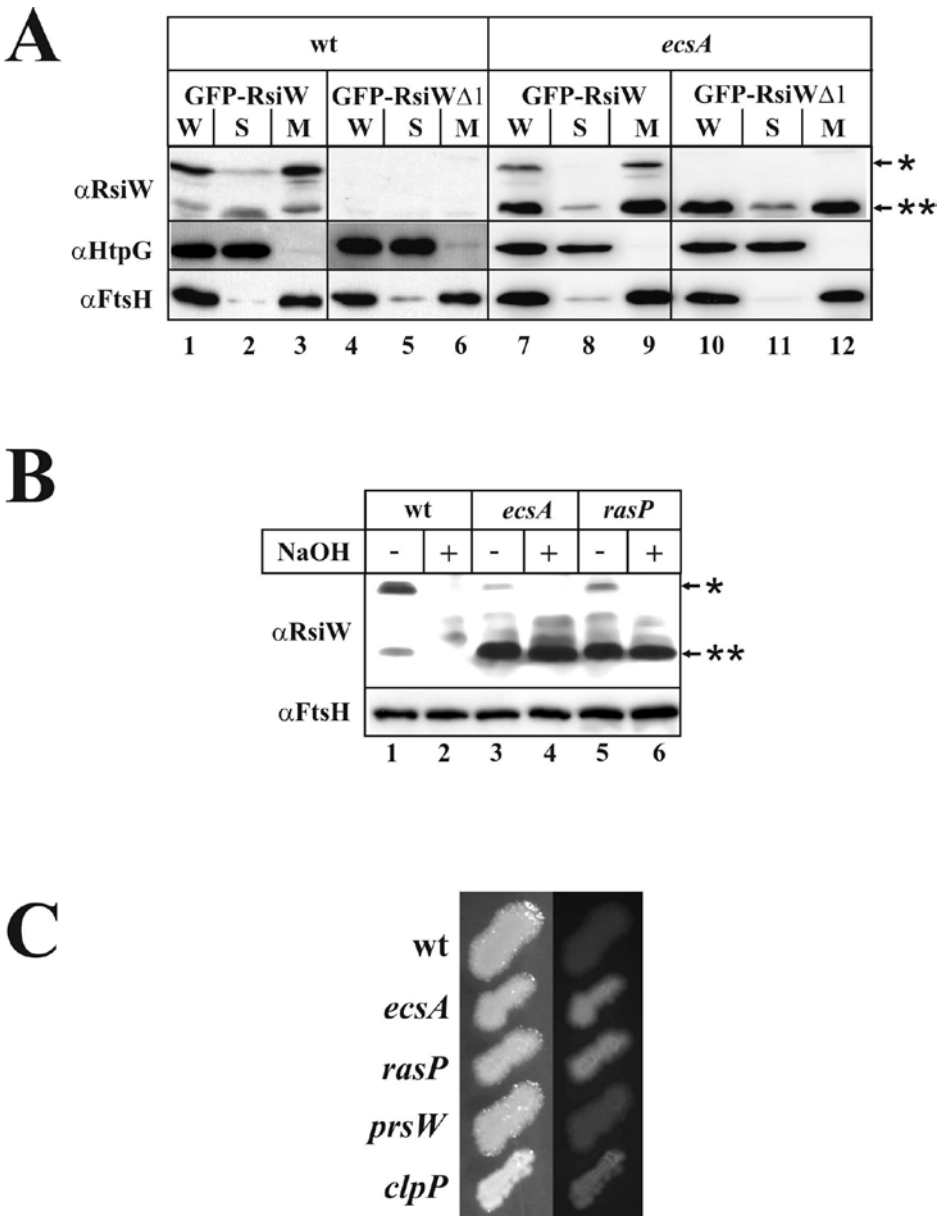

Fig. 3. RasP is inactive in the *ecsA* minus background.
A. Western blots of *B. subtilis* strains expressing reporter proteins of GFP fused to the amino terminus of RsiW and RsiWΔ1 (extracytoplasmic domain of RsiW missing) in wild-type (strains TW705, TW706) and *ecsA* minus background (TW705 *ecsA::spec*, TW706 *ecsA::spec*). Samples of cultures in the late exponential growth phase were withdrawn and cells were disrupted by sonication. To localize fusion proteins, whole cell extracts (W) were further fractionated to the membrane- (M) and soluble-protein fraction (S) by ultracentrifugation. Blots were developed with polyclonal antibodies against RsiW and, as a loading control, with polyclonal antibodies against FtsH and HtpG. The full length GFP-RsiW protein is marked with a star, the site-1 proteolysis product or RsiWΔ1 with two stars. **B.** Western-blot analysis of *B. subtilis* strain 1012 wild-type (1, 2), 1012 *ecsA::spec* (3, 4) and 1012 *rasP::tet* (5, 6). Cells were alkaline shocked as described above and samples were withdrawn 10 minutes after the shock and at the same time points for unshocked cells. Membrane fractions were prepared and analysed with antibodies against RsiW and FtsH. **C.** GFP-fluorescence of *B. subtilis* colony patches encoding the gene for a GFP-FtsL reporter protein in wild-type (JAH66), *ecsA* minus (JAH66 *ecsA::spec*), *rasP* minus (JAH66 *rasP::tet*), *prsW* minus (JAH66 *prsW::bleo*) and *clpP* minus (JAH66 *clpP::spec*) background. Left side: patches under normal light; right side: patches under UV light to monitor GFP fluorescence. The knockout strains for *ecsA* and *rasP* show enhanced fluorescence due to the block in FtsL degradation.

There might be two different explanations for this finding. First, RasP activity itself is directly or indirectly dependent on EcsAB. For example, EcsAB is known to influence correct localisation of secretory proteins (Leskela *et al.*, 1999) and, therefore, could be involved in correct membrane insertion of RasP. Second, an EcsAB substrate mislocated in the absence of the ABC-transporter activity inhibits RasP. To address this question, we overexpressed *rasP* under control of a strong xylose inducible promoter in the *ecsA::spec* background and analysed alkali induction of the σ^W-controlled *yuaF-lacZ* fusion. In the *ecsA* wild-type background, alkali induction of the reporter fusion was detectable already in the absence of xylose (Fig. 3D, columns 9-12), which is due to leakiness of the *xylA* promoter (Schöbel *et al.*, 2004). As it was expected, the isogenic *ecsA* knockout strain did not induce *lacZ*. However, after overproduction of RasP in the presence of xylose, there was a significant increase of β-galactosidase activity after alkaline shock (Fig. 1A, columns 15 - 18). One possible explanation for these findings is RasP being competitively inhibited by an EcsAB substrate, which is alleviated by increasing the concentration of RasP.

B. subtilis ecsA and rasP minus strains display similar pleiotropic phenotypes.

A point mutation defective for ATPase activity of EcsA (*ecsA26*) had originally been isolated in a screen for *B. subtilis* mutants unable to secrete overproduced α-amylase (AmyQ) (Kontinen & Sarvas, 1988). Further characterisation of this mutation revealed a pleiotropic phenotype. The mutant is impaired in processing of pre-AmyQ and three other secretory proteins, and its ability to sporulate and to become competent is decreased (Leskela *et al.*, 1999; Pummi *et al.*, 2002). In addition, a mutant for *ecsB* was described to be unable to produce a biofilm (Branda *et al.*, 2004). As RasP seems to be inactive in the *ecsA* minus strain, we wondered whether at least some of the defects listed above are correlated to *rasP*. The *rasP::tet* deletion, *sigW::erm* (Huang *et al.*, 1998) and *ecsA::spec* as controls, were introduced to strain IH6531 that harbours plasmid pKTH10 for *amyQ* overexpression (Kontinen & Sarvas, 1988). AmyQ activity of culture supernatants was determined.

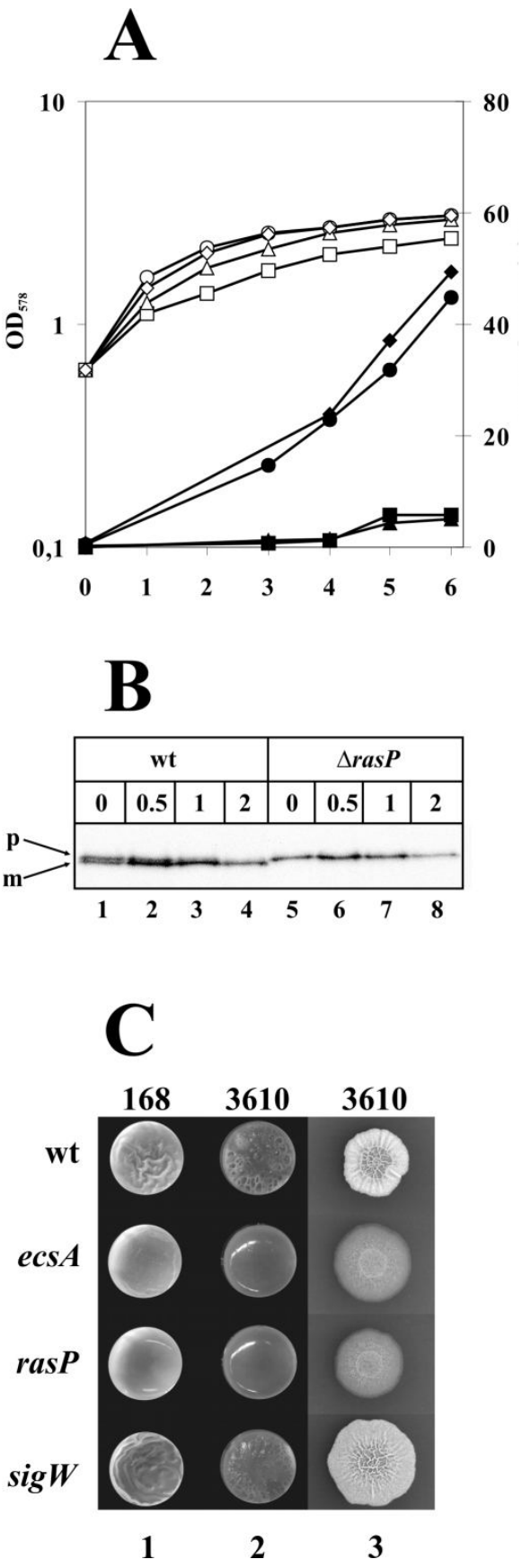

Fig. 4. *B. subtilis rasP* and *ecsA* minus strains display the same pleiotropic phenotype.

A. Effect of mutations of the *rasP*, *ecsA* and *sigW* genes on the secretion of α-Amylase (AmyQ). Cells of IH6531 (wild-type, pKTH10; ○), IH6531 *sigW::erm* (*sigW::erm*, pKTH10; ◊), IH6531 *ecsA::spec* (*ecsA::spec*, pKTH10; □), and IH6531 *rasP::tet* (*rasP::tet*, pKTH10; Δ) were grown in modified 2x LB broth, and culture medium samples were taken for the amylase assay. Open and solid symbols show growth and the concentration of AmyQ, respectively.

B. Effect of the *rasP* knockout on the rate of processing of AmyQ. The strains were subjected to pulse-chase labelling during exponential phase, samples were withdrawn at chase times as indicated (0 min, 0.5 min, 1 min, and 2 min). Each lane was loaded with material immunoprecipitated from 330 µl of culture. Precursor (p) and mature (m) forms of AmyQ are indicated.

C. Mutant alleles (*ecsA::spec*, *rasP::spec*, *sigW::bleo*) were introduced into *B. subtilis* strains 168 and NCIB3610. To assay pellicle formation (first and second columns), each mutant was inoculated at a low density into a standing culture consisting of 3 ml of MSgg medium in a microtiter plate well, and the cultures were incubated at 28°C for 60 h without shaking. To assay colony development (third column), each mutant derived from NCIB3610 was grown overnight in LB and a 3-µl sample of the culture was spotted onto MSgg agar and incubated at 28°C for 96 h.

For the wild-type strain and the isogenic *sigW* minus strain, high AmyQ activity is detectable, whereas the *rasP* as well as the *ecsA* minus strains show only about 10 % of the wild-type level (Fig. 4A).

In pulse chase experiments it becomes obvious that almost no processing of preAmyQ to its mature form takes place when *rasP* is deleted (Fig. 4B), as it has been described for the *ecsA*26 mutant strain (Leskela *et al.*, 1999). Next, transformation efficiency of respective strains was determined. For both *rasP::tet* and *ecsA::spec*, transformation efficiency was only about 0.2 % of the wild-type, for the *sigW* knockout strain 10 % efficiency of the wild-type was measured. Sporulation tests were performed by plating serial dilutions of samples of cells grown in sporulation medium without and with heat treatment. To our surprise, the sporulation rate of the *ecsA::spec* strain, and also for *rasP::tet*, was similar to the wild-type in three individual experiments. An additional control of an *ftsH::cat* strain, which is known to be defective for sporulation (Deuerling *et al.*, 1997), displayed a very low sporulation rate, confirming validity of the test.

Therefore, earlier observations of a sporulation defect of an *ecsA* minus strain (Kontinen & Sarvas, 1988) have to be revised. However, we observed rapid lysis of the *ecsA::spec* and the *rasP::tet* strains upon prolonged incubation in LB medium in stationary phase (data not shown), which might have been the reason for misinterpreting sporulation rates. The last phenotype we checked was the ability of respective strains to form structured multicellular communities, known as biofilm. To that purpose, the knockout mutations were introduced to the *B. subtilis* strains 168 and to the undomesticated NCIB3610, that is described to form a characteristic pellicle on liquid medium and a complex colony morphology on solid medium (Branda *et al.*, 2004). Both the *rasP* and the *ecsA* knockouts are clearly handicapped in forming structured biofilms and colonies (Fig. 4C). In summary, a *rasP* deletion strain displays the same pleiotropic phenotype as an *ecsA* deletion strain, making it reasonable that at least some of the defects of an *ecsA* mutant is related to the inactivity of RasP.

Discussion

ABC transporters translocate a great variety of substances into or out of cells and organelles and constitute one of the largest protein super families, representatives of which can be found in all organisms. Typically, they consist of two subunits of a hydrophobic protein with 6 transmembrane domains, and of two subunits of a hydrophilic protein that couples ATP hydrolysis to the transport process (Davidson & Chen, 2004). In bacteria, ABC-transporters either function as import systems, usually assisted by a soluble or membrane anchored solute binding protein, or as exporters of surface components (capsular polysaccharides, lipo-polysaccharides, teichoic acid), proteins involved in bacterial pathogenesis (hemolysin, heme-binding protein, alkaline protease), peptide antibiotics, heme, drugs and siderophores. For *B. subtilis*, at least 78 ABC transporters can be discerned, with 38 importers and 40 exporters. EcsAB was grouped to a subfamily of extruders related to antibiotic resistance systems, with similarities to possible efflux pumps of peptide antibiotics (Quentin *et al.*, 1999). The actual substrate for EcsAB and its function is unknown. The most interesting phenotype of an EcsA minus mutant is its inability to properly secrete AmyQ. The preAmyQ precursor protein remains cell associated but is accessible to tryptic digestion in protoplasts, pointing to a defect in its processing by signal peptidases. Moreover, overexpression of the *sipT* signal peptidase gene enhanced secretion of AmyQ in the *ecsA26* strain (Pummi *et al.*, 2002).

There is a clear defect of the intramembrane cleaving protease RasP activity in the *ecsA* knockout background, but the molecular basis for this effect remains enigmatic and at that stage we are only able to speculate. As there are no antibodies for RasP available and we were not able to detect epitope tagged RasP in membrane fractions of *B. subtilis* at all, it is not clear whether this defect is due to proper membrane insertion of RasP in the *ecsA* minus strain. However, the fact that *rasP*, controlled by a xylose inducible promoter, does not restore full RasP activity reveals that the absence of RasP activity is not due to a regulatory effect *ecsA* might have on the transcriptional level (Pummi *et al.*, 2002). In addition, the fact that RasP activity is restored when overexpressed favours a model of inhibition of existing RasP in the absence of the EcsAB ABC transporter. It will be an intriguing question what

substance(s) EcsAB transports, and it is conceivable that it will be peptides, possibly peptides that insert into the cytoplasmic membrane and therefore could interfere with RasP. Note that it has been suggested that ABC transporters are able to remove peptides like lantibiotics out of the cytoplasmic membrane (Otto & Götz, 2001), and a 'hydrophobic vacuum cleaner model' has been proposed (Otto & Götz, 2001; Stein *et al.*, 2005).

Another observation is that a *rasP* minus strain, like the *ecsA* knockout, does not process preAmyQ, which is not caused by their common inability to induce the σ^W regulon. It is not clear what impact RasP might have on processing of overexpressed AmyQ, and whether the processing defect in an *ecsA* minus strain is due to the inactivity of RasP. The only direct role RasP could play in secretion is a possible function as a signal peptide peptidase, as it has been proposed for the RasP ortholog RseP of *E. coli* because of its ability to cleave the β-lactamase signal peptide (Akiyama *et al.*, 2004). For prokaryotes in general, little is known about removal of signal peptides from the membrane. *E. coli* SppA (Protease IV) was shown to degrade the processed signal sequence of the major lipoprotein, but it is not the only protease that is responsible for signal peptide digestion in the cell envelope (Suzuki *et al.*, 1987). For *B. subtilis*, three proteins similar to SppA have been described (TepA, SppA, YqeZ; Bolhuis *et al.*, 1999; Helmann, 2002). Both, SppA and YqeZ are σ^W-controlled (Huang *et al.*, 1999; Wiegert *et al.*, 2001), and for YqeZ a function for immunity against sublancin rather than degradation of signal peptides has been shown (Butcher & Helmann, 2006). For SppA (YteI) and TepA (YmfB), a function in degradation of proteins or (signal) peptides that are inhibitory to protein translocation has been proposed (Bolhuis *et al.*, 1999). Signal peptide peptidases described for eukaryotes belong to the group of intramembrane cleaving proteases and attack certain signal peptides after they have been clipped from newly synthesized secretory or membrane proteins (Weihofen & Martoglio, 2003). It might be one possible function of RasP to degrade certain signal peptides in the membrane, and that in the absence of its activity accumulation of these peptides inhibit signal sequence processing by leader peptidases, as it has been shown *in vitro* for synthetic signal peptides (Wickner *et al.*, 1987). We have tried to discern (signal) peptides in isolated

membranes of *B. subtilis rasP* minus and other strains using different methods for several times, but so far methods proved to be problematic.

The inability of the *rasP* minus strains to produce a biofilm is another interesting feature. Biofilm formation in *B. subtilis* is a complex program that requires a variety of regulatory proteins and differential regulation of σ^D- and σ^H-dependent autolysins expressed at specific stages during pellicle formation (Kobayashi, 2007). RasP might play an important role in that process and points to a central role of the iClip. Noteworthy, a triple mutant of the ECF sigma factors σ^W, σ^X, and σ^M is also unable to produce structured communities (Mascher *et al.*, 2007), and it will be interesting to see whether the induction of σ^X and σ^M is dependent on regulated intramembrane proteolysis by RasP as well.

Taken together, we are at an early stage and a lot of questions remain to be answered. However, it is an intriguing question to unravel the role and connection of EcsA and RasP, as both of these proteins can be found in a great variety of prokaryotes. Both might represent a good target for new antimicrobial agents, as for example orthologs of RasP are involved in pathogenic processes (Makinoshima & Glickman, 2005; Makinoshima & Glickman, 2006).

Experimental Procedures

Bacterial strains, plasmids, and growth conditions.

Bacterial strains as well as plasmids used in this study are listed in Table 1. *E. coli* and *B. subtilis* strains were grown aerobically at 37 °C in Luria-Bertani medium (LB). When necessary, LB was supplemented with ampicillin (100 µg/ml), phleomycin (1µg/ml), neomycin (10 µg/ml), spectinomycine (100 µg/ml), chloramphenicol (10 µg/ml), tetracycline (10 µg/ml) or erythromycine (1 µg/ml or 100 µg/ml). Xylose was added at a concentration of 2 % and vancomycine at a concentration of 2 µg/ml when indicated. Pellicle formation experiments were performed according to a published procedure (Branda *et al.*, 2004). 50 µl mid-log phase culture in LB was inoculated into 10 ml minimal MSgg medium (5 mM potassium phosphate, pH 7, 100 mM MOPS, pH 7, 2 mM $MgCl_2$, 700 µM $CaCl_2$, 50 µM $MnCl_2$, 50 µM $FeCl_3$, 1 µM $ZnCl_2$, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, 50 µg ml^{-1} tryptophan, 50 µg ml^{-1} phenylalanine and

50 µg ml^{-1} threonine) and incubated at 28°C. For colony architecture analysis, 3 µl of LB-precultures were spotted onto minimal MSgg agar plates and incubated at 28°C.

β-Galactosidase and α-Amlyase assays, Northern and Western blot analysis.

β-Galactosidase activities and α-Amylase activities of culture supernatants were measured as described elsewhere (Wiegert *et al.*, 2001; Pummi *et al.*, 2002). Northern blot analysis was performed according to a published procedure (Homuth *et al.*, 1997) with antisense RNAs against *yuaG*, *pbpE* and *yhaU* (Zellmeier et al., 2006). Western blots were performed as described previously (Homuth *et al.*, 1996) using a semi-dry blotting procedure (Biorad, Trans Blot SD). Blots were developed with polyclonal antibodies against RsiW at a dilution of 1 : 4000, *B. subtilis* FtsH and HtpG (1:10000), and the FLAG epitope (1:5000).

NaOH- shock experiments and preparation of B. subtilis cell fractions.

NaOH- shock experiments were performed as previously described (Schöbel *et al.*, 2004). Cells were harvested by centrifugation, washed and suspended in 1 ml of cold disruption buffer (50 mM Tris/HCl, 100 mM NaCl, pH 7.5) containing the Complete protease inhibitor cocktail (Roche). Samples were adjusted to the same OD_{578} by dilution with cold disruption buffer. 1 ml of cell suspensions were sonicated (Cell Disrupter B15, Branson) on ice. 100 µl were removed (whole cell fraction, W) and the remaining 900 µl were centrifuged at 5000 x g for 15 min at 4 °C to remove cell debris. Then, 800 µl of the supernatant was ultracentrifuged at 45000 x g for 1 h at 4 °C. The supernatant (soluble fraction, S) was removed and the resulting membrane pellet (membrane fraction, M) was washed with 500 µl of disruption buffer, ultracentrifuged again (45000 x g, 0.5 h, 4 °C), dissolved in 100 µl of Laemmli buffer and heated for 5 min at 95 °C. The protein content of the W and S fractions was estimated according to Bradford, and 10 µg of total protein was loaded on each lane for SDS-PAGE and Western blotting. A volume equivalent to the S fraction was loaded for the M fraction, i.e. 1/8 of the volume of the S fraction containing 10 µg soluble protein.

TABLE 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80dlacZ ΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*) 7697 *gal*U *gal*K λ⁻ *rps*L *nup*G	(Grant *et al.*, 1990)
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	(Saito *et al.*, 1979)
168	*trpC2*	laboratory stock
NCIB 3610	undomesticated *B.subtilis* strain	(Branda *et al.*, 2001)
1012 *rasP::tet*	*1012 yluC::tet* (Tet^R)	(Schöbel *et al.*, 2004)
1012 *rasP::spec*	*spec*- insertion in *rasP* ($Spec^R$)	This work
1012 *prsW::bleo*	*1012 ypdC::bleo* ($Bleo^R$)	(Heinrich & Wiegert, 2006)
1012 *sigW::spec*	1012 with *spec*- insertion in *sigW-rsiW* ($Spec^R$)	(Schöbel *et al.*, 2004)
1012 *ecsA::spec*	1012 with *spec*- insertion in *ecsA* ($Spec^R$)	This work
1012 *clpP::spec*	1012 with *spec*- insertion in *clpP* ($Spec^R$)	(Zellmeier *et al.*, 2006)
IH6531	*glyB133 hisA1 trpC2* pKTH10 (Km^R)	(Leskela *et al.*, 1999)
IH8209	*hisA1 thr-5 trpC2* (IH6513 cured for pKTH10)	(Kontinen & Sarvas, 1988)
BFS233	pMUTIN strain with transcriptional fusion P*yuaF-lacZ*	(Schumann *et al.*, 2001)
TW705	*1012 amyE::gfp rsiW* (Cm^R)	(Zellmeier *et al.*, 2006)
TW706	*1012 amyE::gfp rsiW Δ1* (Cm^R)	(Zellmeier *et al.*, 2006)
JAH66	*1012 amyE::gfp ftsL* (Cm^R)	This work
JAH67	*1012 ecsA::spec lacA::pAL-FLAGecsA amyE::*P*yuaF-lacZ*	This work
JAH68	JAH67 with *lacA::pAL-FLAGecsA-E160Q*	This work
TW601	BFS233 *rasP::tet amyE::*P_x*rasP*	(Schöbel *et al.*, 2004)
JAH12	empty plasmid pX transformed to BFS233 as a control (BFS233 *amyE::*P_x)	This work
JAH14	BFS233 *rasP::tet ecsA::spec amyE::*P_x*rasP*	This work
Plasmids		
pJH07	pBR322 standard cloning vector with *ecsA* up- and down-stream regions flanking *spec* resistance gene	This work
pJH08	pBR322 standard cloning vector with *rasP* up- and down-stream regions flanking *spec* resistance gene	This work
pTW700	plasmid for translational gfp^+ fusions	(Zellmeier *et al.*, 2006)
pJH66	pTW700 with a gfp^+*-ftsL* fusion	This work
pJH67	encodes EcsA with FLAG epitope tag (pAL-FLAG*ecsA*)	This work
pJH68	pJH67 with Walker B mutation E160Q in *ecsA*	This work

Pulse-chase experiments, sporulation- and competence-tests.

Pulse-chase experiments were performed as described elsewhere (Leskela *et al.*, 1999) using strain IH6531 and derivatives thereof obtained by transformation of chromosomal DNA of knockouts of *ecsA* (1012 *ecsA::spec*), *rasP* (1012 *rasP::tet*), and *sigW* (*sigW::erm* of HB4246; Huang *et al.*, 1998). Competence was analysed as the transformation efficiency (Kontinen & Sarvas, 1988) and the sporulation frequency according to a standard procedure (Nicholson & Setlow, 1990) using strain IH8209 and respective knockouts (see above).

Construction of B. subtilis ecsA and rasP minus strains and SPP1 phage transduction.

The *ecsA* and *rasP* genes were inactivated by a deletion and insertion of a spectinomycine resistance gene. To delete *ecsA* , the gene and 5'/3'-flanking regions were PCR amplified using primers (1) 5'- GGCCATGTCGACCA CCTCATTTGACAATTTGCTTCA-3' and (2) 5'- GGC CATAAGCTT CCTGCTTCAAGTAAGGCTCCAT-3' and chromosomal DNA of *B. subtilis* 1012 as a template. The PCR product was restricted with *Sal*I / *Hin*dIII and ligated to the pBR322 vector fragment cut with the same enzymes, yielding plasmid pBR*ecsA*. An internal 450 bp *ecsA* fragment was replaced by restricting pBR*ecsA* with *Bam*HI / *Sac*I and inserting the spectinomycine resistance gene cut with the same enzymes that was PCR amplified with primers (3) 5'-GGCCATAAGCTT GGATCCATCGATTTGACATTTTTCT TGTGGA-3' and (4) 5' GGC CATAAGCTTGAGCTCGTAAGCA CCTGTTATTGCAATAAAA-3' and plasmid pK2-*spec* (Härtl *et al.*, 2001) as a template, resulting in plasmid pJH07. The *ecsA::spec* construct was PCR amplified with above primers (1) and (2), and the product was used to transform *B. subtilis* 1012. Chromosomal DNA of transformants (1012 *ecsA::spec*) resistant to spectinomycine were checked by PCR and by Southern blotting using a DIG labelled DNA probe of *ecsA* according to standard procedures (Sambrook & Russell, 2005). The *ecsA* knockout was combined with a transcriptional fusion of the σ^W-controlled *yuaF* promoter to *lacZ* by transforming chromosomal DNA of 1012 *ecsA::spec* to *B. subtilis* JAH12, which is a derivative of BFS233 containing the empty xylose control system of plasmid pX (Kim et al. 1996) integrated in *amyQ* .

As it was not possible to transduce the *rasP::tet* construct to *B. subtilis* strain NCIB3610, a *rasP::spec* construct was cloned by replacing the *ecsA*-5' and *ecsA*-3' regions of pJH07 with respective *rasP* up- and downstream regions that were PCR amplified using primers (5) 5'-GGCCATGTCGACTGTGATCGC ACAGCTCGGAAC-3', (6) 5'-GGCCAT GGATCCTATAACTGTATTCACGAA CATACCA-3', (7) 5'-GGCCATGAG CTCTTGTCACATGGAACGATATC CAG-3' and (8) 5'-GGCCATGAATTC CCTCATCGCGAACAAGCGAAG-3', resulting in plasmid pJH08. *B. subtilis* 1012 *rasP::spec* was constructed as described above. *B. subtilis* NCIB3610 does not become competent, therefore, respective knockouts were introduced via SPP1 phage transduction as described earlier (Kearns & Losick, 2003).

Complementation and site-directed mutagenesis of ecsA

To complement the *B. subtilis ecsA::spec* deletion strain, the *ecsA* gene was ectopically expressed under IPTG control. The *ecsA* gene was PCR amplified with primers (9) 5'-GGCCAT AGATCTATGTCTCTGCTATCGGTA AAAGAC-3' and (10) 5'-GGCCAT GCATGCTTATTCATGGCCAGCGT CTTCC-3', restricted with *Bgl*II and *Sph*I, and ligated to the vector fragment of plasmid pAL-FLAG*rsiW* (Schöbel *et al.*, 2004) cut with *Bam*HI and *Sph*I. The resulting plasmid pJH67 encodes a translational fusion of the 3xFLAG epitope tag to the aminoterminus of EcsA, which was integrated at the *lacA* locus using *B. subilis* strain 1012 *amyE::*P_{yuaF}*-lacZ lacA::spec* (Schöbel *et al.*, 2004). For a double cross-over event, transformants were screened for erythromycin resistance and spectino-mycine sensitivity, resulting in strain 1012 *amyE::*P_{yuaF}*-lacZ lacA::*pAL-FLAG*ecsA*. Finally, the *ecsA* gene was deleted by transformation of chromsomal DNA of the 1012 *ecsA::spec* strain. The resulting strain was named JAH67. An allele of *ecsA* with a mutation of the catalytic glutamate residue of the Walker B ATPase motif to glutamine (E160Q) was constructed by a two step PCR mega-primer method essentially as described earlier (Schöbel *et al.*, 2004), using primer (11) 5'-CCTGCG CTCTACATTATTGATCAGCCTTTTC TAGGGCTTGATC-3' and primers (9) and (10), yielding plasmid pJH68, which was then transformed to *B. subtilis* as described above for pJH67. The resulting strain (1012 *ecsA::spec amyE::*P_{yuaF}*-lacZ lacA::*pAL-FLAG*ecsA*E160Q was named JAH68.

Construction of a B. subtilis GFP-FtsL reporter fusion.

To test for stability of FtsL, a fusion of GFP to the aminoterminus of FtsL was constructed, essentially as described elsewhere (Zellmeier *et al.*, 2006). The *ftsL* coding region was PCR amplified using oligonucleotides (12) 5'-GGCCATAGATCTATGAGCAA TTTAGCTTACCAACCAGAG-3' and (13) 5'- GGCCATAGATCTAGCG CTTCATTCCTGTATGTTTTTCAC TTT-3' and chromosomal DNA of *B. subtilis* 1012 as a template, the product was restricted with Bgl*II* and ligated to plasmid pTW700 cut with the same enzyme. Correct orientation of the insert was checked by restriction analysis and DNA-sequencing, the resulting plasmid was named pJH66.

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Chapter 5

Regulated Intramembrane Proteolysis in the Control of ECF Sigma Factors

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SUMMARY

There is growing evidence that proteolytic degradation of membrane spanning regulatory proteins like anti-sigma factors is involved in a variety of important transmembrane signaling processes in bacteria. This mechanism of regulated intramembrane proteolysis (RIP) enables them to respond to extracellular signals and stresses. Here, we summarize the current knowledge on RIP controlling extracytoplasmic sigma factors.

1. Introduction

The cell envelope is one of the most important inventions of life. It does not only enclose the cell's content but protects it from environmental stresses and regulates transport and communication. Therefore, maintenance of the envelope's integrity and functionality in changing conditions is a major task for the cell to survive. To that purpose, transmembrane signaling pathways that sense and transduce environmental signals in order to regulate gene expression are of crucial importance. There are two major transmembrane signaling pathways in bacteria that regulate gene expression in response to envelope stress, which are two component systems and alternative sigma factors of the extracytoplasmic function (ECF) family (Raivio and Silhavy, 2001; Jordan *et al.*, 2008). In this review we will focus on the regulation of ECF sigma factor activity via regulated intramembrane proteolysis of a corresponding transmembrane anti-sigma factor.

2. ECF sigma factors

The existence of a subfamily of eubacterial RNA polymerase sigma factors that seemed to regulate extracytoplasmic functions was first described in 1994 (Lonetto *et al.*, 1994). These so called ECF sigma factors are classified as Group IV of the σ^{70} subfamily of sigma factors and constitute the largest and most divergent subclass (Helmann, 2002; Butcher *et al.*, 2008). Their structure, in comparison to the archetypal Group I, is characterized by a two-domain architecture of the highly conserved regions 2 and 4 that provide the minimal structure required for sigma factor activity; region 1.1 and most of region 3 are missing (Campbell *et al.*, 2003).

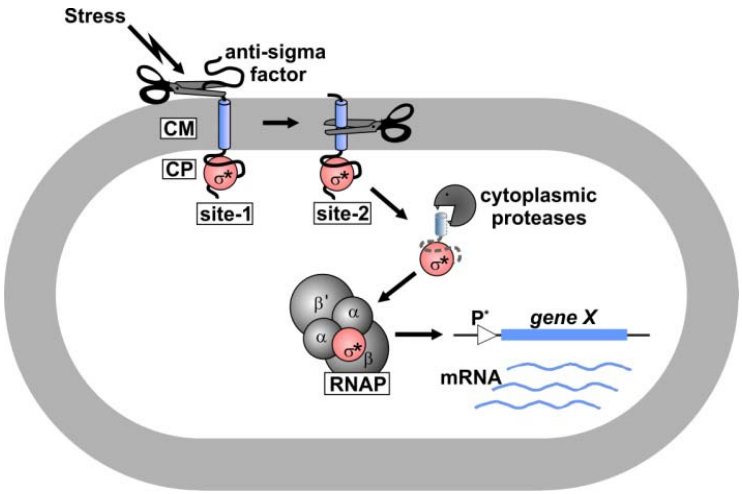
ECF sigma factors have been identified in a variety of Gram-negative and Gram-positive bacteria, and their number seems to increase with the complexity of lifestyle. Whereas *E. coli* encodes two of them (σ^E and FecI), *Bacillus subtilis* encodes 7 (σ^M, σ^V, σ^W, σ^X, σ^Y, σ^Z, YlaC), and e.g. for *Streptomyces coelicolor* about 50 are described (Helmann, 2002). Genes controlled by ECF sigma factors are often involved in processes related to transport, cell envelope integrity, and antibiosis, but also to certain stresses like oxidative conditions and heat (Helmann, 2002). ECF sigma factors are e.g. important to provide intrinsic immunity against antimicrobial compounds produced by

other microorganisms (Butcher and Helmann, 2006).

A minority of ECF sigma factors, like the well investigated σ^R of *S. coelicolor* (Kang *et al.*, 1999) and σ^E of *Rhodobacter sphaeroides* (Anthony *et al.*, 2004), regulates cytoplasmic stress responses, and, therefore, is controlled by a cytoplasmic anti-sigma factor. However, due to the fact that most ECF sigma factors respond to extracytoplasmic stimuli and stresses, their activity is regulated by one or more co-transcribed transmembrane anti-sigma factors that consist of an extracytoplasmic domain and an intracellular inhibitory domain that sequesters the ECF sigma factor from interaction with the RNA polymerase core enzyme (Helmann, 2002). Only for the Gram-negative and the Gram-positive model bacteria *E. coli* and *B. subtilis* the mechanism of ECF sigma factor liberation has been unraveled in detail. In both models, i.e. *E. coli* σ^E and *B. subtilis* σ^W, an environmental signal results in the complete proteolytic degradation of the corresponding transmembrane anti-sigma factor by the mechanism of regulated intramembrane proteolysis (Fig. 1).

3. Regulated intramembrane proteolysis

Transmembrane signaling pathways that involve the proteolytic cleavage of a membrane-spanning regulatory protein have been well investigated for eukaryotic models, where membrane tethered transcription factors are activated or factors involved in intercellular communication are emitted (Brown *et al.*, 2000; Urban and Freeman, 2002; Wolfe and Kopan, 2004; Ehrmann and Clausen, 2004). Because the signaling process is tightly regulated and triggered by intramembrane proteolysis, the mechanism was termed ‘regulated intramembrane proteolysis’ (RIP). In the past decade, emerging evidence revealed that RIP also plays a prominent role in a variety of important and highly controlled bacterial transmembrane signaling processes in stress response (Ades, 2008; Schöbel *et al.*, 2004), sporulation Rudner *et al.*, 1999; Yu and Kroos, 2000), cell division (Bramkamp *et al.*, 2006), cell cycle regulation (Chen *et al.*, 2005), quorum sensing (Stevenson *et al.*, 2007), pheromone and toxin production (An *et al.*, 1999; Matson and DiRita, 2005), and biofilm formation (Qiu *et al.*, 2007; Heinrich *et al.*, 2008).



Organsim	σ / anti-σ	Site-1	Site-2	Cytoplasm	Ref.
Escherichia coli	σ^E / RseA	DegS	RseP (YaeL)	ClpXP	[1; 2; 5; 11; 26]
Pseudomonas aeruginosa	AlgU / MucA	AlgW	MucP	ClpXP	[19; 63; 64; 66]
Bordetella bronchiseptica	HurI / HurR	n.d.	HurP	n.d.	[46]
Myxococcus xanthus	CarQ / CarR	n.d.	n.d.	n.d.	[13; 54]
Bacillus subtilis	σ^W / RsiW	PrsW (YpdC)	RasP(YluC)	ClpXP (ClpEP)	[27; 35; 68; 82]

Fig. 1: Model for the regulation of ECF sigma factor activity through transmembrane signaling that involves reg ulated i ntramembrane proteolysis (RIP). 'site-1' and 'site-2' represent the proteases implicated in that process. The table represents the ECF sigma factors that are reported to be regulated by RIP. There are strong indications that σ^E-homologous systems are present in pathogenic enterobacteriaceae [*Vibrio cholerae* (Kovacikova and Skorupski, 2002)*; Salmonella enteric* serovar *Typhimurium* (Kovacikova and Skorupski, 2002)] CM: cytoplasmic membrane; CP: cytoplasm; RNAP: RNA polymerase; n.d.: not determined to that time point.

Also, recent progress has established that RIP executes pivotal roles in the virulence of pathogens (Urban, 2009). The key step in RIP is catalyzed by a family of peptidases named intramembrane cleaving proteases (I-CLiPs) that catalyze cleavage of transmembrane domains of substrate proteins (Weihofen and Martoglio, 2003; Erez *et al.*, 2009). I-CLiPs described so far are multispanning membrane proteins and belong to three mechanistic groups of proteases. These are zinc metalloproteases (site-2 proteases; S2P), aspartyl proteases (presenilins and signal peptide peptidases), and serine proteases (rhomboids) (Weihofen and Martoglio, 2003).

For bacteria, only members of the S2P- and rhomboid families have been found in a great variety of species. With the exception of TatA activation by a rhomboid I-CliP (Stevenson *et al.*, 2007), all other bacterial transmembrane signaling pathways that involve RIP described so far make use of proteases of the S2P family. S2Ps possess the classical consensus HExxH motif of a metalloprotease and a conserved sequence (LDG) with an aspartate as the

third coordinating residue of the catalytic zinc atom. Rhomboids contain a GxSG motif which is characteristic for serine proteases of the chymotrypsin / trypsin / elastase family. The crystal structures of the rhomboid GlpG of *E. coli* and *Haemophilus influenzae* and of the S2P of the archaebacterial species *Methanocaldococcus jannaschii* have been resolved recently [reviewed in (Urban and Shi, 2008)]. The active sites are located within a folded, proteinaceous domain and sequestered from the surrounding lipids. GlpG from *E. coli* holds a water-filled cavity where the active-site residue Ser is placed, and it opens to the extracellular side. In contrast, *M. jannaschii* S2P has a polar channel that allows water entry from the cytoplasm to the catalytic zinc atom. In virtually all cases of transmembrane signaling by RIP, the intramembrane cleavage event is initiated by a preceding proteolytic processing in the extracytoplasmic part of the substrate protein, also referred to as site-1 proteolysis, which makes the substrate competent for site-2 proteolysis, the intramembrane cleavage step in RIP. Moreover, additional cytoplasmic proteases can be involved, so that in summary an extracytoplasmic signal triggers a concerted proteolytic cascade in three cellular compartments with participation of at least three different proteases to transmit information and elicit cellular responses (Fig. 1). The signals that trigger RIP and the type of responding site-1 proteases seem to be specific for respective processes; whereas site-2 proteases described so far are zinc metalloproteases of the S2P family.

4. Regulation of the *E. coli* σ^E-dependent envelope stress response by RIP

The regulation of the *E. coli* ECF sigma factor σ^E by RIP has been reviewed in a number of excellent articles (Ades, 2004; Alba and Gross, 2004; Ehrmann and Clausen, 2004; Brooks and Buchanan, 2008; Ades, 2008). Therefore, we will give only a short overview, and the reader might refer to these articles for further details.

σ^E was discovered as an essential alternative sigma factor in *E. coli* that responds to severe heat stress Erickson and Gross, 1989). Later it became evident that it controls an envelope stress response that is uniquely induced by disruption of protein folding in the periplasm, in particular by misfolded outer membrane proteins (Mecsas *et al.*, 1993). About 20 promoters are σ^E-controlled, among them the second

promoter of its own gene (*rpoE*), one of the four promoters of *rpoH* (*rpoH3*) encoding the heat shock sigma factor σ^{32}, promoters of four genes acting directly on the folding of *E. coli* envelope proteins (*dsbC*, *fkpA*, *skp*, and *surA*), the promoter of the periplasmic protease gene *degP*, and the promoter of the I-CLiP gene *rseP*. The *rpoE* gene encoding σ^E is cotranscribed with *rseA*, *rseB* and *rseC* (box in Fig. 2A). RseA is a transmembrane anti-sigma factor with a type II topology (N-in, C-out), whereas RseB is a soluble periplasmic protein that interacts with the extracytoplasmic part of RseA (Fig. 2A). RseC seems to exert no direct effect on modulating the σ^E -mediated stress response (Missiakas *et al.*, 1997; De Las Penas *et al.*, 1997). σ^E forms a complex with the anti-sigma factor RseA and binds to it with approximately 300-fold greater affinity than to core RNA polymerase (RNAP) (Campbell *et al.*, 2003). To release σ^E for interaction with RNAP, RseA is sequentially cleaved via RIP. Site-1 proteolysis is performed by DegS (Ades *et al.*, 1999), whereas site-2 cleavage is catalyzed by RseP (RseP was formerly named YaeL or EcfE) (Alba *et al.*, 2002; Kanehara *et al.*, 2002). Furthermore, cytoplasmic proteases like ClpXP are involved in complete degradation of RseA (Flynn *et al.*, 2004) (Fig. 2A).

4.1. Site-1 proteolysis of RseA by DegS

DegS is a homotrimeric serine-protease with each subunit anchored to the cytoplasmic membrane by an N-terminal transmembrane domain, the catalytic domain and a C-terminal PDZ-domain that controls proteolytic activity facing the periplasm (Hasselblatt *et al.*, 2007). Binding of peptides that derive from C-terminal residues of non-native outer membrane proteins (OMPs) like OmpC (consensus sequence -YXF-COOH) to the PDZ domain allosterically shifts equilibrium from an inactive state of DegS, in which the active site is unreactive, to a functional proteolytic conformation (Sohn and Sauer, 2009; Sohn *et al.*, 2007) to cleave RseA in a site-specific manner (Fig. 2A). RseB inhibits proteolysis by DegS *in vitro* by binding tightly to a conserved region near the C-terminus of the poorly structured RseA periplasmic domain, but the RseA sequences that mediate DegS recognition and RseB binding do not overlap directly. In addition, RseB inhibition is independent from the OMP signal that activates DegS (Cezairliyan and Sauer, 2007).

The *in vivo* role of RseB and probable inputs into the envelope-stress response that regulate RseB activity remain to be elucidated. The crystal structure of RseB has been resolved recently (Kim *et al.*, 2007; Wollmann and Zeth, 2007) and solution structures of RseB in complex with RseA were determined (Jin *et al.*, 2008). It is suggested that RseB might respond to additional cell envelope stress signals, e.g. to mislocalized lipoproteins.

4.2. Site-2 proteolysis of RseA by RseP

RseA periplasmatically truncated by DegS becomes substrate for the membrane-embedded RseP Zn-metallo-protease (Fig. 2A), which belongs to the S2P family of I-CLiPs. RseP cleaves site-1 processed RseA within its transmembrane domain (Alba *et al.*, 2002; Kanehara *et al.*, 2002). RseP has the potential ability to cut a broad range of membrane protein sequences, provided that there are residues of low helical propensity in the transmembrane regions (Akiyama *et al.*, 2004) that stabilize the substrate–RseP interaction. The third of four transmembrane segments of RseP (TM3) seems to directly bind to the substrate (Koide *et al.*, 2008). However, RseA is the only known *in vivo* substrate for RseP at present.

To ensure strict dependence of the site-2 proteolysis on the site-1 cleavage, negative regulatory elements suppress proteolytic action of RseP against full-length RseA. These elements include the tandem, circularly permutated PDZ domains (PDZ-N and PDZ-C) of RseP that are believed to interact with another component, which might be the Gln-rich sequences of RseA itself (Kanehara *et al.*, 2003; Inaba *et al.*, 2008). Also, in cells with both *degS* and *rseB* deleted, full length RseA is degraded in a RseP dependent manner, suggesting that RseB displays a role in modulating RseP activity (Grigorova *et al.*, 2004).

4.3. Complete degradation of truncated cytoplasmic RseA

Site-2 clipped RseA (RseA-S2) that is released to the cytoplasm still sequesters σ^E from interaction with RNAP unless it will be completely removed by cytoplasmic proteases. RseA-S2 ends with the amino acid sequence -VAA at its C-terminus, which represents a C-motif 1 for recognition by the ClpXP AAA^+ protease (Flynn *et al.*, 2003). Therefore, site-2 cleavage of RseA exposes a cryptic proteolytic tag that resembles the C-terminal SsrA-tag. The VAA-tag is recognized by the SspB adaptor protein, and delivers site-2

clipped RseA to proteolysis by ClpXP and probably other proteases (Flynn *et al.*, 2004; Chaba *et al.*, 2007).

The ATP-dependent unfolding activity of ClpX tears RseA-S2 apart from σ^E that now is free to bind to core RNAP and to initiate transcription of σ^E-controlled genes of the σ^E regulon (Fig. 2A).

5. ECF sigma factor regulation via RIP in other Gram-negative bacteria

The investigation of stress responses in pathogenic bacteria is of particular interest because these regulatory mechanisms enable them to survive host defense mechanisms. A significant role of σ^E-homologous envelope stress responses has been determined for the enterobacterial pathogens *Vibrio cholerae* (Kovacikova and Skorupski, 2002) and *Salmonella enterica* serovar *Typhimurium* (Humphreys *et al.*, 1999; Testerman *et al.*, 2002), *Haemophilus influenza* (Craig *et al.*, 2002), and *Pseudomonas aeruginosa* (Martin *et al.*, 1994; DeVries and Ohman, 1994), however, in most cases the role of RIP has not been investigated directly. σ^E of *V. cholera* and *S. Typhimurium* is induced by cationic antimicrobial peptides and confers resistance to them. For *V. cholerae* it was shown that the induction is dependent on the outer membrane protein OmpU that contains a C-terminal YDF-motif (Crouch *et al.*, 2005; Mathur *et al.*, 2007).

P. aeruginosa is an opportunistic pathogen that is one of the major causes of morbidity in cystic fibrosis (CF). It is able to produce large amounts of the polysaccharide alginate that causes the mucoid colony morphology of the bacterium. It is believed that alginate production is responsible for biofilm formation that enables the bacterium to survive host defenses and antibiotic treatment. Transcription of the large alginate biosynthetic operon *algD-algA* is controlled by the ECF sigma factor AlgU and its anti-sigma factor MucA, and clinical isolates from CF patients of alginate overproducing strains of *P. aeruginosa* are usually mutated in *mucA* (Ramsey and Wozniak, 2005). Recent work showed that the AlgU / MucA sigma factor / anti-sigma factor system is homologous to *E. coli* σ^E / RseA and is controlled by RIP by the same components (Fig. 1), i.e. AlgW (DegS), MucP (RseP) and MucB (RseB). In addition, a small secretory protein (MucE) that might represent an outer membrane protein was identified that, upon overexpression, induce mucoidy of *P. aeruginosa*. Like *E. coli* OmpC, its

C-terminal amino acid residues are believed to activate AlgW through interaction with its PDZ domain, although there are some variations in the recognition motif (Qiu *et al.*, 2007).

It also became evident that ClpXP is crucial to degrade the cytoplasmic portion of truncated MucA (Qiu *et al.*, 2008). Biochemical data point to differences in the control of AlgW activity by its PDZ domain in comparison to DegS (Cezairliyan and Sauer, 2009). In *Bordetella bronchiseptica*, a pathogen of humans and animals that colonizes the respiratory tract, an S2P (HurP) is essential for heme-dependent induction of the gene for an outer membrane heme receptor (BhuR) and downstream genes. HurP, which is able to complement *V. cholerae* YaeL, is believed to function in RIP of HurR, an anti-sigma factor of the ECF sigma factor HurI (King-Lyons *et al.*, 2007) (Fig. 1).

The Gram-negative model bacterium *Myxococcus xanthus* has a complex life cycle. Under starvation conditions cells swarm together to build mounds and intricate fruiting bodies, and it is also able to form heat resistant spores. One of its features is the light induced synthesis of carotenoids as a protection mechanism against damages caused by light, regulated by the *carQRS* operon. It was shown that CarQ is an ECF sigma factor (Martinez-Argudo *et al*., 1998) and CarR the corresponding transmembrane anti sigma factor (Browning et al., 2003) (Fig. 1). CarR becomes unstable upon exposure of the cells to light, and it was speculated that it is degraded by RIP in analogy to *E. coli* RseA (Browning et al., 2003).

6. ECF sigma factor regulation via RIP in Gram-positive bacteria: the *B. subtilis* σ^W regulon

Since the *B. subtilis* genome sequencing project revealed seven ECF sigma factors (Kunst *et al.*, 1997), intensive work has been performed to define regulons and to assign function for the genes controlled by these alternative sigma factors. Each of the ECF sigma factors proved to be dispensable under standard laboratory growth conditions, and even a sevenfold deletion mutant is viable (Asai *et al.*, 2008). In general, the investigations are mainly hampered by the fact that for all theses ECF sigma factors there is no clear inducing signal and the molecular basis for induction is largely unknown. One of the best characterized ECF sigma factor regulons of *B. subtilis* is the σ^W regulon. Consensus searches with the autoregulated promoter of the *sigW*

operon and a method of *in vitro* run off transcription combined with DNA macroarray analysis (ROMA) identified about 60 genes controlled by σ^W (Helmann, 2002). In addition, global analyses of the *B. subtilis* transcriptome after pH stress revealed the σ^W regulon to be strongly induced by alkaline shock (Wiegert *et al.*, 2001). To date, for about half of the genes of the σ^W regulon a function could be assigned. As these σ^W-controlled genes display a prominent function in the defense of antibiotics like fosfomycin and antimicrobial peptides, but also in detoxification and cell envelope synthesis, the genes are believed to constitute an antibiosis regulon that reacts on cell envelope stress (Helmann, 2006). Besides alkaline stress, which most probably acts in an artificial manner, phage infection (Wiegert *et al.*, 2001), salt stress (Petersohn *et al.*, 2001), antimicrobial peptides (Pietiäinen *et al.*, 2005; Butcher and Helmann, 2006) and certain antibiotics like vancomycin (Cao *et al.*, 2002) induce σ^W. The *sigW* gene is cotranscribed with a second gene (box in Fig. 2B) that encodes a single-pass transmembrane protein with type II-topology and that negatively regulates σ^W-activity through direct interaction of its N-terminal cytoplasmic region with σ^W (Schöbel *et al.*, 2004). Because of this classical anti-sigma factor activity, it was named *rsiW* (regulation of σ^W) (Helmann, 2002). Our group could show that RsiW undergoes stress-induced RIP in a proteolytic cascade finally resulting in the complete degradation of RsiW and in the release of σ^W (Fig. 2B). The general mechanism, which is the first example of RIP regulating ECF sigma factor activity in a Gram-positive bacterium, resembles the *E. coli* σ^E / RseA system; however there are striking differences that will be outlined below.

6.1. Site-1 proteolysis of RsiW by PrsW

When it became evident that RsiW undergoes stress-induced RIP to release σ^W comparable to *E. coli* RseA, it seemed likely that also a DegS-like serine protease contributes to site-1 proteolysis of RsiW (Schöbel *et al.*, 2004). *E. coli* DegS belongs to the family of HtrA proteases, three of which are encoded in the *B. subtilis* genome [HtrA / YkdA, HtrB / YytA, HtrC / YyxA (Noone *et al.*, 2000)]. However, single and multiple knockouts of these proteases were not impaired in inducing σ^W following alkaline shock (Schöbel *et al.*, 2004; Heinrich and Wiegert, 2006).

Finally, two different experimental approaches identified the monocistronic *prsW* gene (protease responsible for activating σ^W; formerly *ypdC*) most probably encoding a novel site-1 protease (Ellermeier and Losick, 2006; Heinrich and Wiegert, 2006). First, *prsW* was mapped as a gain-of-function mutant constitutively activating the σ^W regulon (Ellermeier and Losick, 2006), which is known to provide intrinsic resistance to the SdpC-toxin in cells lacking the SdpI immunity protein (Butcher and Helmann, 2006). Second, a transposon screen with a reporter consisting of the Green Fluorescent Protein (GFP) fused to the amino terminus of RsiW was performed. Several transposon insertions in the *prsW* gene were identified that stabilize GFP-RsiW and prevent site-1 cleavage of the anti-sigma factor (Fig. 2B) (Heinrich and Wiegert, 2006).

Both investigations could show that *prsW* is the only determinant of site-1 proteolysis of RsiW in a reconstituted *E. coli* system. In the database of Clusters of Orthologous Groups of proteins (COGs) PrsW belongs to COG2339, where multispanning membrane proteins of unknown function found in Gram-positive and Gram-negative bacteria as well as some Archaea are grouped. On the basis of the HHpred algorithm, Ellermeier and Losick (2006) found similarities of PrsW to the COG1266 group. This group shares three conserved sequence motifs with the eukaryotic type II CAAX prenyl endopeptidase family. One member of the type II CAAX prenyl endopeptidase family is the yeast RCE1 protease (Ras and a-factor converting enzyme) that removes the last three amino acid residues of the C-terminal CAAX motif of prenylated a-factor and Ras in *Saccharomyces cerevisiae*. COG1266 and the type II CAAX prenyl endopeptidase family had been grouped earlier to a superfamily of probable membrane embedded metalloproteases, in the following abbreviated as MEM-superfamily, that were suspected to be potentially involved in protein and / or peptide modification and secretion (Pei and Grishin, 2001).

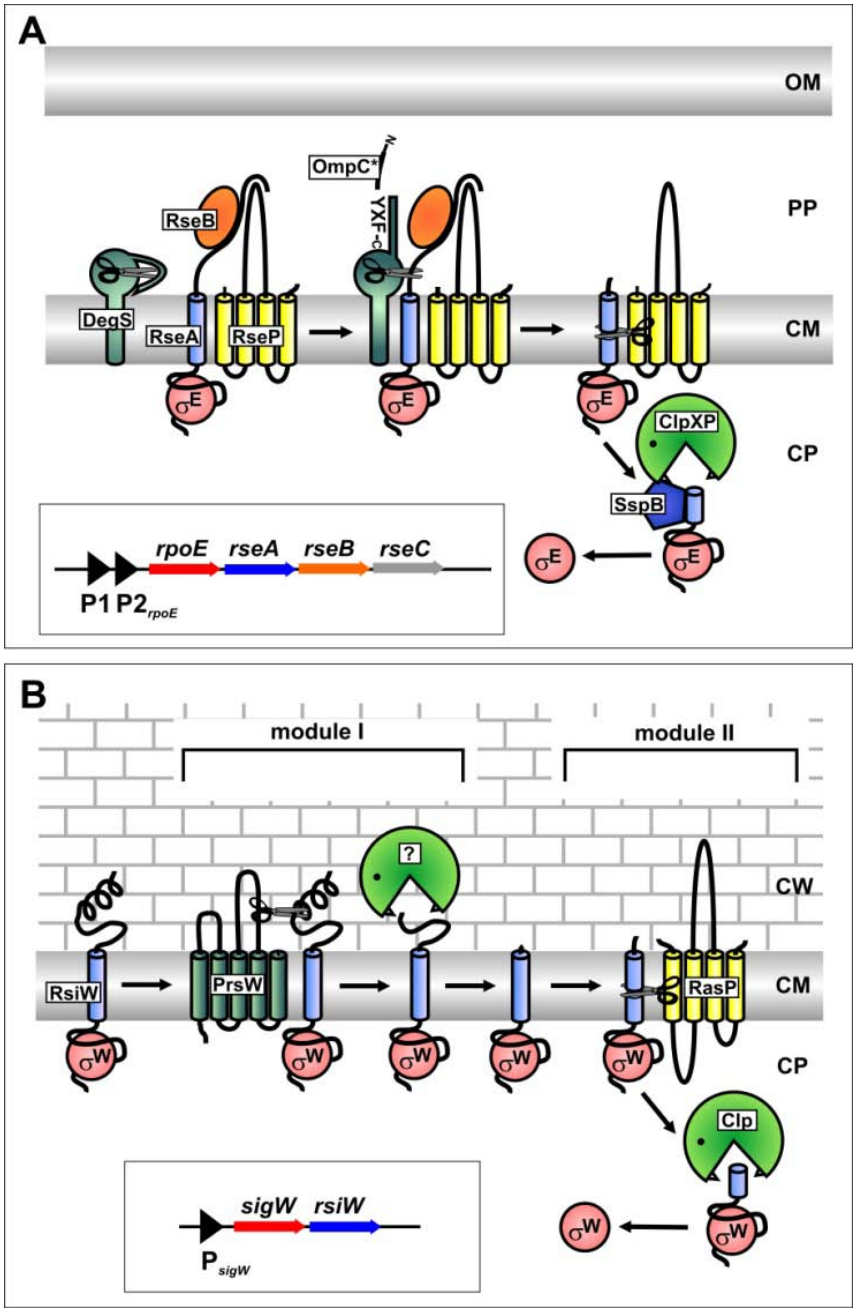


Fig. 2: Model for regulated intramembrane proteolysis of anti-sigma factors.
A. *E. coli* RseA is cleaved by DegS (site-1 proteolysis). DegS is a periplasmic serine protease that is activated by non-native outer membrane proteins like OmpC (OmpC*) that emerge e.g. upon heat shock. Truncated RseA is then cleaved by the intramembrane cleaving protease RseP (site-2 proteolysis). This step releases RseA to the cytoplasm and uncovers a cryptic proteolytic tag at the newly formed C-terminal end of the truncated anti-sigma factor. Under participation of the SspB adaptor protein, truncated RseA is then completely degraded by the ClpXP protease. **B.** For *B. subtilis* RsiW, two proteolytic modules are involved. In module I, the stress induced PrsW-catalyzed site-1 clipping of the 40 extracytoplasmic C-terminal amino acid residues makes RsiW a substrate for degradative, so far undefined, extracytoplasmic bulk proteases (labeled with a question mark). Only then, RsiW that is trimmed for nearly the entire extracytoplasmic part becomes a substrate for proteases of module II, which are first the processing site-2 protease RasP that uncovers a cryptic proteolytic tag, which is recognized by degradative cytoplasmic proteases like ClpXP. Genetic organization of the genes of the *rpoE* operon and the *sigW* operon are depicted in the boxes. For more details see text. CP: cytoplasm; CM: cytoplasmic membrane; PP: periplasm; OM: outer membrane; CW: cell wall.

As members of COG2339 including PrsW posses the highly conserved potential active site glutamate and histidine residues, they seem to belong to the large MEM-superfamily.*In vitro* assays to directly demonstrate protease activity of PrsW were not possible so far, mainly because purification of the protein failed. The molecular signal(s) that activates the site-1 proteolytic step and other regulatory factors that might be involved in degradation of RsiW remain elusive to that time point. Our recent results suggest that site-1 cleavage catalyzed by PrsW takes place at position 40 amino acids apart from the C-terminal end of RsiW, between Ala168 and Ser169, and that further proteases trim this site-1 clipped form of RsiW in an unspecific manner to make it a substrate for intra-membrane proteolysis (Heinrich *et al.*, unpublished).

6.2. Site-2 proteolysis of RsiW by RasP

RasP (regulating anti-sigma factor protease, formerly YluC) was the first protease that was identified influencing RsiW stability (Fig. 2B), because in a *rasP* deletion strain alkaline stress induced degradation of RsiW and induction of σ^W was abolished (Schöbel *et al.*, 2004).

RasP belongs to the S2P-family of I-CLiPs. As well as for *B. subtilis* SpoIVFB, which is involved in processing the sporulation sigma factor pro-σ^K, its homology to human S2P and a possible role in RIP had been recognized earlier (Brown *et al.*, 2000).

Like *E. coli* RseP, RasP is predicted to contain four transmembrane helices and an extracytoplasmic PDZ domain. It cleaves site-1 truncated RsiW within the transmembrane segment adjacent to conserved alanine residues (Zellmeier *et al.*, 2006). Truncated RsiW missing the entire extracytoplasmic domain is a constitutive substrate for RasP (Schöbel *et al.*, 2004). The mechanism by which proteolytic action of RasP against full-length RsiW is suppressed is not known so far, but sterical occlusion of full length RsiW is most likely. Remarkably, in a *rasP* minus background site-1 degradation of RsiW seems to take place in the absence of an inducing signal, suggesting that RasP modulates PrsW activity. In addition to RsiW, the cell division protein FtsL was identified being a RasP substrate (Bramkamp *et al.*, 2006).

For unknown reasons, the ABC transporter EcsAB is crucial for RasP activity, as in the absence of its activity site-2 cleavage of RsiW does not take

place (Heinrich *et al.*, 2008). The function of EcsAB is still unknown, but it belongs to a subfamily of extruders related to antibiotic-resistance systems, with similarities to possible efflux pumps for peptide antibiotics. Both, *B. subtilis rasP* and *ecsA* minus strains are viable, but display a similar pleiotropic phenotype, i.e. a defect in processing and secretion of α-amylase, a defect in competence development, and a defect in the formation of multicellular structures known as biofilms.

6.3. Other proteases involved in stress-induced degradation of RsiW

In the same manner as described for *E. coli* RseA, the intramembrane proteolysis step catalyzed by RasP uncovers a cryptic proteolytic tag at the C-terminal end of site2-clipped RsiW that ensures complete degradation of the inhibitory domain in the cytoplasm mainly by ClpXP (Fig. 2B) (Zellmeier *et al.*, 2006). Conserved alanine residues in the transmembrane part of RsiW are of crucial importance for this process, and a triple mutation (A91L, A92L, A93L) prevents cytoplasmic degradation but not site-2 proteolysis. ClpE seems to be able to replace the the ClpX ATPase subunit in a *clpX* minus strain background. Overexpression of SsrA-tagged GFP as a ClpXP substrate reduces induction of σ^W-controlled genes following alkaline shock, suggesting that a titration mechanism is able to tune the σ^W-mediated stress response to the cellular state, e.g. down regulation of σ^W-induction when other severe stress conditions produce non-native proteins as ClpXP substrates.

As outlined above, recent results show that PrsW cleaves RsiW more distal to the transmembrane helix between amino acid residues Ala168 and Ser169. Furthermore, most of the PrsW-processed extracytoplasmic part of RsiW has to be removed from the C-terminal end by one or more other proteases to make truncated RsiW a substrate for RasP. These protease(s) are not identified for *B. subtilis* so far. However, in the reconstituted *E. coli* system the tail-specific protease Tsp (Prc) is responsible (Heinrich *et al.*, unpublished). Therefore, RIP of RsiW seems to involve two proteolytic modules, each consisting of one site-specific processing protease (site-1: PrsW; site-2: RasP) that uncovers a cryptic proteolytic recognition signal, and more unspecific degrading proteases (site-1: unknown peptidase(s); site-2: Clp peptidases).

7. Conclusions

In contrast to eukaryotic systems, where the process of RIP has been studied for a variety of different vital processes, the important role of intramembrane proteases in bacterial transmembrane signal transduction pathways now becomes more and more manifested. Recent progress has established that intramembrane proteases execute crucial functions in the pathogenicity of microorganisms (Urban, 2009; Makinoshima and Glickman, 2006). For example, an S2P-family protease has been connected to the regulation of cell envelope composition and *in vivo* growth and persistence of *Mycobacterium tuberculosis* in its host (Makinoshima and Glickman, 2005). It seems likely that there is a link of intramembrane proteolysis to the control of one or more of the ten ECF sigma factors *M. tuberculosis* encodes. Also, recent data revealed that an as yet uncharacterized ECF sigma factor (σ^S) of *Staphylococcus aureus* appears to be an important component of the stress and pathogenic responses of this organism. However, if and how σ^S activity is regulated via RIP remains to be elucidated (Shaw *et al.*, 2008). It will be an exciting question whether others of the numerous ECF sigma factors prokaryotes encode, especially in Gram-positive bacteria, are regulated via RIP.

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Chapter 6

General discussion
(Synopsis)

Janine Heinrich

6.1 Identification of genes affecting RIP of the anti-sigma factor RsiW

The major scope of this thesis was to identify and characterize the so far unknown site-1 protease involved in RIP of the anti-sigma factor RsiW of *B. subtilis*. In my preceding diploma thesis, I had established a reporter-system consisting of an N-terminal fusion of the Green Fluorescent Protein (GFP) to RsiW that enabled to monitor stability of the anti-sigma factor. Because the GFP-RsiW fusion protein is constantly degraded in the *B. subtilis* wild-type background, it proved to be a suitable reporter to search for factors that influence RIP of RsiW. With a transposon screen, several gene loci were identified where an insertion of the mobile element resulted in the stabilization of the reporter, visualized by enhanced fluorescence of colonies. Two of these gene loci were of particular interest, *ypdC* and *ecsAB*, because site-1 and site-2 proteolysis was affected, respectively.

6.2 Identification of the site-1 protease

Transposon insertions at several positions in the *ypdC* gene all resulted in a stabilization of the GFP-RsiW fusion protein. Together with other multispanning membrane proteins of unknown function, found in Gram-positive and Gram-negative bacteria as well as in Archaea, YpdC belongs to the COG2339 cluster of Orthologous groups of proteins. However, as YpdC does not display obvious similarities to proteins of known function, it was not sure whether it represents a protease or an additional factor involved in RIP of RsiW.

Western blot experiments clearly showed that YpdC is a determinant of site-1 cleavage. In the absence of *ypdC*, the full length RsiW is stabilised in the membrane and not degraded upon alkaline shock. As a consequence, σ^W-controlled genes are not induced and the intrinsic resistance of *B. subtilis* cells to antimicrobial peptides like sublancin is abolished. These *ypdC* knockout phenotypes could be complemented by ectopic expression of *ypdC*. A direct proof for proteolytic activity of YpdC was not possible, because purification of the membrane protein for *in vitro* assay failed. Therefore, a reconstituted system in *E. coli* was designed. Here, induction of *ypdC* led to site-1 cleavage of RsiW without a stress signal.

At the same time, Ellermeier and Losick (2006) found YpdC as a determinant of site-1 proteolysis in a completely different experimental approach. Induction of the σ^W-regulon is known to provide intrinsic immunity against the SdpC toxin. They isolated gain of

function mutants of *ypdC* that constitutively activates σ^W-controlled genes. They also executed experiments in a reconstituted *E. coli* system. Moreover, they performed database searches with a specific algorithm that revealed similarities of YpdC to COG1266. This group shares three conserved sequence motifs with the eukaryotic type II CAAX prenyl endopeptidase family. The only member of this family with known function is the yeast RCEI protease (Ras and a-factor converting enzyme) that removes the last three amino acid residues of the C-terminal CAAX-motif of prenylated a-factor and Ras in *Saccharomyces cerevisiae*. Both, COG1266 and the type II CAAX prenyl endopeptidase family, belong to a superfamily of probable membrane-embedded metalloproteases (MEM-superfamily) which are proposed to be involved in protein and / or peptide modification and secretion (Pei and Grishin, 2001). YpdC itself possesses the highly conserved potential active site glutamate and histidine residues of COG2339. Based on these data, they proposed YpdC to belong to the MEM-family of more distantly related membrane-embedded metalloproteases. It was concluded that YpdC represents a novel site-1 protease, renamed PrsW (protease responsible for activating σ^W) (Ellermeier and Losick, 2006).

In eukaryotes, the subtilisin-like serine protease S1P was the first site-1 protease described. S1P is anchored in the membrane of the endoplasmatic reticulum (ER) with the active site facing the lumen. It is involved in the activation of dormant transcription factors like the chaperone inductor ATF6 of *Saccharomyces cerevisiae* and the membrane-bound SREBP (Brown and Goldstein, 1999). In the absence of site-1 processing by S1P, mice die during embryogenesis (Yang et al, 2001). Other examples for site-1 proteases are signal peptidases that also employ a catalytically active serine residue. They are e.g. important for the processing of the signal peptide in maturation of the major histocompatibility complex C (MHC) class I via RIP (Lemberg et al, 2001). In the brain, the aspartyl protease β-secretase removes the bulk of the β-amyloid precursor protein before γ-secretase is able to cleave the protein (Selkoe, 2001). This process plays a key role in Alzheimer's disease.

At present, only few examples for peptidases that catalyse site-1 cleavage in the process of RIP are described for bacteria. Whereas corresponding I-CliPs almost exclusively belong to the S2P family, there is a greater variability for the type of site-1 proteases that are specialized to a certain signal. Examples are the serine proteases DegS of *E. coli* and its homolog AlgW of the Gram-negative pathogen *Pseudomonas aeruginosa*, and the

aspartic acid protease PerP of *C. crecentus* (Chen *et al.*, 2006). Lipoprotein signal peptidase SPaseII, which is believed to belong to an unusual class of aspartic acid proteases, serves as a site-1 protease in a process to release the octapeptide pheromone cAD1 that mediates conjugation of *E. faecalis* (An and Clewell, 2002).

With PrsW, we have identified a metalloprotease catalyzing a site-1 cleavage of an ECF anti-sigma factor that substantially differs from the well investigated site-1 cleavage of *E. coli* RseA by DegS [chapter 2 (Heinrich and Wiegert, 2006)]. DegS belongs to the HtrA family of oligomeric, single-pass transmembrane serine proteases that are anchored to the membrane by the N-terminus. The larger C-terminal soluble part of the monomer extends into the periplasm and forms a cooperative trimer with three proteolytic domains (Clausen *et al.*, 2002). The protease activity of each monomer is allosterically inhibited by a regulatory C-terminal PDZ-domain (Hasselblatt *et al.*, 2007; Wilken *et al.*, 2004). Envelope stress causes the occurrence of non-native outer membrane proteins like OmpC in the periplasm. The C-terminal ends of these proteins (i.e. the Omp-signal) interact with the PDZ domains of DegS and induce a conformational change that uncovers the catalytic site of DegS to become active to cleave RseA in a site-specific manner (Walsh *et al*, 2003; Sohn *et al.*, 2007).

In contrast, PrsW is predicted to be a metalloprotease without a PDZ domain, and the nature of PrsW activation is still unknown. Two observations suggest that a negatively regulating element is involved. PrsW in the reconstituted *E. coli* system is constitutively active without a stress signal and overexpression of *prsW* in *B. subtilis* results in a deregulated activation of σ^W. Therefore, it seems that it is the extracytoplasmic domain of RsiW that interacts with a factor that prevents site-1 proteolysis, and it has to be assumed that this interaction is abrogated upon a stress signal. So far, we were not able to identify this negatively regulating element, but experimental data show that it is not the RasP PDZ domain (our own unpublished results).

6.3 Characterisation of PrsW

PrsW is the first member of the MEM-superfamily of putative membrane-embedded metalloproteases with a defined function and substrate. Therefore, structural and functional analyses were performed to investigate its membrane topology and properties in detail. PrsW contains highly conserved twin glutamate residues and an additional histidine residue that most probably coordinate a catalytically active metal-ion.

Site-directed mutagenesis of these residues abolish site-1 cleavage of RsiW (Ellermeier and Losick, 2006; our own unpublished results). The orientation of these active site residues and the topology of PrsW is still not clear, and different models for PrsW topology were predicted. In all cases, PrsW is a polytopic membrane protein with four to six transmembrane helices.

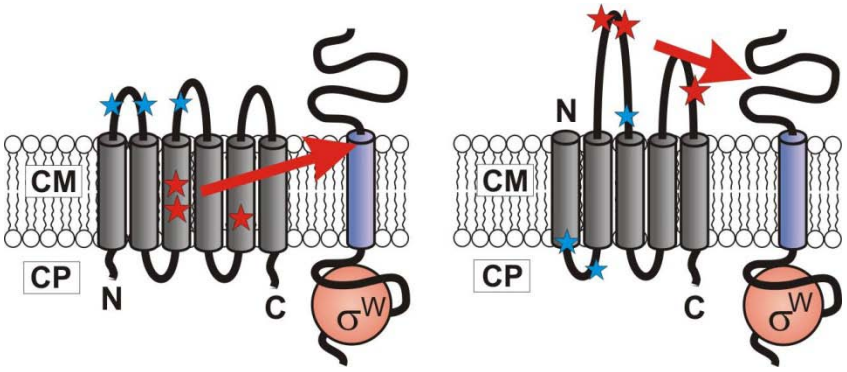

Fig.1 Two different models of membrane topology of PrsW and location of active site residues. Predicted transmembrane domains of PrsW are illustrated in grey, the anti-sigma factor RsiW-transmembrane helix is depicted in blue. Red arrows point to the positions of cleavage in RsiW that are suggested by the two models. Red stars indicate active site residues conserved in COG2339 superfamily members (E75A, E76A, and H175A) that are predicted to coordinate zinc. Shown as blue stars are the amino acid substitutions that rendered PrsW constitutively active (D23G, E28A, and E95K). The first model is adapted from Ellermeier and Losick (2006). CM: cytoplasmic membrane, CP: cytoplasm.

Ellermeier and Losick (2006) postulated the active site residues within the lipid environment of the membrane. In their model (Fig.1), the 'gain of function' mutations that constitutively activate proteolytic activity of PrsW are facing the extracytoplasm. They speculated that these residues constitute a receptor site for antimicrobial peptides such as SdpC, in which electrostatic contacts are made with a complementary cluster of positively charged amino acids.

In order to estimate membrane topology of PrsW in an experimental approach, I constructed fusions of the alkaline phosphatase (PhoA) to different positions in PrsW. Only in the reducing environment of the extracytoplasm PhoA is active. Therefore, PhoA serves as a topology marker. Results obtained propose a model with five transmembrane helices, and the active site residues are located in one of two larger extracytoplasmic loops of PrsW (Fig.1).

To analyze site-1 cleavage and substrate requirements of PrsW in more detail, a novel reconstituted system for *E. coli* was designed, described in chapter 3 (Heinrich *et al.*, 2009). With this system it became obvious that in *E. coli* PrsW-catalysed site-1 proteolysis produces a truncated form of RsiW, which differs in size compared to the site-1 cleavage product detectable in *B. subtilis*. In addition, a random screen identified point mutations in the extracytoplasmic domain of RsiW that render RsiW to RIP in a PrsW independent manner. As one mutation encodes for a stop-codon, some successive C-terminal truncations of RsiW were constructed. Deletion of more than five amino acid residues of the extracytoplasmic domain resulted in an activation of σ^W independently of an alkaline shock. For both, C-terminal truncations and point-mutations in the extracytoplasmic domain of RsiW, the assumed site-1 cleavage product was detectable in the *rasP* minus background. We concluded that the designated site-1 product is not necessarily the direct cleavage product of PrsW, and possibly additional proteases are involved in further trimming of PrsW-cleaved RsiW.

To examine such an involvement of additional peptidases between site-1 and site-2 cleavage of RsiW, the reconstituted system was combined with different knockouts of *E. coli* periplasmic proteases. For a *tsp*-minus strain, PrsW induction resulted in the detection of an RsiW site-1 cleavage product of higher molecular mass. For the reconstituted system, it became evident that RsiW site-1 cleaved by PrsW is indeed further degraded by the tail-specific protease (Tsp). The PrsW processing site was identified between amino acid residues Ala168 and Ser169 of the extracytoplasmic domain of RsiW. Site-directed mutagenesis showed that site-1 cleavage is abolished by mutating Ala168 to Gln.

The PrsW site-1 cleavage site in RsiW more distal to its transmembrane helix is in favour of our model of an extracytoplasmic location of catalytically active amino acid residues in PrsW (Fig. 1). The short peptide of 40 amino acids removed by PrsW is predicted to form an α-helix, and it will be the aim of future work to analyze whether this helix interacts with another RseB-like component to shield RsiW from PrsW attack.

6.4 Requirements for site-2 proteolysis

In the reconstituted *E .coli* system, described in chapter 3, it was shown that RsiW site-1 cleaved by PrsW is further degraded by Tsp. The direct PrsW site-1 product was not attacked by RasP, revealing that further trimming of the residual extracytoplasmic part of RsiW is essential to make site-1 clipped RsiW a substrate for the site-2 protease RasP.

Tsp is a periplasmic endoprotease of *E. coli* that recruits a peptide or protein substrate via the nonpolar C-terminus of the substrate (Beebe *et al.*, 2000). *B. subtilis* encodes two Tsp homologous proteins, CtpA and CtpB. Both are not crucial for trimming site-1 clipped RsiW in *B. subtilis*, and we could show that other bulk proteases, so far not defined, are involved.

Tsp-like proteases were shown to be implicated in RIP earlier. First, CtpB was shown to indirectly trigger σ^K activation. CtpB is able to cleave a protein called SpoIVFA, thereby activating the S2P SpoIVFB that cuts pro-σ^K to release mature σ^K into the cytoplasm (Campo and Rudner, 2007). Second, mutational analysis of the AlgU / MucA sigma factor / anti-sigma factor system of *Pseudomonas aeruginosa* showed that a Tsp-like protease plays a role in the constitutive expression of the AlgU regulon under certain conditions (Wood *et al.*, 2006). This system is homologous to *E. coli* σ^E / RseA, with MucA as the anti-sigma factor undergoing RIP catalyzed by AlgW (DegS) for site-1 cleavage, and MucP (RseP) for site-2 proteolysis. Induction of the AlgU regulon results in massive production of the extracellular polysaccharide alginate, which enables the pathogen to survive in its host. Tsp was shown to be responsible for constitutive alginate production of mucoid strains isolated from cystic fibrosis patients. These strains express a truncated mutant form of MucA [e.g. *mucA22* (Reiling *et al.*, 2005)], that can neither be attacked by AlgW, nor by MucP directly. The truncated forms of MucA have to be trimmed by Tsp to make them a substrate for site-2 cleavage.

In contrast, deletion studies on *E. coli* RseA showed that RseP is able to process truncated RseA lacking 51 and 75 C-terminal residues of the 98 residue extracytoplasmic part without prior action of DegS. As additional deletion of *rseP* stabilized different C-terminal truncated forms of RseA, there seems to be no participation of Tsp or other proteases in *E. coli* (Kanehara *et al.*, 2003).

Recently, it was shown that the C-terminus of RseA exposed after DegS cleavage plays an essential role in the activation of RseP (Li *et al.*, 2009).

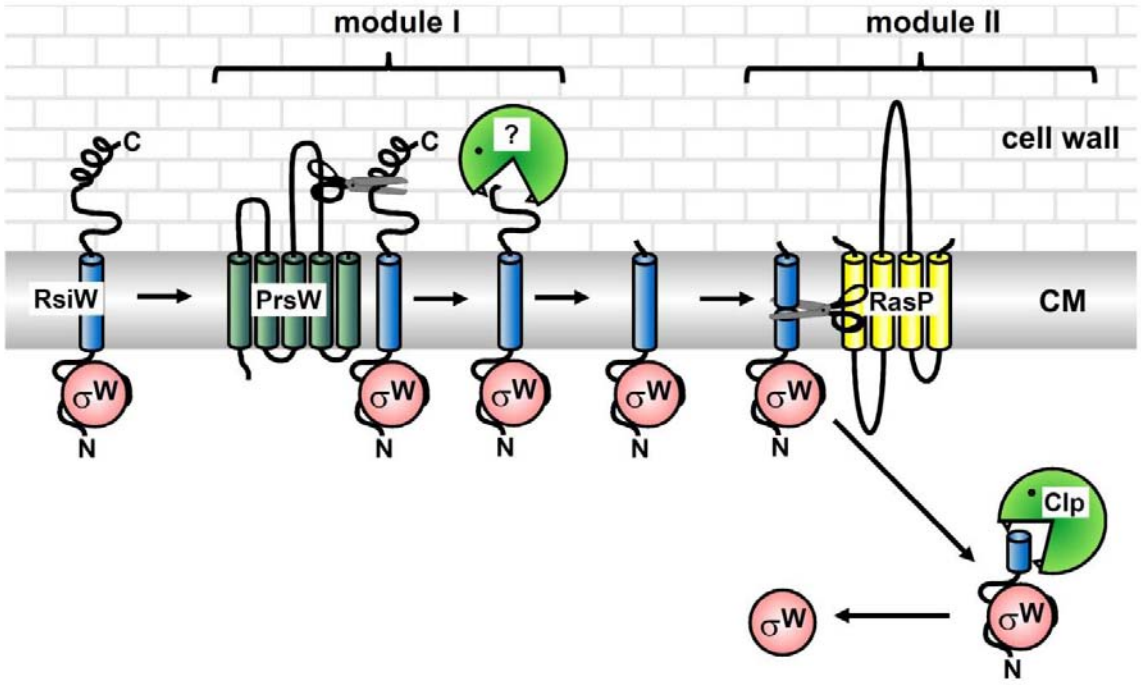

Fig.2 Model for regulated intramembrane proteolysis of RsiW. Two proteolytic modules are involved. In module I, the stress induced PrsW catalyzed site-1 clipping of the 40 extracytoplasmic C-terminal amino acid residues makes RsiW a substrate for degradative extracytoplasmic bulk proteases like Tsp. Only then, RsiW that is trimmed for nearly the entire extracytoplasmic part becomes a substrate for proteases of module II, which is first the processing site-2 protease RasP that uncovers a cryptic proteolytic tag, which is recognized by degradative cytoplasmic proteases like ClpXP. (Heinrich and Wiegert, in press)

It will be interesting to investigate whether PrsW cleaved, and further trimmed RsiW also requires a specific amino acid residue at its C-terminus for subsequent recognition and cleavage by RasP.

In summary, we propose a new model for RIP of RsiW. The process involves two proteolytic modules (Fig. 2), each activated by a site- specific processing proteases (site-1: PrsW; site-2: RasP) that uncovers a proteolytic tag as a recognition signal for more unspecific degrading proteases (site-1: unknown peptidase(s); site-2: Clp peptidases). It is not known whether PrsW senses extracytoplasmic stresses directly, or an associated factor is needed. However, RIP of RsiW is an amazing example of transmembrane signaling in bacteria that involves the concerted action of at least four different proteases to activate one regulatory protein.

6.5 Factors affecting site-2 proteolysis

In our initial transposon screen we also found the ABC transporter EcsAB to have an influence on RsiW stability. As outlined in chapter 4, a strain deleted for *ecsA* is not able to induce σ^W after alkaline shock. This was attributed to a clear defect of the intramembrane cleaving protease RasP in the *ecsA* knockout background. However, the molecular basis for this effect remains unknown. It is conceivable that in the absence of EcsAB, its substrates accumulate and competetively inhibit RasP activity. This is supported by the fact that overexpression of *rasP* partially suppresses its defect in an *ecsA* minus strain. It has to be shown what substance(s) EcsAB transports. Probably it will be peptides that insert into the cytoplasmic membrane and therefore could interfere with RasP. It has been suggested earlier that ABC transporters are able to remove peptides like lantibiotics out of the cytoplasmic membrane (Otto and Gotz, 2001).

Strains of *B. subtilis* deleted for *ecsAB* show a pleiotropic phenotype, e.g. a defect in sporulation and the inability to secrete the α-amylase AmyQ (Leskela *et al.*, 1996), or a defect in forming structured multicellular communities known as biofilms (Branda *et al.*, 2004). The *rasP* minus strain displays exactly the same pleiotropic phenotype, suggesting that the defects of an *ecsAB* minus strain could be due to the inactivity of RasP. A triple mutant of the ECF sigma factors σ^W, σ^X, and σ^M is also unable to produce biofilms (Mascher *et al.*, 2007), and it will be interesting to see whether there is a relation of these observations. Our current hypothesis is that the induction of σ^X and σ^M is dependent on regulated intramembrane proteolysis by RasP as well.

The defect of the *rasP* minus strain to secrete AmyQ is of particular interest, because *Bacillus* species are extensively used for the biotechnological production of degradative enzymes that are secreted by the bacteria. A possible role of RasP as a signal peptide peptidase, as it was proposed for *E. coli* RseP (Akiyama *et al.*, 2004), has to be elucidated in the future.

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Appendix

Publikationsliste

Chapter 2

Heinrich, J. and Wiegert, T. (2006). YpdC determines site-1 degradation in regulated intramembrane proteolysis of the RsiW anti-sigma factor of *Bacillus subtilis*. *Mol Microbiol* **62**, 566-579.

Chapter 3

Heinrich, J., Hein, K., Wiegert, T. (2009). Two proteolytic modules are involved in regulated intramembrane proteolysis of *B. subtilis* RsiW. *Mol Microbiol*, in press.

Chapter 4

Heinrich, J., Lundén, T., Kontinen, V.P., and Wiegert, T. (2008). The *Bacillus subtilis* ABC transporter EcsAB influences intramembrane proteolysis through RasP. *Microbiology* **154**, 1989-1997

Chapter 5

Heinrich, J. and Wiegert, T. (2009) Regulated intramembrane proteolysis in the control of ECF sigma factors. *Research in Microbiology*, in press.

Darstellung des Eigenanteils

Chapter 2

Alle enthaltenen Arbeiten wurden von mir durchgeführt.

Chapter 3

Alle enthaltenen Versuche wurden von mir durchgeführt. Kerstin Hein hat im Rahmen ihrer Diplomarbeit die Plasmide pKH01-5 konstuiert.

Chapter 4

Die Bestimmung der Aktivität der α-Amylase im Kulturüberstand (Fig.4: A), zur Untersuchung des Einflusses von RasP- und EcsA-deletion auf die Sekretion, wurde von Tuula Lundén (Infection Pathogenesis Laboratory, Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland) durchgeführt. Ebenso das Pulse-Chase Experiment (Fig.4: B) zur Untersuchung des Einflusses von RasP-deletion auf die Prozessierung von α-Amylase. Alle anderen dargestellten Ergebnisse wurden von mir erarbeitet.

Chapter 5

Das Manuskript wurde in gleichen Anteilen von PD Dr.Thomas Wiegert und mir verfasst.

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Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbstständig verfasst zu haben und keine anderen als die von mir angegebenen Quellen oder Hilfsmittel verwendet zu haben.

Ferner habe ich weder an der Universität Bayreuth, noch an einer anderen Hochschule versucht eine Dissertation einzureichen, oder mich einer Promotionsprüfung zu unterziehen.

Janine Heinrich
Bayreuth; März 2010