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

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The *Bacillus subtilis* σ^W regulon is induced by different stresses that most probably affect integrity of the cell envelope. The activity of the extracytoplasmic function (ECF) sigma factor σ^W is modulated by the transmembrane anti-sigma factor RsiW, which 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, which encodes an ATP binding cassette (ABC) 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*-negative strain. The defect in σ^W induction of an *ecsA*-knockout strain could be partly suppressed by overproducing RasP. A *B. subtilis* *rasP*-knockout strain displayed the same pleiotropic phenotype as an *ecsA* knockout, namely defects in processing α -amylase, in competence development, and in formation of multicellular structures known as biofilms.

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INTRODUCTION

Genes of *Bacillus subtilis* controlled by the alternative extracytoplasmic function (ECF) sigma factor σ^W constitute an antibiosis regulon (Helmann, 2002, 2006) that responds to a variety of envelope stresses such as certain antibiotics (Cao *et al.*, 2002) and antimicrobial peptides (Pietäininen *et al.*, 2005; Butcher & Helmann, 2006), and also 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 by the 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 zinc-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 orthologue of *E. coli* RseP, and the concept of a cryptic proteolytic tag recognized by ClpXP was first described 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 into the sporulation programme, were mapped to a gene now renamed as *prsW* (protease responsible for activating σ^W , formerly *ypdC*) (Ellermeier & Losick, 2006). It has been shown that suppression is 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 GFP fused to the amino terminus of RsiW was performed, and several transposon insertions in the *prsW* gene were identified that stabilize GFP–RsiW and prevent site-1 cleavage of the anti-sigma factor (Heinrich & Wiegert, 2006). In addition to *prsW*, transposon insertions in a second gene locus, *ecsAB*, show marked stabilization of the fluorescent reporter and prevent 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.

Abbreviations: ABC, ATP binding cassette; ECF, extracytoplasmic function; iClip, intramembrane cleaving protease; RIP, regulated intramembrane proteolysis.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and 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 (LB) medium. When necessary, LB medium was supplemented with ampicillin (100 µg ml⁻¹), phleomycin (1 µg ml⁻¹), neomycin (10 µg ml⁻¹), spectinomycin (100 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), tetracycline (10 µg ml⁻¹) or erythromycin (1 µg ml⁻¹ or 100 µg ml⁻¹). Xylose was added at a concentration of 2 % and vancomycin at a concentration of 2 µg ml⁻¹ where indicated. Pellicle formation experiments were performed according to a published procedure (Branda *et al.*, 2004). A 50 µl volume of mid-exponential-phase culture in LB medium was inoculated into 10 ml minimal MSgg medium [5 mM potassium phosphate (pH 7), 100 mM MOPS (pH 7), 2 mM MgCl₂, 700 µM CaCl₂, 50 µM MnCl₂, 50 µM FeCl₃, 1 µM ZnCl₂, 2 µM thiamine, 0.5 % (v/v) glycerol, 0.5 % glutamate, 50 µg tryptophan ml⁻¹, 50 µg phenylalanine ml⁻¹ and 50 µg threonine ml⁻¹] and incubated at 28 °C. For colony architecture analysis, 3 µl LB precultures were spotted onto minimal MSgg agar plates and incubated at 28 °C.

β-Galactosidase and α-amylase assays, and Northern and Western blot analysis. β-Galactosidase activities and α-amylase activities of culture supernatants were measured as described previously (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*, *phpE* and *yhaU* (Zellmeier *et al.*, 2006). Western blots were performed as described previously (Homuth *et al.*, 1996) using a semi-dry blotting procedure (Bio-Rad, Trans Blot SD). Blots were developed with polyclonal antibodies against RsiW at a dilution of 1:4000, *B. subtilis* FtsH and HtpG (1:10 000), and the FLAG epitope (1:5000).

NaOH-shock experiments and preparation of *B. subtilis* cell fractions. NaOH-shock experiments were performed as described previously (Schöbel *et al.*, 2004). Cells were harvested by centrifugation, washed, and suspended in 1 ml 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 OD₅₇₈ by dilution with cold disruption buffer. Cell suspensions (1 ml) were sonicated (Cell Disrupter B15, Branson) on ice. A 100 µl aliquot was removed (whole-cell fraction,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strain		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara, leu</i>) 7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i>	Grant <i>et al.</i> (1990)
<i>B. subtilis</i> strains		
1012	<i>leuA8</i> <i>metB5</i> <i>trpC2</i> <i>hsrM1</i>	Saito <i>et al.</i> (1979)
168	<i>trpC2</i>	Laboratory stock
NCIB 3610	Undomesticated <i>B. subtilis</i> strain	Branda <i>et al.</i> (2001)
1012 <i>rasP::tet</i>	1012 <i>yluC::tet</i> (Tet ^R)	Schöbel <i>et al.</i> (2004)
1012 <i>rasP::spec</i>	<i>spec</i> insertion in <i>rasP</i> (Spec ^R)	This work
1012 <i>prsW::bleo</i>	1012 <i>ypdC::bleo</i> (Bleo ^R)	Heinrich & Wiegert (2006)
1012 <i>sigW::spec</i>	1012 with <i>spec</i> insertion in <i>sigW-rsiW</i> (Spec ^R)	Schöbel <i>et al.</i> (2004)
1012 <i>ecsA::spec</i>	1012 with <i>spec</i> insertion in <i>ecsA</i> (Spec ^R)	This work
1012 <i>clpP::spec</i>	1012 with <i>spec</i> insertion in <i>clpP</i> (Spec ^R)	Zellmeier <i>et al.</i> (2006)
HB4246	<i>sigW::erm</i> (MLS ^R)	Huang <i>et al.</i> (1998)
IH6531	<i>glyB133</i> <i>hisA1</i> <i>trpC2</i> pKTH10 (Km ^R)	Leskela <i>et al.</i> (1999)
IH8209	<i>hisA1</i> <i>thr-5</i> <i>trpC2</i> (IH6513 cured for pKTH10)	Kontinen & Sarvas (1988)
BFS233	pMUTIN strain with transcriptional fusion <i>P_{yuaF}-lacZ</i>	Schumann <i>et al.</i> (2001)
TW705	1012 <i>amyE::gfp</i> <i>rsiW</i> (Cm ^R)	Zellmeier <i>et al.</i> (2006)
TW706	1012 <i>amyE::gfp</i> <i>rsiW</i> Δ1 (Cm ^R)	Zellmeier <i>et al.</i> (2006)
JAH66	1012 <i>amyE::gfp</i> <i>ftsL</i> (Cm ^R)	This work
JAH67	1012 <i>ecsA::spec</i> <i>lacA::pAL-FLAGecsA</i> <i>amyE::P_{yuaF}-lacZ</i>	This work
JAH68	JAH67 with <i>lacA::pAL-FLAGecsA-E160Q</i>	This work
TW601	BFS233 <i>rasP::tet</i> <i>amyE::P_xrasP</i>	Schöbel <i>et al.</i> (2004)
JAH12	Empty plasmid pX transformed to BFS233 as a control (BFS233 <i>amyE::P_x</i>)	This work
JAH14	BFS233 <i>rasP::tet</i> <i>ecsA::spec</i> <i>amyE::P_xrasP</i>	This work
Plasmids		
pJH07	pBR322 standard cloning vector with <i>ecsA</i> up- and downstream regions flanking <i>spec</i> resistance gene	This work
pJH08	pBR322 standard cloning vector with <i>rasP</i> up- and downstream regions flanking <i>spec</i> resistance gene	This work
pTW700	Plasmid for translational <i>gfp</i> ⁺ fusions	Zellmeier <i>et al.</i> (2006)
pJH66	pTW700 with a <i>gfp</i> ⁺ - <i>ftsL</i> fusion	This work
pJH67	Encodes <i>EcsA</i> with FLAG epitope tag (pAL-FLAGecsA)	This work
pJH68	pJH67 with Walker B mutation E160Q in <i>ecsA</i>	This work

W) and the remaining 900 µl was centrifuged at 5000 g for 15 min at 4 °C to remove cell debris. Then, 800 µl of the supernatant was ultracentrifuged at 45000 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 disruption buffer, ultracentrifuged again (45000 g, 30 min, 4 °C), dissolved in 100 µl Laemmli buffer (Sambrook & Russell, 2005) and heated for 5 min at 95 °C. The protein content of the W and S fractions was estimated by the Bradford method, and 10 µg total protein was loaded in each lane for SDS-PAGE and Western blotting. A volume equivalent to the S fraction was loaded for the M fraction, i.e. one-eighth of the volume of the S fraction, containing 10 µg soluble protein.

Pulse-chase experiments, and 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*-negative strains, and SPP1 phage transduction. The *ecsA* and *rasP* genes were inactivated by a deletion, and by insertion of a spectinomycin-resistance gene, respectively. To delete *ecsA*, the gene and 5'/3'-flanking regions were PCR-amplified using primers (1) 5'-GGCCATGTCGACCACCTCATTTGACAATTTGCTTCA-3' and (2) 5'-GGCCATAAGCTTCCTGCTTCAAGTAAGGCTCCAT-3' and chromosomal DNA of *B. subtilis* 1012 as a template. The PCR product was restricted with *Sall*/*HindIII* and ligated to the pBR322 vector fragment cut with the same enzymes, yielding plasmid pBRecsA. An internal 450 bp *ecsA* fragment was replaced by restricting pBRecsA with *Bam*HI/*Sac*I and inserting the spectinomycin-resistance gene cut with the same enzymes that had been PCR-amplified with primers (3) 5'-GGCCATAAGCTTGGATCCATCGATTTGACATTTTCTTGTTGA-3' and (4) 5'-GGCCATAAGCTTGAGCTCGTAAGACCTGTTATTGCAATAAAA-3' and plasmid pK2-*spec* (Härtl *et al.*, 2001) as a template, resulting in plasmid pJH07. The *ecsA::spec* construct was PCR-amplified with the above primers (1) and (2), and the product was used to transform *B. subtilis* 1012. Chromosomal DNA of transformants (1012 *ecsA::spec*) resistant to spectinomycin was checked by PCR and by Southern blotting using a DIG-labelled DNA probe for *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* into *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 into *B. subtilis* strain NCIB3610, a *rasP::spec* construct was cloned by replacing the *ecsA*-5' and *ecsA*-3' regions of pJH07 with the respective *rasP* up- and downstream regions that were PCR-amplified using primers (5) 5'-GGCCATGTCGACTGTGATCGACAGCTCGGAAC-3', (6) 5'-GGCCATGGATCCTATAACTGTATTCACGAACATACCA-3', (7) 5'-GGCCATGAGCTCTTGTCACATGGAACGATATCCAG-3' and (8) 5'-GGCCATGAATCCCTCATCGCAACAAGCGAAG-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 elsewhere (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'-GGCCATAGATCTATGCTCTGCTATCGGTAAGAC-3' and (10) 5'-GGCCATGCATGCTTATTCATGGCCAGCGTCTTCC-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 3 × FLAG epitope tag to the amino terminus of EcsA, which was integrated at the *lacA* locus using *B. subtilis* strain 1012 *amyE::P_{yuaF}-lacZ lacA::spec* (Schöbel *et al.*, 2004). For a double crossover event, transformants were screened for erythromycin resistance and spectinomycin sensitivity, resulting in strain 1012 *amyE::P_{yuaF}-lacZ lacA::pAL-FLAGecsA*. Finally, the *ecsA* gene was deleted by transformation of chromosomal 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 previously (Schöbel *et al.*, 2004), using primer (11) 5'-CCTGCGCTCTACATTATTGATCAGCCTTTTCTAGGGCTTGATC-3' and primers (9) and (10), yielding plasmid pJH68, which was then transformed into *B. subtilis* as described above for pJH67. The resulting strain (1012 *ecsA::spec amyE::P_{yuaF}-lacZ lacA::pAL-FLAGecsAE160Q*) was named JAH68.

Construction of a *B. subtilis* GFP-FtsL reporter fusion. To test for stability of FtsL, a fusion of GFP to the amino terminus of FtsL was constructed, essentially as described previously (Zellmeier *et al.*, 2006). The *ftsL* coding region was PCR-amplified using primers (12) 5'-GGCCATAGATCTATGAGCAATTTAGCTTACCAACCAGAG-3' and (13) 5'-GGCCATAGATCTAGCGCTTCATTCTGTATGTTTTCACCTT-3' and chromosomal DNA of *B. subtilis* 1012 as a template, and the product was restricted with *Bgl*II and ligated into plasmid pTW700 cut with the same enzyme. Correct orientation of the insert was checked by restriction analysis and DNA sequencing, and the resulting plasmid was named pJH66.

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-R*siW* 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 stabilization of the GFP-R*siW* reporter and abolishing induction of a transcriptional fusion of *lacZ* to the strong σ^W -controlled *yuaF* promoter were obtained. These genes encode the ABC and the transmembrane part of an ABC transporter that has been characterized previously (Leskela *et al.*, 1996), orthologues 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 R*siW*, a *B. subtilis ecsA*-knockout mutant was constructed by replacing the major part of *ecsA* by a spectinomycin-resistance gene (*ecsA::spec*). When compared with the wild-type, the *ecsA*-knockout strain did not induce the *yuaF-lacZ* reporter following alkaline shock, a stress condition that strongly activates the σ^W regulon. (Fig. 1a, lanes 1 and 2 compared

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 consisting 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 Methods. Strains were BFS233 (wt; 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 (wt; 1, 2) and isogenic knockouts of *ecsA* (3, 4), *rasP* (5, 6) and *sigW* (7, 8). An alkaline shock was performed at OD₅₇₈ 0.7 by the addition of NaOH to a final concentration of 24 mM (pH 8.9) where indicated. Samples were withdrawn 10 min after NaOH addition and at the same time point for unshocked cells. Total RNA (2 μ g) was loaded into each lane. Riboprobes against σ^W -controlled *pbpE* and *yuaG*, and alkaline-inducible *yhaU* were used.

with 7 and 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, similar to *rasP*- or *sigW*-knockout strains (Fig. 1b). In contrast, alkali induction of the σ^W -independent *yhaU* gene remained unaffected.

To exclude the possibility 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 3 $\times$ FLAG epitope-tagged protein under IPTG control. Alkali induction of the *yuaF* reporter fusion could be measured in the absence of IPTG (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 catalytic activity or the absence of EcsA protein, an *ecsA* allele mutated in the catalytic Walker B ATPase domain (E160Q) was expressed. Whereas both 3 $\times$ FLAG-EcsA and 3 $\times$ FLAG-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 the corresponding *ecsA* wild-type strain, indicating that σ^W -mediated sublancin immunity is absent in the *ecsA*-negative strain. Similarly, treatment with vancomycin, 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*-negative 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 of induction is not limited to specific σ^W -inducing stress signals.

The *B. subtilis* *ecsA* mutant 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 in transposon-insertion strains, suggested that stress-induced intramembrane proteolysis of RsiW is impaired in the absence of EcsAB activity. Therefore, the *ecsA::spec* mutation was introduced into strains expressing GFP-RsiW and a GFP-RsiW Δ 1 reporter, respectively. GFP-RsiW Δ 1 is a constitutive substrate for RasP and not dependent on site-1 proteolysis for degradation (Zellmeier *et al.*, 2006). Colonies of both strains were highly fluorescent, whereas in the *ecsA* wild-type background

Fig. 2. Complementation of the *ecsA::spec* deletion. NaOH-shock experiments to induce σ^W were performed as described in Methods. 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.

only weak fluorescence was visible (data not shown). In Western blots, the site-1-clipped truncated form of GFP-RsiW was the main form detectable in membrane fractions (Fig. 3a, compare lanes 3 and 9). GFP-RsiW Δ 1, which is absent in the wild-type due to proteolysis by RasP and further cytoplasmic proteases (Zellmeier *et al.*, 2006), was stabilized in the *ecsA::spec* background (Fig. 3a, lanes 6 and 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, the full-length RsiW was detectable only in non-shocked cells (Fig. 3b, lanes 1, 2). In conclusion,

Fig. 3. RasP is inactive in the *ecsA*-negative background. (a) Western blots of *B. subtilis* strains expressing reporter proteins consisting of GFP fused to the amino terminus of RsiW and RsiW Δ 1 (RsiW lacking the extracytoplasmic domain of the wild-type) (strains TW705, TW706), and *ecsA*-negative backgrounds (TW705 *ecsA::spec*, TW706 *ecsA::spec*). Samples of cultures were obtained during the late-exponential growth phase and cells were disrupted by sonication. To localize fusion proteins, whole-cell extracts (W) were further fractionated into membrane (M) and soluble (S) protein fractions 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 indicated with an asterisk, the site-1 proteolysis product or RsiW Δ 1 with two asterisks. (b) Western blot analysis of *B. subtilis* strain 1012 wild-type (wt; 1, 2), 1012 *ecsA::spec* (3, 4) and 1012 *rasP::tet* (5, 6). Cells were alkaline-shocked as described above, and samples were taken 10 min 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 carrying the gene for a GFP-FtsL reporter protein in wild-type (wt; JAH66), *ecsA*-negative (JAH66 *ecsA::spec*), *rasP*-negative (JAH66 *rasP::tet*), *prsW*-negative (JAH66 *prsW::bleo*) and *clpP*-negative (JAH66 *clpP::spec*) backgrounds. Left-hand panel, patches under normal light; right-hand panel, 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.

RasP-catalysed intramembrane site-2 proteolysis of RsiW does not take place in the absence of EcsAB activity, concomitantly with deregulated site-1 proteolysis that takes place without an external stress.

The intramembrane cleaving protease RasP is inactive in a *B. subtilis* *ecsA*-negative 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 using the fluorescence of whole cells grown on LB agar plates. Fluorescence in the wild-type and *prwW*-knockout background was low, indicating that GFP-FtsL is unstable and degraded in a PrwW-independent manner (Fig. 3c). The *rasP*-negative and *ecsA*-negative strains were 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 was also stabilized in a *clpP*-negative background, meaning that the alanine residues in its transmembrane domain constitute a cryptic proteolytic tag, as has been described previously for RsiW (Zellmeier *et al.*, 2006). Taken together, the failure to degrade both RsiW and FtsL suggests that, in the absence of EcsAB activity, RasP is not functional.

There might be two different explanations for this finding. First, that RasP activity itself is directly or indirectly dependent on EcsAB. For example, EcsAB is known to influence correct localization of secretory proteins (Leskela *et al.*, 1999), and therefore could be involved in correct membrane insertion of RasP. Second, that an EcsAB substrate mislocated in the absence of the ABC transporter activity inhibits RasP. To address this question, we overexpressed *rasP* under the 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 already detectable in the absence of xylose (Fig. 1a, columns 11–14), which is due to leakiness of the *xylA* promoter (Schöbel *et al.*, 2004). As 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 in β -galactosidase activity after alkaline shock (Fig. 1a, columns 15–18). One possible explanation for these findings is that RasP is competitively inhibited by an EcsAB substrate, and that this is alleviated by increasing the concentration of RasP.

B. subtilis *ecsA*- and *rasP*-negative strains display similar pleiotropic phenotypes

A point mutation causing defective 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 characterization 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* has been described that is unable to produce a biofilm (Branda *et al.*, 2004). As RasP seems to be inactive in the *ecsA*-negative strain, we wondered whether at least some of the defects listed above are correlated with *rasP*. The *rasP::tet* deletion, and *sigW::erm* (Huang *et al.*, 1998) and *ecsA::spec* as controls, were introduced into strain IH6531, which harbours plasmid pKTH10 for *amyQ* overexpression (Kontinen & Sarvas, 1988). The AmyQ activity of culture supernatants was determined. For the wild-type strain and the isogenic *sigW*-negative strain, high AmyQ activity was detectable, whereas the *rasP*-negative and *ecsA*-negative strains showed only about 10 % of the wild-type level (Fig. 4a). In pulse-chase experiments it became obvious that almost no processing of preAmyQ to its mature form took place when *rasP* was deleted (Fig. 4b), as has been described for the *ecsA26* mutant strain (Leskela *et al.*, 1999). Next, the transformation efficiency of the respective strains was determined. For both *rasP::tet* and *ecsA::spec*, transformation efficiency was only about 0.2 % of that of the wild-type; for the *sigW*-knockout strain, an efficiency 10 % of that of the wild-type was measured. Sporulation tests were performed by plating serial dilutions of samples of cells grown in sporulation medium with and without heat treatment. To our surprise, the sporulation rate of the *ecsA::spec* strain, and also of the *rasP::tet* strain, was similar to that of 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 the validity of the test. Therefore, earlier observations of a sporulation defect in an *ecsA*-negative 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 misinterpretation of sporulation rates. The last phenotype that we checked was the ability of respective strains to form structured multicellular communities, known as biofilms. To that purpose, the knockout mutations were introduced into *B. subtilis* strains 168 and into the undomesticated NCIB3610 isolate, which has been described as forming a characteristic pellicle on liquid medium and having a complex colony morphology on solid medium (Branda *et al.*, 2004). Both the *rasP* and the *ecsA* knockouts were clearly handicapped in forming structured biofilms and

Fig. 4. *B. subtilis* *rasP*⁻ and *ecsA*-negative 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; circles), IH6531 *sigW::erm* (*sigW::erm*, pKTH10; diamonds), IH6531 *ecsA::spec* (*ecsA::spec*, pKTH10; squares) and IH6531 *rasP::tet* (*rasP::tet*, pKTH10; triangles) were grown in modified 2 $\times$ LB broth, and culture medium samples were taken for the amylase assay. Open and filled 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, and samples were withdrawn at the chase times indicated (0, 0.5, 1 and 2 min). Each lane was loaded with material immunoprecipitated from 330 μ l 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 MSgg medium in a microtitre plate well, and the cultures were incubated at 28 $^{\circ}$ C for 60 h without shaking. To assay colony development (third column), each mutant derived from NCIB3610 was grown overnight in LB medium, and a 3 μ l sample of the culture was spotted onto MSgg agar and incubated at 28 $^{\circ}$ C for 96 h.

largest protein super-families, representatives of which can be found in all organisms. Typically, they consist of two subunits of a hydrophobic protein with six 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 function either as import systems, usually assisted by a soluble or membrane-anchored solute-binding protein, or as exporters of surface components (capsular polysaccharides, lipopolysaccharides, teichoic acid), proteins involved in bacterial pathogenesis (haemolysin, haem-binding protein, alkaline protease), peptide antibiotics, haem, drugs and siderophores. For *B. subtilis*, at least 78 ABC transporters can be discerned, with 38 importers and 40 exporters. EcsAB has been grouped in a subfamily of extruders related to antibiotic-resistance systems, with similarities to possible efflux pumps for peptide antibiotics (Quentin *et al.*, 1999). The actual substrate for EcsAB and its function is unknown. The most interesting phenotype of an EcsA-negative 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 enhances secretion of AmyQ in the *ecsA26* strain (Pummi *et al.*, 2002).

There is a clear defect in the intramembrane cleaving protease RasP activity in the *ecsA*-knockout background, but the molecular basis for this defect remains enigmatic and at this stage we are only able to speculate. As there are no antibodies to RasP available and we were not able to detect epitope-tagged RasP in membrane fractions of

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 in the *ecsA* mutant are 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

B. subtilis at all, it is not clear whether this defect is due to improper membrane insertion of RasP in the *ecsA*-negative 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 that *ecsA* might have at the transcriptional level (Pummi *et al.*, 2002). In addition, the fact that RasP activity is restored when the protein is overexpressed favours a model of inhibition of existing RasP in the absence of the EcsAB ABC transporter. Which substance(s) EcsAB transports is an intriguing question, and it is conceivable that it transports peptides, possibly peptides that insert into the cytoplasmic membrane and which therefore could interfere with RasP. Note that it has been suggested that ABC transporters are able to remove peptides like lantibiotics from 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*-negative strain, like the *ecsA* knockout, does not process preAmyQ, and that this is not caused by their common inability to induce the σ^W regulon. It is not clear what effect RasP might have on the processing of overexpressed AmyQ, and whether the processing defect in the *ecsA*-negative strain is due to the inactivity of RasP. The only direct role that RasP could play in secretion is a possible function as a signal peptide peptidase, as has been proposed for the RasP orthologue 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 the removal of signal peptides from the membrane. *E. coli* SppA (protease IV) has been 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 and 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). One function of RasP might be to degrade certain signal peptides in the membrane, and in the absence of RasP activity the accumulation of these peptides might inhibit signal sequence processing by leader peptidases, as 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*-negative and other strains

several times using different methods, but so far the methods have proved to be problematic.

The inability of the *rasP*-negative strains to produce a biofilm is another interesting feature. Biofilm formation in *B. subtilis* is a complex programme 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 this points to a central role of the iClip. It is noteworthy that 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 roles of and connections between EcsA and RasP, as both of these proteins can be found in a great variety of prokaryotes. Both might represent good targets for new antimicrobial agents, as, for example, orthologues of RasP are involved in pathogenic processes (Makinoshima & Glickman, 2005, 2006).

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