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pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*

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Summary

The type III secretion system of *Salmonella* pathogenicity island 2 (SPI-2) is required for bacterial replication inside macrophages. SseB has been considered a putative target of the secretion system on the basis of its similarity with EspA, a protein secreted by the type III secretion system of enteropathogenic *Escherichia coli* (EPEC). EspA forms a filamentous structure on the bacterial cell surface and is involved in translocation of proteins into the eukaryotic cytosol. In this paper, we show that SseB is a secreted protein that associates with the surface of the bacterial cell and might, therefore, also be required for delivery of SPI-2 effector proteins to the eukaryotic cell cytosol. SseB begins to accumulate inside the bacterial cell when the culture enters early stationary phase. However, SseB is only secreted if the bacteria are grown at low pH or if the pH is shifted after growth from 7.0 to below pH 5.0. The secretion occurs within minutes of acidification and is totally dependent on a functional SPI-2 type III secretion system. As the pH of the *Salmonella*-containing vacuole inside host cells has been shown to acidify to between pH 4.0 and 5.0, and as SPI-2 gene expression occurs inside host cells, low pH might be a physiological stimulus for SPI-2-mediated secretion *in vivo*.

Introduction

Type III secretion systems of pathogenic bacteria comprise a large number of proteins that transfer specific effector proteins from bacterial into eukaryotic cells. This process typically involves a secreton for exporting proteins from the bacterial cell, a translocon for transferring effector proteins into host cells, various regulators that control gene transcription and protein secretion, chaperones that bind to and are needed for secretion of translocators and effector proteins and the effector proteins themselves (Hueck, 1998). Effector proteins can modulate host cell signalling pathways in a variety of ways leading, for example, to bacterial uptake in the case of *Shigella* (Adam *et al*., 1995; Ménard *et al*., 1996) or the blocking of phagocytosis in the case of *Yersinia* (Cornelis and Wolf-Watz, 1997).

Salmonella typhimurium is unusual in that it contains two type III secretion systems. One, called Inv/Spa, controls bacterial invasion of epithelial cells (Galán and Curtiss, 1989; Galán, 1996). Most of the genes associated with Inv/Spa are encoded within a 40 kb pathogenicity island (SPI-1) located at 63 centisomes (cs) on the chromosome (Mills *et al*., 1995). Genes for the second type III secretion system are found within a second pathogenicity island (SPI-2) at 30 cs on the chromosome. This secretion system plays a crucial role in systemic growth of *Salmonella* in its host (Hensel *et al*., 1995; Shea *et al*., 1996) and is required for bacterial growth in macrophages (Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998).

The identification and characterization of type III-secreted proteins of *Yersinia* spp., *Shigella* spp., pathogenic *Escherichia coli* and Inv/Spa of *Salmonella* spp. has been made possible by the discovery of *in vitro* growth conditions in which the secreted proteins are exported (reviewed by Hueck, 1998). Although it has been demonstrated that SPI-2 genes are expressed inside host cells (Valdivia and Falkow, 1997) and can be regulated by certain environmental conditions, including phosphate starvation and low Mg^{2+} concentrations (Deiwick *et al*., 1999), no secreted protein of the SPI-2 system has yet been identified, and the conditions that trigger protein secretion by this system have not been defined.

On the basis of similarities of sequence and organization to genes of other bacterial pathogens, several genes located in a 9 kb region within SPI-2 have been proposed to encode secreted proteins (Hensel *et al*., 1998). Proteins encoded by *sseB*, *sseC* and *sseD* display weak similarity to EspA, EspD and EspB respectively. These Esp proteins are secreted by the type III secretion system of the locus of enterocyte effacement (LEE) of enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) (Donnenberg *et al*., 1993; Kenny *et al*., 1996; Lai *et al*., 1997; Ebel

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et al., 1998) and are essential for virulence (Frankel *et al*., 1998). Recently, it has been demonstrated that EspA is involved in the translocation of EspB (Knutton *et al*., 1998; Wolff *et al*., 1998) and another LEE-encoded protein, Tir (Kenny *et al*., 1997), into the plasma membrane and cytoplasm of epithelial cells. EspA is a component of a filamentous surface appendage with a cylindrical structure that forms a contact between the bacterium and the eukaryotic cell surface (Ebel *et al*., 1998; Knutton *et al*., 1998) and could act as a channel through which proteins are delivered into the host cells (Frankel *et al*., 1998).

An *S. typhimurium* strain carrying a deletion of *sseB* is strongly attenuated in mice and is defective in replication within macrophages (Hensel *et al*., 1998). In view of this observation and the similarity between the *sseB* gene product and EspA, SseB seemed a likely candidate for a protein that might function as part of a SPI-2 translocon and could be used to establish *in vitro* conditions inducing SPI-2-mediated protein secretion. Using antibodies raised against recombinant SseB, we show here that SseB is rapidly secreted onto the bacterial cell surface by the SPI-2 type III secretion system when the pH of the culture medium is shifted to 5.0. This pH-based induction may be physiologically relevant, as the pH of the *Salmonella*-containing vacuole of infected cells has been reported to drop to 4.0–5.0 within 1 h after bacterial uptake (Rathman *et al*., 1996). Furthermore, as the Inv/Spa system is induced at pH 8.0 (Daefler, 1999), it appears that *Salmonella* regulates the two secretion systems independently in a way that prevents the potentially harmful consequences of activating two structurally related, but functionally independent, secretion systems in the same cell.

Results

Surface localization of SseB

To investigate SseB secretion, polyclonal antiserum was raised against recombinant SseB, and this was used to detect SseB by immunoblot analysis. Initial experiments showed that the intracellular levels of SseB increased when bacteria were grown in low-Mg^{2+} or low-phosphate media (data not shown), as has been reported for SsaP and SscA (Deiwick *et al*., 1999). However, under these conditions, no secretion of SseB was detected using the methods described below (data not shown). As SPI-2 gene expression is induced inside host cells (Valdivia and Falkow, 1997), we grew *S. typhimurium* wild-type strain 12023 at 37°C overnight in intracellular salt medium (ISM), which reflects the salt conditions inside eukaryotic cells (Headley and Payne, 1990). The pH of the medium was adjusted to 5.0, as the pH of the *Salmonella*-containing vacuole decreases to between 4.0 and 5.0 shortly after bacterial entry (Rathman *et al*., 1996).

To determine whether SseB is an intracellular protein, a protein secreted to the cell surface or is secreted into the culture medium, we treated bacterial cultures to obtain three fractions. The supernatant fraction contains proteins recovered by precipitation of the culture supernatant after centrifugation to isolate the bacterial cells. The second fraction consists of proteins detached from the surface of pelleted cells by the hydrophobic agents *p*-xylene or *n*-hexadecane (Michiels *et al*., 1990; Ménard *et al*., 1994; Iriarte *et al*., 1998). These agents allow efficient recovery of proteins weakly associated with the bacterial cell surface, but not of known membrane proteins (Michiels *et al*., 1990). The cell pellet fraction contains proteins remaining in the cell pellet after these treatments and includes intracellular proteins.

When proteins in the three fractions obtained from a culture of strain 12023 after growth in ISM pH 5.0 were analysed by immunoblotting for the presence of SseB, a polypeptide of the size predicted for SseB was detected in the xylene and cell pellet fractions, but not in the supernatant fraction, suggesting that SseB is mainly secreted onto the bacterial cell surface (Fig. 1A). Identical results were obtained using hexadecane as an alternative to xylene (data not shown). Constitutive expression of SseB from a plasmid resulted in an increased intensity of the signal in the xylene and cell pellet fractions but, again, it was not detected in the supernatant fraction (Fig. 1A).

To verify that the observed signals correspond to the SseB protein, we analysed fractions from cultures of strain 12023 and an isogenic Δ*sseB* strain by immunoblotting. A single polypeptide was detected in the pellet and hexadecane fractions from the wild-type strain, but not from the Δ*sseB* strain (Fig. 1B). The antibody specificity was also confirmed by immunoprecipitation experiments and by expression of SseB in *E. coli* DH5α (data not shown).

To determine whether hexadecane treatment had a significant effect on bacterial cell integrity, serial dilutions of a culture of strain 12023 were plated to LB medium before and after extraction with hexadecane. There was no decrease in the numbers of bacterial colony-forming units (cfu) after treatment, indicating that exposure to hexadecane did not adversely affect cell viability (data not shown).

We also found that SseB was removed from the bacterial cell surface by vigorous vortexing, by passing the culture through a 26-gauge needle and by treating cells with 1 M Tris-Cl, pH 7.5 (data not shown).

A recent report has shown that SipC and InvJ, two Inv/Spa-secreted proteins, could be recovered from the plastic surface of the vessel in which the bacterial cells were cultured (Daefler, 1999). When strain 12023 was grown under the conditions described above, a small amount of SseB was also found on the surface of the growth vessel (Fig. 1C). Therefore, detection of extracellular SseB does not

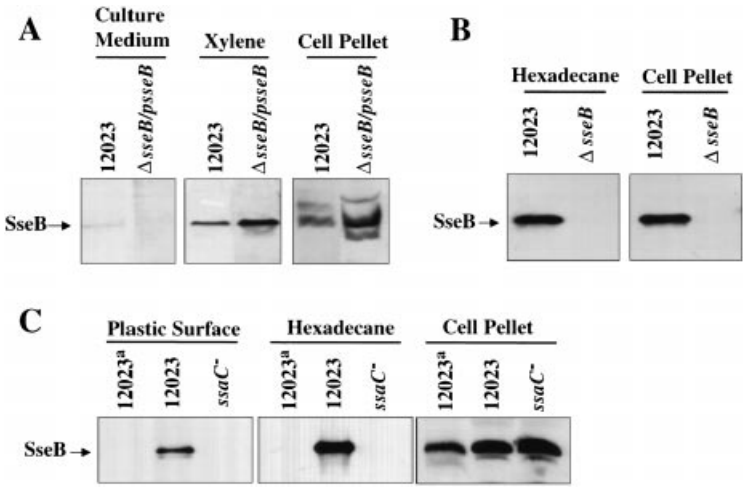

Fig. 1. Intracellular and cell surface localization of SseB. *S. typhimurium* 12023 (wild type), HH102 (Δ*sseB*) and HH103 (Δ*sseB*/p*sseB*) were grown in ISM medium at pH 5.0 overnight and analysed as follows.
A. Culture supernatant, xylene and cell pellet fractions of the indicated strains were prepared and analysed by immunoblotting.
B. Immunoblot analysis of SseB in hexadecane and cell pellet fractions of 12023 strain and its Δ*sseB* derivative HH102.
C. Immunoblot analysis of strains 12023 and P7D2 (*ssaC*::mTn*5*) grown in MgM-MES medium at pH 5.0. 12023[a] indicates the immunoblot analysis of strain 12023 grown in MgM-MES medium at pH 7.5.

require prior treatment of the bacterial cells. These results indicate that the extracellular location of SseB is not an artifact of the fractionation methods.

Approximately half the total amount of SseB was detected in the hexadecane fraction, but other means of detaching it from the bacterial cell surface were less efficient. Approximately 10% of total SseB was recovered from the plastic surface of the growth vessel after overnight growth. Therefore, in subsequent experiments, hexadecane was routinely used to assay secreted SseB.

As a further test for the presence of SseB on the cell surface, bacterial cells grown overnight under inducing conditions were treated with proteinase K. Proteinase K has been widely used to discriminate between intracellular and extracellular proteins, because its addition to the bacterial cell culture results in degradation of extracellular but not intracellular proteins (Ménard *et al*., 1994; Iriarte *et al*., 1998). After proteinase K incubation, the enzyme was inactivated, and cells were treated with hexadecane. In this case, SseB was no longer recovered, and the amount of SseB detected in the cell pellet fraction was not affected (Fig. 2A). Hence, the hydrophobic agents, xylene and hexadecane, can efficiently separate surface-localized SseB from that in the remainder of the cell.

In contrast to SseB, the SPI-2-encoded proteins SscA and SsaP were detected in the cell pellet but not in the hexadecane fractions (Fig. 2B). SscA is a putative chaperone (Hensel *et al*., 1998), and SsaP is similar to SpaN, an outer membrane protein of the type III secretion system of *Shigella flexneri*, and to InvJ, a Inv/Spa-secreted protein (Hueck, 1998).

Collectively, these data show that secretion of SseB can be assayed by hexadecane fractionation, that this secretion is induced during growth in laboratory medium and that the majority of the secreted protein remains weakly bound to the bacterial cell surface.

Effect of different mutations on SseB secretion

To establish whether SseB secretion was dependent on the type III secretion system encoded by SPI-2, several strains

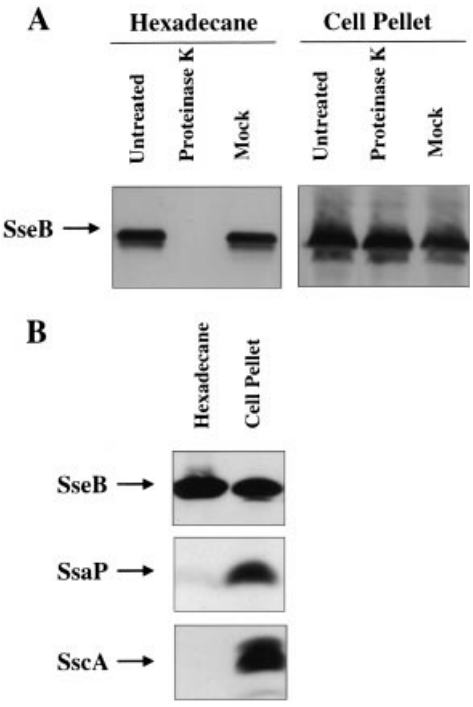

Fig. 2. A. Cell surface-associated, but not intracellular SseB is degraded by proteinase treatment of bacterial cells. *S. typhimurium* 12023 was grown in ISM pH 5.0 medium overnight, and the cells were treated with proteinase K. Hexadecane and cell pellet protein fractions were prepared and analysed by immunoblotting. Lanes: untreated, a cell aliquot was held on ice before fractionation; proteinase K, a second aliquot was treated with proteinase K; mock, a third aliquot followed the proteinase K protocol, but the enzyme was not added.
B. Immunoblot analysis of SseB, SscA and SsaP in hexadecane and cell pellet fractions of strain 12023 grown in MgM medium at pH 5.0.

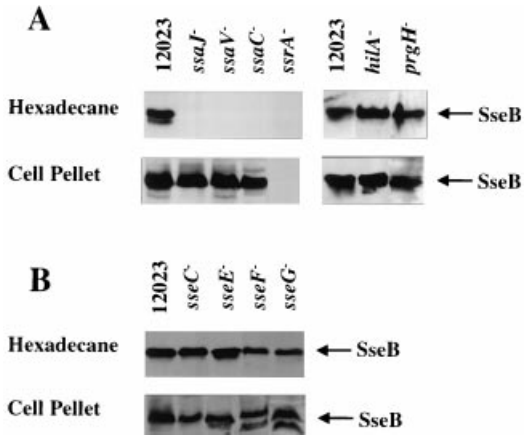

Fig. 3. Production and secretion of SseB in different mutant strains. *S. typhimurium* strains were grown in MgM medium at pH 5.0. Hexadecane and cell pellet fractions were prepared and analysed by immunoblotting as described in *Experimental procedures*.
A. Strains 12023 (wild type), P11D10 (*ssaJ*::mTn*5*), HH109 (*ssaV*::*aphT*), P7G2 (*ssaC*::mTn*5*), P3F4 (*ssrA*::mTn*5*), HH129 (*hilA*::Tn*10*), HH130 (*prgH*::Tn*phoA*).
B. Strains HH104 (*sseC*::*aphT*), HH106 (*sseEΔ*), HH107 (*sseFΔ*::*aphT*) and HH108 (*sseG*::*aphT*)

carrying mutations in genes encoding putative secretion apparatus proteins were analysed for SseB secretion. SsaV is predicted to be an inner membrane protein (Hensel *et al*., 1997) and shares strong similarity with the LcrD family of proteins (Galán *et al*., 1992). SsaJ is a predicted lipoprotein (Hensel *et al*., 1997) that may link inner and outer membranes based on its similarity with the YscJ/Mxi family of lipoproteins (Michiels *et al*., 1991). SsaC is similar to InvG (Ochman *et al*., 1996; Shea *et al*., 1996), a member of the PulD family of translocases (Kaniga *et al*., 1994) and an outer membrane protein (Crago and Koronakis, 1998). Mutations in the genes encoding these proteins prevented secretion of SseB, because no SseB was detected on the bacterial cell surface or on the surface of the growth vessel (Figs 1C, 3A and data not shown). However, these mutations did not significantly affect the intracellular pool of SseB (Figs 1C and 3A). The *ssrA* gene is predicted to encode the sensor protein of a two-component regulatory system (Shea *et al*., 1996) and is required for efficient transcription of other SPI-2 genes (Hensel *et al*., 1998). As expected, in the *ssrA* mutant, SseB was not detected in either fraction (Fig. 3A).

To confirm that SseB secretion under inducing conditions was occurring solely through the SPI-2 secreton, we analysed its secretion in a strain carrying a mutation in *prgH*, which encodes a structural protein of the Inv/Spa apparatus (Behlau and Miller, 1993), and a strain carrying a mutation in *hilA*, a gene encoding a regulator of the *inv/spa* genes (Bajaj *et al*., 1995). The levels of secreted SseB in these strains were similar to that of the wild-type strain (Fig. 3A).

To determine if other SPI-2 genes might play a role in SseB expression or secretion, we analysed SseB location in several other mutant strains. Non-polar mutations in *sseC*, *sseE*, *sseF* and *sseG* had no effect on levels of SseB in the hexadecane or pellet fractions (Fig. 3B). SseC has sequence similarity to a class of proteins involved in the translocation of effectors into the target host cell and contains three hydrophobic regions that could represent membrane-spanning domains (Hensel *et al*., 1998). SseE, SseF and SseG have no significant similarities to other known proteins. The three proteins have predicted transmembrane helices varying between one for SseE to four for SseF (Hensel *et al*., 1998). These proteins might represent part of a translocon and not be involved with SseB secretion onto the bacterial cell surface, or they might be effectors that are delivered into the host cell.

SseB secretion is induced by acid pH

Different media were tested for SseB secretion in an attempt to identify the component(s) responsible for the induction of secretion. Growth in media with either low phosphate (ISM-MES) or low Mg^{2+} (MgM medium) increased the total amount of SseB detected at a pH of either 5.0 or 7.5, compared with growth in ISM (Fig. 4). However, in all the media tested, SseB was only recovered from the cell surface when the bacteria were cultured at pH 5.0. When strain 12023 was grown in MgM-MES medium, SseB was detected on the bacterial cell surface and on the growth vessel at pH 5.0, but not at pH 7.5. There was no significant difference in the levels of intracellular SseB at either pH (Fig. 1C).

To investigate whether this apparent acid-induced secretion merely reflects an increase in *sseB* transcription, the SseB protein was expressed from a plasmid under the control of a constitutive promoter in MgM medium at pH 5.0 and pH 7.5. Secretion of SseB was again only detected

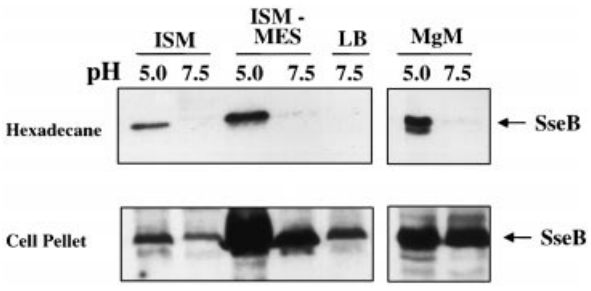

Fig. 4. SseB production and secretion in different media and at different pH. Cultures of strain 12023 were obtained by growing the cells overnight in the indicated media. Hexadecane and cell pellet fractions were prepared and analysed by immunoblotting. The composition of the media is described in *Experimental procedures*.

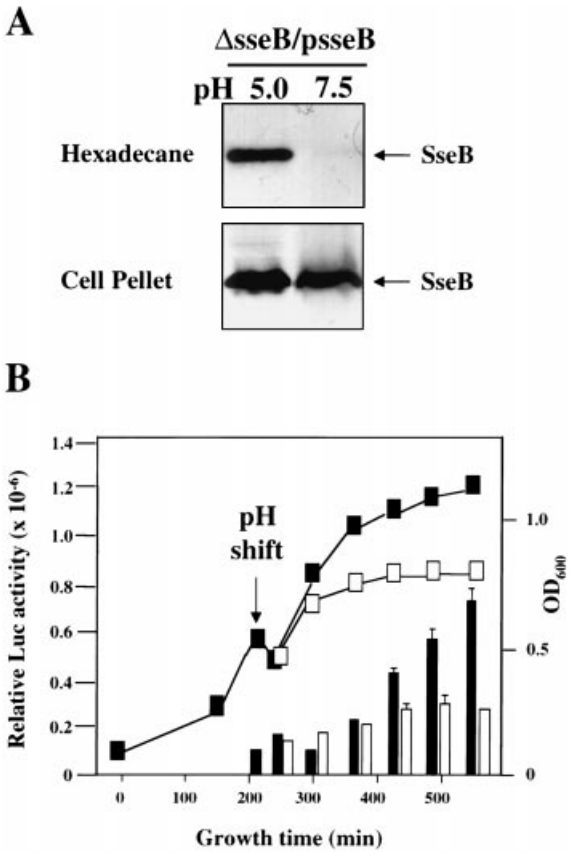

Fig. 5. Acid-induced secretion of SseB is independent of *sseB* transcription.
A. Immunoblotting of hexadecane and cell pellet fractions of HH103 (Δ*sseB*/p*sseB*) after overnight growth in MgM medium at pH 5.0 or pH 7.5.
B. Luc activities of *sseA*::*luc* in response to low pH. Strain MvP127 (*sseA*::*luc*) was grown in MgM medium, pH 7.0, until the culture reached an OD_{600} of 0.5. The medium was then changed to fresh MgM medium, pH 5.0 or pH 7.0 (OD_{600} – open and solid squares respectively). Luciferase activity was also determined (open and solid bars respectively) and expressed in terms of equivalent bacterial cfu.

at pH 5.0 (Fig. 5A), indicating that the pH-induced secretion of SseB appears to be independent of *sseB* transcription. In addition, transcription of *sseB* was analysed using a transcriptional fusion to *sseA*, which lies immediately upstream and is part of the same operon as *sseB* (Cirillo *et al*., 1998). The *sseA*::*luc* fusion strain was grown in MgM medium at pH 7.0 until the OD_{600} reached ≈0.5. The medium was then changed to fresh MgM medium at pH 5.0 or pH 7.0, and luciferase activity was determined at different time points. Exposure to the lower pH did not increase transcription of the gene fusion (Fig. 5B). We conclude that the pH-based control of SseB secretion is not caused by transcriptional activation. Rather, these observations suggest that SseB delivery to the surface relies on the acidic pH-induced release of preformed molecules of SseB stored in the cytosol.

Growth phase regulation of SseB expression

To investigate the kinetics of the pH-induced secretion, strain 12023 was grown in MgM medium, pH 7.5, at 37°C and, when the OD_{600} of the culture reached ≈0.35, the medium was replaced with fresh MgM medium at pH 5.0, and bacteria were sampled at different time points of growth. Over the first hour, SseB was barely detected in the cell pellet fraction of the culture and was not detected in the hexadecane fraction (Fig. 6A). When the bacteria reached an OD_{600} of ≈0.9 (entering stationary phase), the amount of SseB detected inside the cells increased greatly, and SseB was detected in the hexadecane fraction (Fig. 6A). A mock induction experiment was performed in the same way, except that the pH was kept at 7.5. The same kinetics of intracellular accumulation of SseB were observed, but no secreted SseB was detected (Fig. 6B).

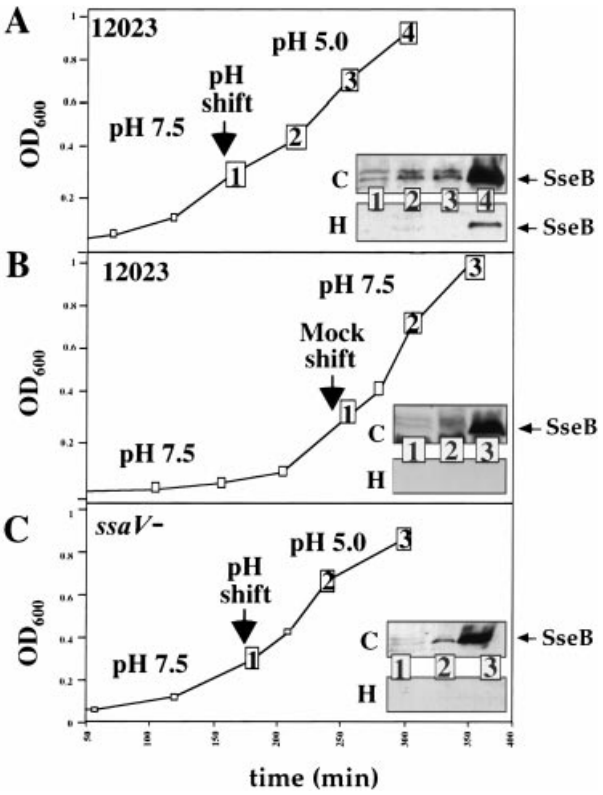

Fig. 6. Effect of growth stage of the bacterial culture on SseB accumulation.
A. Strain 12023 was grown in MgM medium, pH 7.5, until it reached an OD_{600} of 0.35. The medium was then changed to fresh MgM medium, pH 5.0, and samples were taken at different time points thereafter.
B. Strain 12023 was grown in MgM medium, pH 7.5, until it reached an OD_{600} of 0.35. The medium was then changed to fresh MgM medium, pH 7.5, and samples were taken at different time points thereafter.
C. Strain HH109 (*ssaV*::*aphT*) was grown in MgM medium, pH 7.5, until it reached an OD_{600} of 0.35. The medium was then changed to fresh MgM medium, pH 5.0, and samples were taken at different time points thereafter. In all cases, hexadecane (H) and cell pellet (C) fractions were analysed by immunoblotting.

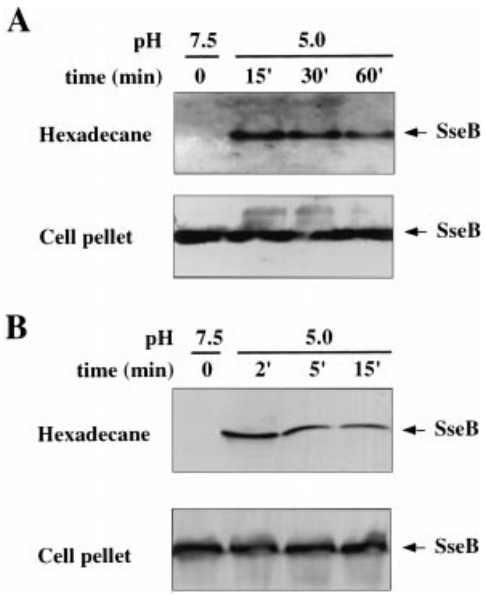

Fig. 7. Secretion of SseB can be detected within minutes of a pH shift.
A. Strain 12023 was grown in MgM medium, pH 7.0, until the culture reached an OD_{600} of ≈1.0, and the pH of the medium was then shifted to pH 5.0 by adding 0.1 M HCl. Samples were taken at different time points after the pH shift. Hexadecane and cell pellet fractions were obtained and analysed by immunoblotting.
B. Strain 12023 was grown in MgM-MES, pH 7.0, until the culture reached an OD_{600} of ≈1.0. The medium was then changed to MgM-MES medium, pH 5.0. Samples were taken at different time points after the pH shift. Hexadecane and cell pellet fractions were obtained and analysed by immunoblotting. For clarity, different exposure times are shown for hexadecane and cell pellet fractions. The amount of secreted SseB corresponds approximately to 10% of total SseB.

To rule out the possibility that the appearance of SseB in the hexadecane fraction resulted from the release of intracellular SseB from lysed cells in the bacterial culture, the same experiment was carried out using a strain carrying a mutation in *ssaV*, encoding one of the components of the secretion apparatus. A similar accumulation of intracellular SseB was observed, but no secreted protein was detected (Fig. 6C).

Rapid secretion of SseB

To investigate the kinetics of SseB secretion in closer detail, strain 12023 was grown in MgM medium, pH 7.0, at 37°C until the cells were entering stationary phase. At this time, the pH of the medium was shifted to 5.0, and bacteria were sampled at different time points thereafter. Measurement of the culture medium pH confirmed that it did not change during the course of the experiment. After 15 min of incubation at pH 5.0, SseB was detected in the hexadecane fraction. There was no obvious change in the amount of intracellular SseB after the pH shift, confirming that a drop in pH does not dramatically affect SseB production. A similar amount of SseB was recovered from the cell surface after 1 h of incubation at acidic pH (Fig. 7A), suggesting that pH induction results in a quick release of a proportion of presynthesized SseB.

To rule out the possibility that pH microchanges might affect SseB secretion, we carried out an induction experiment by transferring bacteria to fresh MgM-MES medium, pH 5.0, a medium with good buffering capacity in this range. Strain 12023 was grown in MgM medium, pH 7.0, until the cells were entering stationary phase, and cell aliquots were transferred to MgM-MES medium, pH 5.0. Bacteria were then sampled at different time points. Two minutes after the pH change, SseB was already detected in the hexadecane fraction, and longer incubation times did not result in greater yields (Fig. 7B). This yield represented ≈10% of the total SseB detected, significantly lower than the amount of secreted protein detected after overnight growth. Transfer of a cell aliquot to pH 7.0 medium did not lead to secretion of SseB (data not shown). The short time in which the secretion of SseB takes place after the pH shift strongly suggests that the secretion apparatus is assembled in the membrane before the pH change.

Effect of different external pH levels on SseB secretion

To characterize acid-induced secretion of SseB further, we analysed the SseB secretion response to different pH levels ranging from 7.5 to 4.5. Secretion of SseB was detected when strain 12023 was shifted to MgM medium at pH 4.5 or pH 5.0 after growth at pH 7.5, but not when it was transferred to medium at a higher pH (Fig. 8A). To confirm that the appearance of SseB in the hexadecane fraction was not the result of non-specific release caused by the acid environment, we included a strain carrying a mutation in *ssaV* as a control. No SseB was detected in the hexadecane fraction of this strain after exposure to pH 4.5, confirming that the secretion of SseB in the wild-type strain was mediated by SPI-2 (Fig. 8B).

Agglutination of bacterial cells

Attempts to identify structures on the surface of strain 12023 similar to those containing EspA in EPEC (Knutton *et al*., 1998) after growth in inducing conditions were not successful. However, a proportion of cells in such cultures were agglutinated by the anti-SseB serum (Fig. 9), indicating that SseB is exposed on the surface of the bacterial cell. It is possible that the SPI-2 secretion system does not produce filamentous structures containing SseB or, if it does, these appendages might be relatively fragile.

Discussion

In this paper, we show that SseB is a component of the SPI-2 type III secretion system of *S. typhimurium* and is

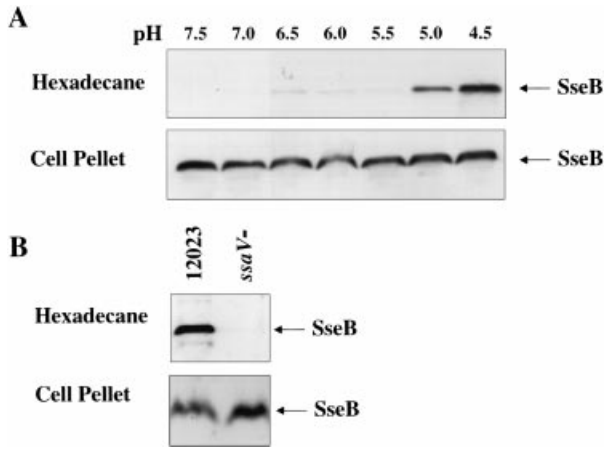

Fig. 8. Effect of different pH levels on SseB secretion.
A. Strain 12023 was grown in MgM-MES medium at pH 7.5 until the culture reached an OD_{600} of ≈1.0. The medium was then changed to MgM-MES media at different pH. Samples were taken 5 min after the pH shift. Hexadecane and cell pellet fractions were obtained and analysed by immunoblotting.
B. Strain HH109 (*ssaV*::*aphT*), was grown in MgM-MES medium at pH 7.5 until an OD_{600} of 1.0 was reached. The medium was changed to fresh MgM-MES medium at pH 4.5 and incubated for 5 min. Hexadecane and cell pellet fractions were obtained and analysed by immunoblotting. For clarity, different exposure times are shown for hexadecane and cell pellet fractions. The amount of secreted SseB corresponds approximately to 10% of total SseB.

secreted onto the bacterial cell surface in response to acidification of the culture medium. Initial evidence implicating SseB as a protein secreted by the SPI-2 system was based on amino acid sequence similarity to EspA of EPEC. Further evidence comes from the similar phenotype of Δ*sseB* and other SPI-2 mutant strains in macrophages and *in vivo*, and the fact that transcription of the *sseB* operon is dependent on *ssrA* (Cirillo *et al*., 1998; Hensel *et al*., 1998). Expression of several other SPI-2 genes, including *sseA*, has been shown previously to be enhanced by low Mg^{2+} and phosphate starvation (Deiwick *et al*., 1999). As *sseB* is part of the same operon as *sseA* (Cirillo *et al*., 1998; T. Kubo and D. W. Holden, unpublished results), it is not surprising that greater amounts of SseB were found in cells growing in media with limiting Mg^{2+} or phosphate. However, like the other SPI-2 genes that have been examined (Deiwick *et al*., 1999), this increase occurred gradually and reached maximal levels after the bacterial cultures had entered stationary growth phase. These results suggest that, although nutrient deprivation is an important prerequisite for SPI-2 gene expression, there are other factor(s) that are present at stationary phase growth and are necessary for SseB accumulation. The identity of these factor(s) and the mechanism of this regulation are presently unknown.

Transfer of SseB to the bacterial cell surface was shown to be dependent on three genes predicted to encode components of the SPI-2 secreton, but mutations in SPI-1 genes did not affect this process. Therefore, SseB is the first secreted protein of the SPI-2 system to be identified. SseB secretion is apparently controlled by the pH of the medium, with maximal secretion occurring rapidly after transfer of cells to pH 4.5–5.0. The levels of secreted SseB in cultures grown overnight under inducing conditions were higher than in those induced by a pH shift. This suggests that the acid environment allows limited secretion and that the cells then enter a stage during which secretion is prevented. The higher levels of secreted SseB detected after overnight culture might result from several rounds of secretion. Acidification of the media did not have a significant effect on *sseB* expression or intracellular SseB levels, but appears to be the external stimulus that triggers the secretion of preformed cytosolic SseB.

Changes in extracellular pH influence production and secretion of components of other type III secretion systems. A decrease in extracellular pH from pH 7.4 to pH 6.0 resulted in a 10-fold decrease in *virF* transcription and reduced invasiveness of *Shigella flexneri* (Nakayama and Watanabe, 1995). High pH has been shown to increase the expression of type III secretion system genes in *Pseudomonas syringae* (Rahme *et al*., 1992; Xiao *et al*., 1992) and *Erwinia amylovora* (Wei *et al*., 1992). Recent work

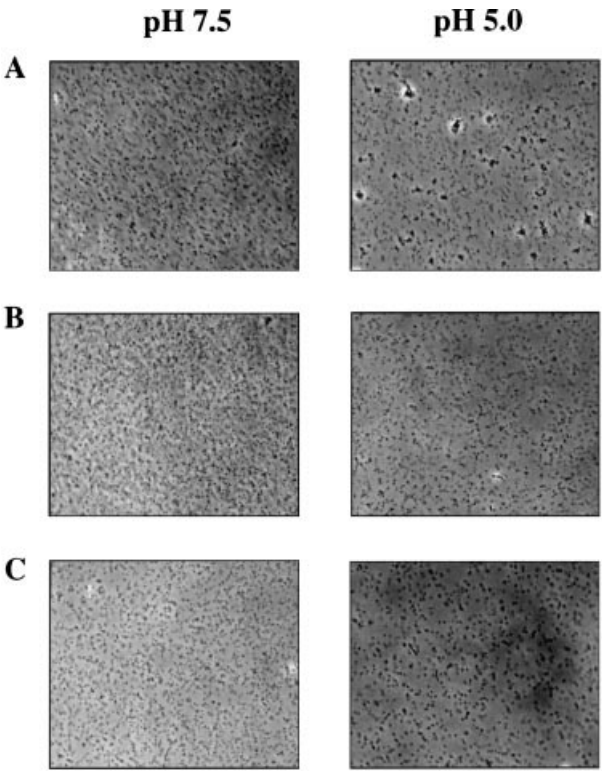

Fig. 9. Agglutination assays with anti-SseB serum of different strains after overnight growth in MgM medium at pH 7.5 or pH 5.0.
A. Strain 12023.
B. Strain HH102 (Δ*sseB*).
C. Strain HH109 (*ssaV*::*aphT*).

has shown that pH is probably the most important factor regulating the expression and secretion of components of the Inv/Spa system of *S. typhimurium* (Daefler, 1999). In this case, however, induction occurred after the pH of the media was shifted from acidic to mildly alkaline, and synthesis of machinery components and secretion of substrates were induced simultaneously after the pH change. Acid-induced secretion of SseB therefore appears to be a novel variation on the theme of pH regulation of type III secretion. Several type III secretion systems have been shown to be induced *in vivo* by intimate contact with the host cell membrane (Ménard *et al*., 1994; Rosqvist *et al*., 1994; Zierler and Galán, 1995; Petterson *et al*., 1996), leading to the concept that type III secretion is contact dependent (Galán, 1996; Cornelis and Wolf-Watz, 1997). The finding that acidic pH is sufficient to induce secretion of SseB by the SPI-2 system in laboratory conditions does not rule out a contact-dependent induction *in vivo*, and further work is required to establish the signals that regulate secretion inside host cells.

The InvA/Spa secretion system is involved in bacterial invasion of the gut epithelium of the small intestine (Galán, 1996), and the alkaline-controlled induction of SPI-1 genes is thought to reflect the increase in physiological pH as the bacteria pass from the stomach to the small intestine (Daefler, 1999). The SPI-2 secretion system is required for bacterial replication inside the macrophages (Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998), and SPI-2 genes are induced over several hours after bacterial entry into host cells (Cirillo *et al*., 1998). After uptake, the bacteria become localized in vacuoles that have been reported to acidify rapidly to pH 4.0–5.0 (Rathman *et al*., 1996). Intracellular bacterial accumulation and SPI-2 gene expression were reduced by inhibition of the acidification process by bafilomycin (Rathman *et al*., 1996; Cirillo *et al*., 1998). We and Cirillo *et al*. (1998) did not observe transcriptional induction of SPI-2 genes by low pH *in vitro*, suggesting that the effect of bafilomycin on SPI-2 transcription may not result from a direct effect on pH. These observations, together with the results presented here, suggest that low pH could be the physiological signal mediating SPI-2 secretion of SseB in host cells. Regulation of SPI-1 and SPI-2 secretion by different ranges of pH might help to ensure that these two functionally distinct systems are activated independently and only when and where they are required.

The ability of hydrophobic reagents to remove SseB from the cell indicates that the protein becomes weakly associated with the cell surface after secretion. EspA, which shares limited sequence similarity with SseB, is part of a filamentous organelle that links the bacterium and the eukaryotic cell and is required for the transfer of other secreted proteins into infected cells (Ebel *et al*., 1998; Knutton *et al*., 1998). Although initial attempts to identify SPI-2-dependent surface structures on *S. typhimurium* have not been successful, the results reported here support the notion that SseB could be part of the SPI-2 translocon delivering effector proteins from *S. typhimurium* into the host cell.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Strain HH129 was constructed by P22 transduction of a *hilA*::Tn*10* mutation from a derivative of SL1344 (a gift from Catherine Lee, Harvard Medical School, USA) to strain 12023. Strain HH130 was constructed by P22 transduction of

Table 1. Plasmids and bacterial strains used in this study.

Plasmid or strain	Description	Reference
Plasmids		
p*sseB*	Cm^r; *sseB* in pACYC184	Hensel *et al*. (1998)
E. coli strains		
DH5α	See reference	Gibco BRL
S. typhimurium strains		
12023	Wild type	NCTC, Colindale, UK
P3F4	*ssrA*::mTn*5*	Shea *et al*. (1996)
P11D10	*ssaJ*::mTn*5*	Shea *et al*. (1996)
P7G2	*ssaC*::mTn*5*	Shea *et al*. (1996)
HH102	*sseB*Δ::*aphT*, Km^r; non-polar mutation	Hensel *et al*. (1998)
HH103	HH102 containing p*sseB*	Hensel *et al*. (1998)
HH104	*sseC*::*aphT*, Km^r; non-polar mutation	Hensel *et al*. (1998)
HH106	*sseE*Δ; non-polar mutation	Hensel *et al*. (1998)
HH107	*sseF*Δ::*aphT*, Km^r; non-polar mutation	Hensel *et al*. (1998)
HH108	*sseG*::*aphT*, Km^r; non-polar mutation	Hensel *et al*. (1998)
HH109	*ssaV*::*aphT*, Km^r; non-polar mutation	Deiwick *et al*. (1999)
HH129	*hilA*::Tn*10*	This study
HH130	*prgH*::Tn*phoA*	This study
MvP127	*sseA*::*luc*	Hensel *et al*. (1998)

a *prgH*::Tn*phoA* mutation from IB040, a derivative of SL1344 (Behlau and Miller, 1993), to strain 12023.

Bacterial growth and media

Intracellular salts medium (ISM) contained 170 mM K_2PO_4/KH_2PO_4, pH 5.0 or 7.5, 0.5 mM $MgSO_4$, 1 μM $CaCl_2$, 6 mM K_2SO_4, 5 mM NH_4Cl, 5 mM NaCl, 0.4% glucose and 2 μg ml^{-1} nicotinic acid (Headley and Payne, 1990); ISM-MES media were identical except that 170 mM 2-[*N*-morpholino] ethane-sulphonic acid at pH 5.0 or 7.5 replaced the KPO_4; magnesium minimal medium (MgM) contained 100 mM Tris-Cl, pH 5.0 or 7.4, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 8 μM $MgCl_2$, 38 mM glycerol and 0.1% casamino acids (Hmiel *et al*., 1986); MgM-MES media were identical except that 170 mM 2-[*N*-morpholino]ethane-sulphonic acid at the corresponding pH replaced the Tris-Cl. Antibiotics kanamycin, ampicillin or chloramphenicol were added at 50 μg ml^{-1} when appropriate. Except where indicated, bacteria were grown at 37°C overnight with aeration.

To investigate SseB production and secretion, the strains were grown in MgM medium at pH 7.5 to two different stages of growth. To assay cells growing in exponential phase, the pH was changed when the OD_{600} was ≈0.35. To assay cells entering stationary phase, pH changes were carried out at an OD_{600} of ≈1.0. The pH changes were carried out in two different ways: by replacing the medium or by shifting its pH with a 0.1 M solution of HCl. In both cases, the pH of the culture was monitored during the incubation period, and the results obtained were similar. Media replacement was done both by centrifuging the culture at 35 000 *g* for 5 min at 4°C and gentle resuspension of the cell pellet in fresh media, and by filtering the cells onto 0.45 μm HA filters (Millipore), transferring the filter to the fresh media and gently resuspending the cells. Both methods gave similar results.

Preparation of cell fractions

Bacterial cell densities were determined by measurement of the OD_{600}. To ensure that protein from equal numbers of cells was analysed, protein samples were adjusted to OD_{600} values in all experiments such that each fraction from a 10 ml culture of OD_{600} 0.6 was taken up in 100 μl of protein denaturing buffer for gel electrophoresis.

Cell cultures were cooled on ice and centrifuged at 35 000 *g* for 5 min at 4°C, and the proteins present in the supernatant were collected by trichloroacetic acid precipitation. After removal of the culture medium, the cell pellet was washed with ice-cold PBS buffer and resuspended in 0.3 ml of the same buffer. The suspension was mixed gently with 0.2 ml of xylene or *n*-hexadecane for 5 min at room temperature and centrifuged at 10 000 *g* for 10 min at room temperature (Michiels *et al*., 1990). The organic layer was discarded, the aqueous layer mixed with 1.2 ml of acetone and held at −20°C to form a precipitate, and the cell pellet was stored at −70°C before analysis. The acetone precipitate was centrifuged, dried and also stored at −70°C before analysis. All fractions were dissolved in protein-denaturing buffer before polyacrylamide gel electrophoresis.

To analyse the presence of SseB on the surface of the plastic growth vessel, and to allow a direct comparison with other fractions of the same sample, an appropriate amount of protein-denaturing buffer was added to the drained growth vessel and incubated for 60 min at 37°C.

For proteinase K treatment, cells were pelleted from a 50 ml culture, washed and suspended in 2.5 ml of PBS buffer. A 500 μl aliquot was held on ice, and 20 μl of 25 mM $CaCl_2$ was added to second and third aliquots. Finally, 2.5 μl of 20 mg ml^{-1} proteinase K was added to the third aliquot, and both the second and the third aliquots were held at 37°C for 20 min, when 6 μl of 200 mM EGTA was added to both. All three aliquots were washed three times with cold PBS buffer. *n*-Hexadecane and cell pellet fractions were then prepared as described above.

Recombinant SseB protein and generation of antibodies

For the generation of recombinant SseB (rSseB), a 493 bp fragment was generated by polymerase chain reaction (PCR) using primers SseB-For (5′-CTCGGATCCCAGCAAAATCCGTTT-3′) and SseB-Rev (5′-TGAAAGCTTCATGAGTACGTTTTCTG-3′) introducing *Bam*HI and *Hin*dIII sites, as indicated by the underlining. After digestion with *Bam*HI and *Hin*dIII, the fragment was ligated to vector pQE30 (Qiagen). The resulting construct pJD13 in *E. coli* M15 [pREP] was used for the expression of rSseB, introducing an N-terminal His-tag fusion.

rSseB was expressed according to standard protocols (Qiagen) and purified by metal-chelating chromatography using 'Hi-trap' columns according to the instructions of the manufacturer (Pharmacia). The 19.3 kDa antigen was administered to rabbits by subcutaneous injection of purified protein (about 1.0 mg) emulsified with complete or incomplete Freund's adjuvant for initial or booster immunizations respectively (Harlow and Lane, 1988). Antibody titres were checked by Western blots using the purified antigen and total-cell fractions of *E. coli* expressing *sseB* under the control of the T7 promoter. The generation of anti-SscA and anti-SsaP sera has been described previously (Deiwick *et al*., 1999).

Polyacrylamide gel electrophoresis and Western analysis of proteins

Protein fractions were dissolved in the appropriate volume of protein-denaturing buffer containing 62.5 mM Tris-Cl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue and held at 100°C for 5 min. Proteins were immediately separated on a 12% SDS–polyacrylamide gel using a tricine buffer system (Schägger and von Jagow, 1987). The gel was stained with Coomassie blue (Sambrook *et al*., 1989) or, for Western analysis, transferred to Immobilon-P membranes (Millipore) using a semi-dry blotting apparatus (Bio-Rad) with the buffer system of Kyhse-Andersen (1984). Western analysis used the ECL detection system under conditions recommended by the manufacturer (Amersham Life Science). Incubation of membranes with primary antibodies (rabbit anti-SseB, anti-SscA and anti-SsaP sera) was followed by incubation with anti-rabbit horseradish peroxidase conjugate (Amersham Life Science) as secondary antibody. Immunoreactive species were quantified as chemiluminescent signals on X-ray film by densitometry scanning.

Assay of reporter gene activity

Luciferase activities of bacterial cultures were determined as described by Deiwick *et al*. (1999).

Agglutination of bacterial cells

Bacterial strains were grown with gentle aeration for 16 h in MgM media at pH 5.0 and pH 7.5. Aliquots (5 μl) of the cultures were mixed with the same volume of a 10-fold dilution of anti-SseB serum and incubated for 5 min at room temperature. The samples were then analysed by phase-contrast microscopy at magnification ×400.

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