Pilotins are mobile T3SS components involved in assembly and substrate specificity of the bacterial type III secretion system

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Short title: Pilotins contribute to T3SS assembly and substrate specificity

Abstract

In animal pathogens, assembly of the type III secretion system injectisome requires the presence of so-called pilotins, small lipoproteins that assist the formation of the secretin ring in the outer membrane. Using a combination of functional assays, interaction studies, proteomics and live-cell microscopy, we determined the contribution of the pilotin to assembly, function and substrate selectivity of the T3SS and identified potential new downstream roles of pilotin proteins. In the absence of its pilotin SctG, *Yersinia enterocolitica* forms few, largely polar injectisome sorting platforms and needles. In line, the majority of export apparatus subcomplexes is mobile in these strains, suggesting the absence of fully assembled injectisomes. Remarkably, the export of late T3SS substrates including the virulence effectors is hardly affected in these bacteria, whereas early T3SS substrates, such as the needle subunits, are only exported to a very low degree. We found that pilotins transiently interact with the secretin and the large export apparatus component SctV, but mostly localize throughout the bacterial membrane, where they form transient mobile clusters, which do not colocalize with assembled injectisomes. In addition, pilotins interact with non-T3SS components, including sugar transporters. Pilotins therefore have additional functions downstream injectisome assembly, which include the regulation of type III secretion and a potential new link to the cellular energy metabolism.

Introduction

Bacteria that live in contact to eukaryotic cells greatly benefit from being able to manipulate those cells. The bacterial type III secretion system (T3SS) allows such manipulation by injecting effector proteins directly from the bacterial cytosol into the host cell cytoplasm [1–5]. Although the T3SS is also used by symbionts and commensals, it is best known for its essential role in infections of important human pathogens such as *Salmonella*, *Shigella* and *Yersinia*. In these pathogens, the T3SS machinery, also called injectisome¹, is often assembled upon entry into the host organism. Once the basal body (membrane rings and export apparatus, Fig. 1a) and the cytosolic components are assembled (reviewed in Diepold and Wagner, 2014), the T3SS starts to secrete its own distal components. Amongst those so-called early secretion substrates are the subunits of the needle and proteins required for its correct assembly, as well as early regulatory proteins. When the required needle length is reached [7,8], middle secretion substrates – the translocon proteins forming the needle tip and two hydrophobic interactors that build a pore in the host membrane – are secreted. At this point, the injectisome is ready for secretion of the late secretion substrates, the virulence effectors, which is initiated by host cell contact.

Injectisomes are assembled at the bacterial surface according to the needs of the bacteria in a given situation. While in some cases, one to few injectisomes are sufficient (such as for the intracellular *Salmonella* SPI-2 T3SS [9]), bacteria that need to actively make contact with the host cells usually build more injectisomes [10,11]. The distribution of injectisomes across the bacterial surface is non-random [12–14], but it is currently unclear what determines where new injectisomes are built.

A prime candidate to establish the site of assembly for new injectisomes is the pilotin, a lipoprotein anchored in the inner leaflet of the outer membrane (OM). Pilotins are required for the proper integration and oligomerization of the secretin protein, which forms a large channel in the OM in the T3SS of animal pathogens, but also type II secretion systems (T2SS) and type IV pili (T4P) [15–27]. Since the secretin ring is one of the nucleation structures of the injectisome [6,28] and anchors the T3SS at its final position in the peptidoglycan mesh [29] (Fig. 1a), the localization of the pilotin may determine the place of secretin ring formation and in consequence the distribution of injectisomes.

¹ The T3SS is at the core of both injectisomes and bacterial flagella [11,96]. In this manuscript, “T3SS” refers to the injectisome-type, virulence-associated system.

Pilotins and their cognate secretin proteins in the T2SS and T3SS have been purified and structurally analyzed in 1:1 complexes [23,30,31]. As a lipoprotein, the pilotin also interacts with the localization of lipoproteins (Lol) protein export machinery [31,32]. Based on these properties, different models for the export of the pilotin itself and its role in secretin ring assembly have been proposed [30,31]. Whether the pilotin stays attached to the secretin after secretin ring formation in the OM and the subsequent assembly of the injectisome is debated and might differ between organisms. For T2SS secretins, such attachment was initially proposed, but later rejected [23,33]; recent analyses of purified secretin rings found pilotins attached stoichiometrically outside the main ring [34,35]. *In situ* structures of the T3SS showed electron densities around the secretin, possibly corresponding to pilotin proteins, in *Shigella flexneri* [36], but not in *Salmonella enterica* [37]. In spite of the importance of pilotins for the localization of the central secretin ring structure, protein secretion by the T3SS was found to be strongly impaired, but not completely prevented in absence of the pilotin in some organisms [15,19,25] – the structural and mechanistic basis for this phenotype is currently unclear.

Despite their central role, many key characteristics of pilotins are poorly understood or debated: Why are pilotins usually not encoded with all other structural components of the T3SS, but upstream the main transcription factor of the system? What determines their localization (and eventually that of the injectisome)? Do pilotins remain part of the injectisome after the assembly and do they adapt a functional role in type III secretion at this point?

To shed light on these questions and the role of pilotins in assembly and function of the T3SS, we studied the role of the *Yersinia enterocolitica* pilotin SctG² with functional assays, fluorescence microscopy, proteomics, as well as protein interaction and dynamics studies. We found that, unexpectedly, SctG interacts with the secretin SctC only temporarily, and mostly localizes in dynamic patches that are dispersed throughout the bacterial membrane. In absence of the pilotin, only a small subset of bacteria assemble single, mostly polar needles which are not stably attached to fully assembled injectisomes, as indicated by the lack of colocalization with the cytosolic sorting platform component SctL. To our surprise, presence of the pilotin not only influenced injectisome assembly and overall secretion levels, but specifically altered the secretion pattern of the T3SS. While effector proteins and other late substrates were efficiently secreted in the absence of the pilotin, the export of early substrates, such as the needle components, was greatly impaired. Based on specific

² This manuscript uses the common nomenclature for T3SS components [97,98]; the species-specific nomenclature of the *Yersinia* pilotin is YscW; other names can be found in Table S1.

interaction studies, we provide possible explanations for this unique phenotype and an extended model for the function of the pilotin.

Results

Deletion of the pilotin specifically reduces secretion of early export substrates

To define the overall role of the pilotin SctG in type III secretion, we tested the effects of a complete *sctG* deletion on the virulence plasmid in *Y. enterocolitica* in a secretion assay. Absence of SctG distinctly altered the secretion pattern. While secretion of effector proteins was partially reduced, but still clearly present, early secretion substrates such as the needle subunit SctF, the ruler protein SctP and translocators SctA, B, E were much more severely affected in secretion or not secreted at all. *In trans* expression of SctG complemented the deletion (Fig. 1b, Fig. S1a).

In order to determine the role of SctG in secretion more precisely, we analyzed the secretome of the wild-type and $\Delta sctG$ strains in minimal medium by label-free quantitative mass spectrometry. The results show that T3SS substrates cluster according to their nature, rather than their overall secretion level (Fig. 1c, Table 1, Fig. S1a): The late substrates, effector proteins YopH, O, P, E, M, T as well as the gatekeeper SctW (YopN) and the negative regulators YscM1/2 (marked green in Fig. 1bc), were exported efficiently in absence of SctG. In clear contrast, export of all early and middle substrates of the T3SS (marked magenta in Fig. 1bc) was strongly reduced in $\Delta sctG$. These T3SS substrates build up the needle (SctF) and the inner rod (SctI) connecting it to the export apparatus, control needle length (SctP) and form its tip structure (SctA) as well as a pore in the host membrane (SctB, E). YscX is an early T3SS substrate specific to the Ysc subgroup of T3SS [38,39], which is required for the export of any other protein by the T3SS of the Ysc subgroup [40,41].

Since most pilotins, including SctG, are expressed in an operon with the main transcription factor of the T3SS, VirF [42], we tested if the observed phenotype is caused by impaired VirF expression in the absence of *sctG*. Expression of VirF from plasmid in a $\Delta sctG$ strain indeed slightly increased the secretion of effectors, but not of early substrates (Fig. S1b), indicating that the specific loss of secretion of these early substrates is not caused by an influence on VirF expression.

Fig. 1 – SctG is required for the secretion of early export substrates, but not of effectors.

a) Schematic illustration of the injectisome (adapted from Diepold and Wagner, 2014). Main substructures indicated on the left; components analyzed in this study, including the pilotin SctG and the secretin SctC, are indicated on the right and shown in color. **b)** Secretion assay showing the export of native T3SS substrates into the culture supernatant in the listed strains ($n=3$). Left, molecular weight in kDa; right, assignment of exported proteins. **c)** Label-free mass spectrometry quantification of secreted proteins in $\Delta sctG$ and wild-type (WT), intensity ratio plotted against mean intensity ($n=3$). In **b)** and **c)**, early/middle T3SS export substrates are marked in magenta; late substrates (effectors) are marked in green; substrate with unknown time point of export marked in yellow.

Table 1 – Influence of SctG on T3SS secretion substrates

Label-free quantitative mass spectrometry of known T3SS substrates in the culture supernatant of different strains, as indicated. Magenta shade, early and middle substrates; green shade, late substrates; yellow shade, unknown. # pept., number of total detected peptides. Hits sorted by ratio of $\Delta sctG$ /wild-type (WT). $n=3$, individual measurements listed in Table S2.

T3SS substrate	# pept.	Intensity ratio $\Delta sctG$ / WT	Mean intensity		
			WT	$\Delta sctG$	$\Delta sctC$
Needle component SctF	7	0.0547	3.95E+09	2.16E+08	1.65E+07
Early substrate / regulator YscX	4	0.1366	3.55E+08	4.85E+07	8.80E+06
Tip protein SctA (LcrV)	24	0.1580	8.72E+09	1.38E+09	1.07E+08
Rod / washer protein SctI	3	0.1621	3.56E+08	5.78E+07	2.07E+07
Needle length regulator / ruler SctP	23	0.2359	1.53E+09	3.60E+08	4.84E+07
Translocator SctE (YopB)	19	0.3213	2.73E+09	8.77E+08	3.44E+08
Translocator SctB (YopD)	46	0.4502	3.26E+10	1.47E+10	9.06E+08
Secreted protein YscH/YopR	4	0.5604	2.90E+08	1.62E+08	1.15E+07
Effector YopQ	16	0.6357	1.90E+09	1.21E+09	6.51E+06
Effector YopO	37	0.9833	1.72E+09	1.69E+09	6.58E+07
Effector YopH	64	1.1095	3.11E+10	3.45E+10	1.23E+09
Effector YopE	10	1.5746	1.16E+10	1.82E+10	2.67E+09
Effector YopP	25	2.1810	7.21E+08	1.57E+09	5.24E+06
Gatekeeper SctW (YopN)	17	2.5811	3.65E+09	9.42E+09	3.18E+07
Effector YopM	6	2.6303	4.81E+09	1.26E+10	7.99E+07
Effector YopT	7	4.1605	1.15E+07	4.77E+07	1.28E+07
Regulator YscM1	12	5.5968	4.84E+08	2.71E+09	9.81E+06
Regulator YscM2	7	10.2833	4.52E+07	4.65E+08	5.43E+06

The pilotin is essential for stable localization of the export apparatus and the cytosolic T3SS components, but few needles can form in its absence

We next investigated the specific role of the pilotin in the assembly of the injectisome, which may provide a clue to its function. To this aim, we deleted *sctG* in strains expressing fluorescently labeled variants of the secretin (SctC-mCherry) [28], the large export apparatus component (SctV-EGFP) [29], the cytosolic sorting platform component SctL (mCherry-SctL) [43], and a strain expressing SctF_{SSC}, a variant of the needle protein that can be labeled by maleimide dyes [44]. Except for SctF_{SSC}, which is expressed from plasmid, all other labeled proteins are expressed from their native genetic environment. As expected, fluorescence microscopy of the respective bacteria showed an

important role of SctG in the assembly of the secretin ring. Far from its usual assembly in >10 mostly lateral foci in the wild-type background, SctC mostly localized in large, often polar clusters in the absence of the pilotin (Fig. 2a, left, Dataset S1). These SctC foci mostly do not correspond to fully assembled injectisomes: SctV foci were motile in the membrane in the absence of SctG, like in the absence of the secretin SctC (Fig. 2b, Fig. S2a) [29]. Moreover, we found that the cytosolic component SctL formed very few foci in the absence of SctG (Fig. 2, center; Dataset S1). Nevertheless, a small subset of bacteria lacking SctG did display needles (Fig. 2, right; Dataset S1). These needles, however, did not colocalize with the sorting platform protein SctL (Fig. S2b). *In trans* complementation of SctG restored wild-type localization of all tested components (Fig. S3). Notably, levels of SctG significantly lower than in the wild-type were already sufficient for complementation of secretion and injectisome assembly (Fig. S3).

The results above indicate that SctG is essential for proper assembly and anchoring of all major subcomplexes of the injectisome, but that a subset of cells forms mostly polar needles in its absence. We therefore next analyzed SctG itself to figure out how it conveys its function.

Fig. 2 – SctG is required for normal localization of SctC and stable assembly of the export apparatus and the sorting platform component SctL, but not fully essential for the formation of needles.

a) Fluorescence micrographs of *Y. enterocolitica* expressing the proteins indicated from their native location on the virulence plasmid (SctC-mCherry and mCherry-SctL) or *in trans* (SctF_{55C}, stained with CF 488A maleimide stain) in an effector-less strain background. Top row displays native localization in wild-type (WT) background, bottom row localization in respective ΔsctG strain background. Scale bar, 2 μm. The fraction of bacteria with foci is listed for the respective ΔsctG strains. See Suppl. Dataset 1 for details. *n*=3-6. **b)** Red/green/blue (RGB) overlay and minimum intensity projection showing the movement of SctV-EGFP over time in the indicated effector-less strain backgrounds in live *Y. enterocolitica*. Red/green/blue channel: micrograph at t=0/15/30 s; white foci indicate stable protein localization, whereas colors indicate movement of foci over time. Minimum projection: micrographs at 0/15/30/45/60/75/90 s; foci indicate stable foci over this time period. *n*=3.

180

181 **Pilotin expression is controlled by temperature, similarly to structural T3SS components**

182 Pilotin proteins are abundant but not omnipresent in secretion systems. Despite their consistent
183 reported role for assembly of the highly conserved secretin, pilotins show low sequence
184 conservation (Fig. S4a). Accordingly, we found that even the pilotin from the closely related T3SS of
185 *P. aeruginosa* could not complement a *sctG* deletion in *Y. enterocolitica* (Fig. S4b).

186 Despite this low sequence conservation, pilotins have a conserved unique genetic location within
187 the virulence plasmids or genetic islands encoding the T3SS. While most other structural and
188 regulatory components of the injectisome are under the control of a main transcriptional regulator
189 (VirF in *Y. enterocolitica*, called LcrF in other *Yersinia* species), pilotins are located upstream of the
190 transcriptional regulator itself (genetic neighborhood analysis, Fig. 3a), indicating a specific role in
191 the T3SS with a potentially distinct expression pattern.

192 To unravel the expression kinetics, as well as the subcellular localization of SctG, we introduced a
193 *sctG-sfGFP* fusion in the native *sctG* locus on the *Y. enterocolitica* virulence plasmid by homologous
194 recombination [45]. The SctG-sfGFP fusion protein was stable and functional (Fig. S5). The
195 expression of SctG, VirF and, in consequence, other T3SS components was moderately increased
196 (1.5-2-fold) in the strain. Additionally, few non-T3SS proteins were affected by the fusions (Table
197 S3).

198 SctG-sfGFP was expressed at a level below the detection limit prior to the shift to 37°C, which
199 activates the VirF-dependent expression of other T3SS components. After the temperature shift,
200 SctG expression increased steadily over the next two hours (Fig. 3bc). Somewhat surprisingly, given
201 their different genetic regulation, the SctG-sfGFP expression profile did not differ significantly from
202 mCherry-SctD, a basal body protein labeled in the same strain (Fig. 3c).

203

Fig. 3 – Despite its unique genetic location, SctG expression is regulated similar to other T3SS components.

a) Genetic neighborhood analysis of pilotin proteins (green) of the different T3SS indicated on the left side. Main T3SS transcription factors (orange), secretins (blue) and other T3SS components (gray) are indicated. The *ysrRS* phosphorelay system activating transcription of the Ysa system [46] is marked in yellow. **b)** Expression of SctG-sfGFP (expected molecular weight 42.2 kDa) over time under non-secreting conditions. Immunoblot using antibodies directed against GFP. Control: Expression of GFP from plasmid. Numbers indicate time in min relative to temperature shift to 37°C, which triggers the expression of T3SS components. Right, molecular weight in kDa. *n*=2. **c)** Expression of SctG-sfGFP and the IM ring component mCherry-SctD over time before and after temperature shift to 37°C in a double-labeled strain under non-secreting conditions. Quantification of fluorescence per bacterium, normalized by the respective 120 min value. Dots indicate individual biological replicates with a total of 52, 100, 128, 128, 217, 185, 143 bacteria (from left to right).

219 **Pilotins localize dynamically within the membrane and do not stably colocalize with other**
 220 **injectisome components**

221 The functional SctG-sfGFP fusion allowed us to assay the cellular localization of SctG in live *Y.*
 222 *enterocolitica*. Stable stoichiometric association with the secretin SctC would result in the formation
 223 of membrane foci colocalizing with other injectisome components [28,43]. However, we detected a
 224 disperse membrane localization of SctG-sfGFP which increased over time similar to overall protein
 225 levels in the cell, in addition to cytosolic background fluorescence (Fig. 4a). While a certain clustering
 226 of SctG-sfGFP in the membrane was visible, these clusters did not significantly colocalize with the
 227 mCherry-labeled T3SS components SctC, SctD, or SctL, in contrast to the clear colocalization
 228 observed for mCherry-SctD and EGFP-SctQ used as positive control (Fig. 4b, Fig. S6).

229

Fig. 4 – SctG predominantly localizes in the membrane, but does not significantly colocalize with other injectisome components.

a) Time course microscopy of SctG-sfGFP in *Y. enterocolitica* under non-secreting conditions. Time with reference to the temperature shift to 37°C. Bottom, intracellular localization of SctG-sfGFP at the respective time points. Line scans across single bacteria, approximate average positions of bacterial membranes (m., dark grey) and cytosol (beige) indicated. $n=78, 90, 81, 90$ cells (left to right) from three independent biological replicates. **b)** Colocalization of labeled T3SS components (all expressed from native genetic locus on virulence plasmid) in double-labeled strains as indicated, 120 min after temperature shift to 37°C. $n=3$, except for colocalization of mCherry-SctD and EGFP-SctQ, which was reported earlier [43] and included here as a positive control ($n=1$). Scale bars = 2 μm.

To analyze the mobility and cluster formation of SctG in more detail, we tracked SctG-sfGFP in individual bacteria. Still images and overlays (Fig. 5a), as well as movies (Suppl. Movie 1) showed that the localization of SctG and its clusters in the membrane strongly fluctuates, indicating diffusion within the membrane. We tested this hypothesis by fluorescence recovery after photobleaching (FRAP) and found that indeed, SctG-sfGFP is highly mobile within the membrane with an average half time of recovery at the bleached pole of 21.5 s (+/- 13.0s) (Fig. 5b).

Fig. 5 – Pilotin proteins move dynamically within the membrane, forming transient patches.

Fluorescence microscopy of a strain expressing SctG-sfGFP and mCherry-SctD from their native genetic locations on the virulence plasmid. **a)** Left, single micrographs of SctG-sfGFP taken with 1 s difference. Right, red/green/blue (RGB) overlay of those micrographs for SctG-sfGFP and mCherry-SctD (mCh-SctD) ($n=3$). **b)** Fluorescence recovery after photobleaching for SctG-sfGFP. Left, representative individual micrographs before and at the indicated time points after the bleach event. Bleach spot indicated by red circle. Right, quantification of fluorescence recovery after photobleaching ($n=21$ cells from 3 independent experiments, displayed in different shades of grey). The recovery half-time $t_{1/2}$ was calculated for each individual recovery curve (see Material and Methods for details) and is displayed as average with standard deviation.

260

261 To investigate if SctG localization requires any other T3SS component or non-T3SS-related
 262 *Y. enterocolitica* protein, SctG or SctG-sfGFP were expressed from plasmid in a *Y. enterocolitica*
 263 strain cured of the virulence plasmid (pYV⁻), as well as *Escherichia coli* Top10 (which has no T3SS)
 264 (Fig. S7a). While SctG-sfGFP displayed a membrane localization in *Y. enterocolitica* pYV⁻, similar to
 265 its native localization, it localized predominantly to the cytosol in *E. coli* (Fig. S7ab). Notably,
 266 expression of SctG negatively influenced the growth of *E. coli*. Strains expressing SctG or SctG-sfGFP
 267 only grew to a low OD and microscopy experiments revealed severe lesions and plasmolysis in the
 268 respective bacteria (Fig. S7ac), indicating that the pilotin localizes independently of other T3SS
 269 components, but can be toxic upon ectopic expression. Taken together, SctG follows a similar
 270 expression pattern as other VirF-controlled T3SS components despite its unique genetic location. It
 271 localizes predominantly throughout the membrane, independently from other T3SS components,
 272 and forms small transient patches.

273

Proteome analysis and specific interactions of the pilotin to T3SS components and proteins not directly linked to the T3SS

The unexpected localization of SctG and the finding that amounts below the wild-type level are sufficient for its function in injectisome assembly (Fig. S3) prompted us to consider additional roles of SctG. To identify such roles in an unbiased way, we first compared the *Y. enterocolitica* proteome in presence and absence of SctG under non-secreting conditions. In Δ sctG, the amount of the main transcriptional activator VirF was reduced to about one third. Consequently, most other T3SS components were also present at lower concentrations (Table S4). Absence of SctG had little impact beyond the T3SS, with the notable exception of the OM protein OmpW, whose amount was reduced by a factor of eight (Table S4).

To better understand the function of the pilotin, both with respect to the T3SS and other pathways, we screened for interaction partners by co-immunoprecipitation with SctG-sfGFP, analyzed by label-free mass spectrometry. As expected, SctG interacted with the secretin SctC, but also with other T3SS components, most prominently the effector YopM and the large export apparatus protein SctV (Fig. 6). Most other T3SS components were enriched to a lower degree (Fig. 6, Table S5).

Interestingly, a number of proteins not directly linked to the T3SS were enriched highly significantly. Most prominently, the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) trehalose transporter subunit EIIBC (PTS-IIBC) consistently interacted very specifically with SctG (Fig. 6), despite low overall expression levels (Table S4). A closer look at the results also showed a significant enrichment of several components of the Lol protein export pathway for the insertion of lipoproteins into the OM, namely LolD and LolE, as well as other non-T3SS proteins, such as Lon protease (termed endopeptidase La in *Y. enterocolitica*) and the prokaryotic tubulin homolog FtsZ, a major player in cell division (Fig. 6, Table S5).

299

306

Fig. 7 – The SctG interactor PTS trehalose transporter subunit EIIBC does not affect assembly or function of the T3SS in rich medium.

a) Left: Micrographs of SctG-sfGFP and mCherry-SctL in double-labeled *Y. enterocolitica* strains otherwise wild-type (WT) or with a deletion of the PTS trehalose transporter subunit EIIBC (Δpts). The experiment was performed under non-secreting conditions ($n=3$). Right: Secretion assay of the PTS trehalose transporter subunit EIIBC (Δpts) deletion with WT and $\Delta sctD$ strains serving as positive and negative controls, under secreting conditions ($n=2$), molecular weight in kDa indicated. **b)** Left: Micrographs of EGFP-SctQ expressed from its native location on the virulence plasmid in *Y. enterocolitica* otherwise WT or overexpressing the PTS trehalose transporter subunit EIIBC (+PTS). Experiments performed under non-secreting and secreting conditions as indicated ($n=3$ each). Right: corresponding secretion assay, molecular weight in kDa indicated. Scale bar for all micrographs, 2 μ m.

Discussion

Pilotin proteins are conserved components of T3SS in many bacteria including most animal pathogenic variants. They have a unique genetic location and a central role for the assembly of the T3SS injectisome. However, little was known about their behavior and role beyond the assistance in the proper localization and oligomerization of the secretin ring of the injectisome in the OM. In this study, we confirm this role, but uncover an additional effect on T3SS export substrate selection. Unexpectedly, we found that pilotins are mobile membrane components which may link the T3SS to other cellular pathways, including sugar metabolism.

Pilotins differentially affect major steps in T3SS assembly

Pilotins have long been known to assist the assembly of the SctC secretin ring in the OM [18–22,24,25]. Our data confirm these reports: In the absence of the pilotin, secretins localized in large foci at one of the bacterial poles (Fig. 2a). The localization of other T3SS components in absence of SctG differed from that of the secretin: SctL, a component of the cytosolic sorting platform of the T3SS, only formed single, mostly polar, foci. Similarly, we observed few, also mostly polar, needles for a subset of bacteria. However, these needles and the sorting platform protein SctL did not colocalize (Fig. 2a, Fig. S2b), indicating an absence of stably assembled injectisomes. Consistent this, the nonameric large export apparatus component SctV [48] assembled, but moved within the membrane in the absence of SctG (Fig. 2b, Fig. S2a), indicating a lack of anchoring by the ring-forming SctCDJ proteins [29]. Since all cytosolic T3SS components, including SctL, are required for protein export under normal conditions, we speculate that transient interactions of SctL and the other cytosolic T3SS components with basal body components at the poles might allow the limited export of the needle subunit, which would then stay stably associated with the bacteria despite the disassembly of the cytosolic sorting platform. In agreement with this hypothesis, we had found earlier that the cytosolic T3SS sorting platform components do exchange between the injectisome-bound sorting platform and a cytosolic pool [43,49] and that the sorting platform components temporarily dissociate from the remaining injectisomes under certain conditions, such as low external pH [50]. The presence of a low number of needles had previously been reported for the closely related *P. aeruginosa* T3SS [19]. Notably, the fraction of bacteria with associated needles and the overall secretion efficiency of the pilotin deletion showed an unusually high variation between individual replication experiments, suggesting an influence of external factors. However, despite our best efforts, we were unable to identify these factors.

Role of pilotins in T3SS substrate specificity

Analysis of the T3SS secretome by SDS-PAGE and quantitative mass spectrometry revealed a specific effect of the presence of the pilotin on export of the so-called early substrates, required for formation of the needle and its tip structure (Fig. 1, Table 1). Notably, while deletion or modifications of *sctG* have an effect on the expression of VirF and its targets (Table S3, 4), most likely due to an influence on the formation of hairpin structures in the *sctG-virF* mRNA that regulate VirF transcription as part of the thermoregulation of T3SS expression [42,51], the role of SctG in secretion substrate is independent from VirF levels (Fig. S1b). What could then be the reason for the striking change of substrate specificity in absence of the pilotin, where early export substrate are much more strongly affected by the absence of SctG than the late substrates, the virulence effectors? Similar phenotypes had been found, although not always explicitly noted, in earlier studies in *Y. enterocolitica* [15,25] and *P. aeruginosa* [19]. Taking into account the limited, probably transient interaction of the pilotin with the secretin that we observed, it is tempting to speculate that presence of the pilotin at the injectisome favors the secretion of early substrates. Subsequent dissociation of the pilotin would then contribute to a switch in export cargo specificity towards the effectors. The specific (albeit not necessarily direct) interaction of SctG with the large export apparatus component SctV (Fig. 6), which is a key player in substrate selection [52–54], supports this hypothesis.

Alternative explanations for the change in export substrate specificity might be (i) the polar localization of the secretin (and consequently the hypothetical transient T3SS), which might change the accessibility for certain substrates, or (ii) a two-step export pathway, with a first transport step across the IM, facilitated by the T3SS export apparatus (which is assembled, albeit not anchored in the peptidoglycan, in the absence of the pilotin (Fig. 2b)) and a subsequent export step across the OM, facilitated by the secretin (without which no export is observed (Fig. S1)). The two-step transport might prevent the export of early substrates, many of which polymerize outside the cytosol. Alternatively, (iii) strains lacking the pilotin might be able to provide less energy due to the missing link to the PTS sugar transporters or membrane stress as a consequence of secretin misassembly. If effector export is less energy costly than the export of early substrates, this might explain the observed bias in export in absence of SctG. However, in the absence of direct evidence for these alternative explanations, we consider a direct influence of SctG on the substrate specificity through interactions with other T3SS components to be the most likely explanation.

Substrate specificity switching is a central, but incompletely understood, feature type III secretion. Our data suggest that pilotin proteins play a key role in this process, together with the export apparatus components SctV and SctU [55], the gatekeeper complex [56,57] and the sorting platform [58]. If and how the interaction of the pilotin with the effector YopM (Fig. 6) contributes to this process is unclear at the moment.

Additional interactions of the pilotin after its dissociation from the injectisome and possible functions

An unexpected finding of our study was that pilotin concentrations significantly below wild-type levels are sufficient to restore normal T3SS assembly and function (Fig. S3). Although it is one of the few T3SS machinery components encoded outside the *VirF*-regulated operons, native SctG expression develops very similarly to other T3SS components (Fig. 3), consistent with the co-transcription of *sctG* and *virF* in one operon [42]. However, the findings that low pilotin levels already were sufficient for injectisome assembly and secretion and that SctG does not significantly colocalize with other T3SS components (Fig. 4), but is highly mobile in the bacterial membrane (Fig. 5) suggests that the pilotin might have additional functions beyond its role in secretin assembly, possibly after its release from the secretin. We therefore screened for additional interactors of SctG by co-immunoprecipitation. Notably, while the pilotin mostly fractionates with the OM in membrane preparations [18,25,59], many of the proteins found to interact with the labeled pilotin in the co-immunoprecipitation assay are integral IM proteins or IM-associated proteins. The used SctG-sfGFP fusion is expressed from its native genetic location, supports native secretion (Fig. S5), and interacts with both known interactors, the secretin SctC and proteins of the Lol pathway, in our assays (Fig. 6). Despite these indications for correct localization and interactions, it is possible that transport of the fusion protein to the OM is impaired and that this at least enhances the fraction of time the pilotin can contact these IM(-associated) proteins. Alternatively, the interactions may be caused by natively soluble pilotin proteins, possibly bound to other proteins such as LolB [30,31]. Finally, the N-terminal lipid anchor of the pilotin might be removed after assembly of the secretin ring or the injectisome, which would render the protein soluble [25] in the periplasm.

The most striking finding of the interaction studies was the highly specific enrichment of several PTS sugar transporter subunits, most significantly the membrane-bound trehalose transporter subunit EIIBC (Fig. 6, Table S5). PTS systems are used for sugar uptake in many bacteria. They create a concentration gradient by transferring the PEP phosphoryl group onto the incoming sugar via

several intermediate phosphate carriers. The EIIC subunit, which can be fused to the EIIB subunit, serves as channel in the IM. While many bacteria express several PTS transporters, PTS are often unspecific and redundant [60]. Notably, PTS were repeatedly found to act as carbohydrate sensors that control bacterial metabolism in response to the environment [61]. Transporters sensing host-specific compounds can regulate expression and activity of virulence systems [62], indicating that SctG and its main interactor, the PTS trehalose transporter subunit EIIBC, might serve to modulate T3SS function according to the bacterial environment. The fact that so far, no pilotin homologs were found in plant pathogens [3] is consistent with this hypothesis. Indeed, PTS systems were repeatedly associated with bacterial virulence (reviewed in [60,63]): amongst others, presence of the PTS glucose transporter subunit EIIBC conferred a competitive advantage to *Y. pestis* in serum-like medium [64], a *Vibrio cholerae* PTS system was found to modulate virulence gene expression [65], and *Salmonella* Typhimurium lacking a PTS EI subunit showed a nearly 1000-fold increase in LD₅₀ [66]. Specifically a PTS trehalose transporter system induces hypervirulence in *Clostridium difficile* [67]. Notably, the PTS glucose transporter EIIA subunit directly interacts with cytosolic components of the *Salmonella* SPI-2 T3SS and activates type III secretion [47]. Overall, the two most likely links between PTS sugar transporters and virulence appear to be energy provision and/or host sensing. Both possibilities are consistent with the lack of a phenotype of the *pts-IIbc* deletion in our experiments (which were performed in rich medium in the absence of host cells, Fig. 7); however, further experiments are required to describe the link in more detail.

Other significant interaction partners of the pilotin outside the T3SS are FtsZ, a prokaryotic tubulin homolog with a key role in cell division, the protease Lon, components of the ATP synthase (subunits α , β , γ), the Sec export pathway (SecF/A), and LolD/E, two components of the ABC transporter of the Lol lipoprotein export pathway in the IM. FtsZ is an interesting interactor, as it might play a role in the long-known, but still incompletely understood link between T3SS activity and cell growth and division (actively secreting bacteria of many species cease growth and division and are often bigger than T3SS-deficient or non-secreting bacteria [44,68–70]). Since to our knowledge, this is the first time that a T3SS component was found to be associated with bacterial cytoskeleton proteins, this might indicate an involvement of SctG in the non-random localization of T3SS around the cell [12,13]. The Lon protease degrades unfolded or misfolded proteins, especially under stress conditions [71], but can also have more specific roles in metabolism, cell cycle control and virulence through proteolysis of specific regulatory proteins [72,73]. While it cannot be ruled out that the specific interaction of SctG-sfGFP with Lon denotes degradation of partially misfolded SctG-sfGFP,

Lon proteases have been shown to influence T3SS activity, both positively via degradation of histone-like proteins like *Yersinia* YmoA that suppress the transcription of T3SS components [74–77] and negatively, most likely by directly degrading key components of the T3SS, mainly in plant pathogens [78–81]. The presence of three components of the IM ATP synthase amongst the less than forty specific interactors of the pilotin protein is remarkable and, in combination with the strong interaction with the PTS sugar transporter, suggests a link with the bacterial energy metabolism. While such a link is more than conceivable, given the high energy expense of type III secretion [82], its details are unclear at the moment. As lipoproteins in the OM, pilotins are expected to interact with components of both the Sec and the Lol pathway [30,32,83]. While most models emphasize the interaction with LolA and LolB in the periplasm and OM, respectively, we detected strong binding to LolD and LolE in the IM (Fig. 6, Table S5), but no significant binding to LolA or B. Of the two models proposed for the role of pilotins in the assembly of the secretin ring by Majewski and colleagues [31], our data therefore supports the second model where the pilotin is released from LolB before interacting with the secretin.

Taken together, our data indicate that, besides their function in the assembly of the secretin ring of the T3SS, pilotin proteins have additional roles linked to type III secretion. In their absence, assembly of all T3SS subcomplexes and specifically the export of early secretion substrates is strongly impeded, while effectors are exported efficiently. Pilotin protein interact with components of the T3SS, most prominently the secretin SctC and the large export apparatus component SctV, but are mobile in the bacterial membrane and do not stay attached to or even significantly colocalize with the injectisome. In line with these findings, the pilotin also interacted with other cell wall-associated proteins such as a PTS sugar transporter subunit.

A potential model integrating these findings is that during injectisome assembly, SctG interacts with SctC, facilitates its oligomerization in the OM, and the formation of needles. At a later time point, SctG dissociates from the fully assembled injectisome, which then favors the export of effectors upon activation of the T3SS. The pilotin is mobile at this stage and may additionally support this function by interacting with other partners, including PTS sugar transporters, which could provide the necessary energy for secretion. Challenging this model in future studies may therefore lead to a better understanding of the energy provision for the T3SS and its link to sugar import.

Material and methods

Bacterial strain generation and genetic constructs

A list of strains and plasmids used in this study can be found in [Supplementary Table 6](#). All *Y. enterocolitica* strains used in this study are based on the *Y. enterocolitica* wild-type strain MRS40 or the strain IML421asd (Δ HOPEMTasd). In IML421asd all major virulence effector proteins (YopH,O,P,E,M,T) are absent. Furthermore, this strain harbors a deletion of the aspartate-beta-semialdehyde dehydrogenase gene which renders the strain auxotrophic for diaminopimelic acid (DAP), making it suitable for work in an biosafety class 1 environment [84].

Bacterial cultivation, secretion assays and protein analysis

Y. enterocolitica day cultures were inoculated from fresh stationary overnight cultures supplemented with the relevant antibiotics and DAP (80 mg/ml), where required, to an OD₆₀₀ of 0.15 for secreting and 0.12 for non-secreting conditions, respectively. As growth medium, BHI (brain heart infusion broth) supplemented with nalidixic acid (35 mg/ml), DAP (80 mg/ml) where required, glycerol (0.4%) and MgCl₂ (20 mM) was used. Where required, ampicillin was added (0.2 mg/ml) to select for the maintenance of expression plasmids. For secreting conditions, 5 mM EGTA were added; for non-secreting conditions, 5 mM CaCl₂ were added to the pre-warmed (approx. 55°C) medium, which was filtered through a 0.45 µm filter before the addition of other supplements and cooled down to ambient temperature for the experiments. After inoculation, day cultures were incubated at 28°C for 90 min to reach exponential growth phase. Expression of the *yop* regulon was then induced by a rapid temperature shift to 37°C in a water bath. Where indicated, protein expression from plasmid (derivates of pBAD-His/B (Invitrogen) in all cases except expression of VirF from pCL5 [85]) was induced at this point by the addition of 0.02–1.0% L-arabinose as indicated in the respective experiments.

For protein secretion assays and analysis of total cellular proteins, bacteria were incubated for 2-3 h at 37°C, as indicated. 2 ml of the culture were collected at 21,000 *g* for 10 min. The supernatant was removed from the total cell pellet and proteins were precipitated by addition of a final concentration of 10% trichloroacetic acid (TCA) and incubation at 4°C for 1–8 h. Precipitated proteins were collected by centrifugation for 15 min at 21,000 *g* and 4°C, the pellet was washed once with 1 ml ice cold acetone and subsequently resuspended and normalized in SDS-PAGE loading buffer. Total cellular protein samples were normalized to 0.3 OD units (ODu, 1 ODu is equivalent to

1 ml of culture at an OD₆₀₀ of 1, corresponding to approximately 5×10^8 *Y. enterocolitica*) and supernatant samples to 0.6 ODU. Before loading, samples were incubated for 5 min at 99°C. Separation was performed on 11%, 15% or gradient 12–20% SDS-PAGE gels, using BlueClassic Prestained Marker (Jena Biosciences) as a size standard. For visualization, the SDS-PAGE gels were stained with InstantBlue (Expedeon). For immunoblots, the separated proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane. Primary mouse antibodies against GFP (1:4000) (ThermoFisher Proteintech 66002-1-Ig, 1:4000) were used in combination with secondary anti-mouse antibodies conjugated to horseradish peroxidase (Sigma A8275, 1:5000). For visualization, ECL chemiluminescence substrate (Pierce) was used in a LAS-4000 Luminescence Image Analyzer.

Fluorescence microscopy

For fluorescence microscopy, bacteria were treated as described above. After 2–3 h at 37°C, 400 µl of bacterial culture were collected by centrifugation (2400 × g, 2 min) and reconstituted in 200 µl minimal medium (100 mM 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES) pH 7.2, 5 mM (NH₄)₂SO₄, 100 mM NaCl, 20 mM sodium glutamate, 10 mM MgCl₂, 5 mM K₂SO₄, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 50 mM glycine). 2 µl of resuspension were spotted on agarose pads (1.5% low melting agarose (Sigma-Aldrich) in minimal medium) in glass depression slides (Marienfeld). Where required, agarose pads and media were supplemented with 80 mg/ml DAP for ΔHOPeMTasd-based strains, 5 mM CaCl₂ for non-secreting conditions, or 5 mM EGTA for secreting conditions.

Microscopy was performed on a Deltavision Elite Optical Sectioning Microscope equipped with an UPlanSApo 100×/1.40 oil objective (Olympus), using an Evolve EMCCD camera (Photometrics). Where applicable, the prepared samples were illuminated first in the mCherry = 0.4 s with a mCherry filter set and afterwards in GFP = 0.2 sec with a GFP filter set. Depending on the experiment, z stacks with 1–15 slices (Δz = 0.15 µm) per fluorescence channel were acquired. The acquired micrographs were subsequently deconvolved using softWoRx 7.0.0 (standard “conservative” settings). Images were further processed with FIJI (ImageJ 1.51f/1.52i/1.52n) [86]. Where necessary, a drift correction with the StackReg Plugin [87] was performed. Fluorescence quantification was performed in FIJI. For presentation of micrographs in a figure, representative fields of view were selected, and brightness and contrast of the micrographs was adjusted identically within the compared image sets.

Maleimide-based needle (SctF) staining.

Maleimide-based staining to visualize the injectisome needles was performed as described in [50]. Briefly, expression of SctF_{S5C} from the pBAD-His/B plasmid was induced by addition of 1.0% L-arabinose at the time of the temperature shift in strains expressing the native SctF at the same time. After 2-3 h, bacteria were collected and resuspended in 0.2 volumes of minimal medium supplemented with 5 µM of CF 488A maleimide dye (Sigma-Aldrich, USA) at 37 °C for 5 min. After staining, cells were washed once with 1500 µl of minimal medium and resuspended in 200 µl of minimal medium. 2 µl of bacterial suspension were spotted on 1.5% agarose pads in minimal medium and visualized under the microscope as described above.

Classification and quantification of fluorescent foci

To determine the number of stable YscV-EGFP foci, seven micrographs were taken in 15 s intervals and the minimal intensity for each location was determined from the deconvoluted micrographs. Foci were then identified in FIJI using an intensity threshold of 700 (determined in images of the wild-type background strain) and the “analyze particles” function with a minimal size of 4 pixels and a particle circularity of 0.5-1.0. To evaluate the fraction of bacteria with foci for SctC-mCherry, mCherry-SctL and SctF_{S5C} in the absence of SctG, foci were manually identified and classified as polar, subpolar and lateral based on their subcellular localization in deconvoluted micrographs. In case a cell displayed multiple foci in different localization classes, bacteria were classified as containing a lateral, subpolar, polar focus in descending order (e.g. a bacterium with a lateral and a polar focus was assigned “lateral”). The overall number of bacteria in a field of view was determined by using the deep learning based network, DeepBacs [88], that was previously trained with differential interferometry contrast (DIC) micrographs of *Y. enterocolitica*. The overall percentage of cells with foci is displayed in the figure.

Fluorescence distribution analysis

For analysis of the fluorescence distribution across bacteria, line scan analysis was performed on deconvoluted micrographs in FIJI. With the straight line freehand tool, measurements areas were defined from one lateral membrane to the other at a 90° angle. Per condition, 3 fields of view with 18-30 cells were analyzed and the background was measured. The line scans were individually corrected for background fluorescence, normalized (fluorescence fraction relative to overall fluorescence over background) and centered at the position closest to center of the total fluorescence over background.

Fluorescence recovery after photobleaching (FRAP)

Cells were grown in non-secreting medium as described above. Samples were taken after 3 h at 37°C. To minimize unnecessary bleaching, no z stacks were acquired. Three pre-bleach images were acquired and locations near selected bacterial cell poles were photobleached by individual 30 ns pulses of a 488 nm laser. Recovery of the bleached foci was followed by time-lapse microscopy. Deconvolved micrographs were used for the fluorescence quantification.

The relative fluorescence of the bleached spot was calculated as follows:

$$\text{Relative fluorescence} = \frac{(\text{Intensity bleached cell pole} - \text{Intensity background})}{(\text{Intensity total cell} - \text{Intensity background})}$$

The relative fluorescence was then normalized (average pre-bleach value set to 1; post-bleach value set to 0) and an exponential recovery was fitted using the equation:

$$y = A(1 - e^{-bx})$$

where y stands for the relative fluorescence, x for the time, A for the overall ratio of fluorescence recovery, and b for the inverse of the time constant $\tau_{1/2}$. For the fit, A was restricted to values between 0.7 and 1.1. Each bleach curve was fitted individually, and fits with $R^2 < 0.7$ were excluded from further analysis. The half-time of recovery was calculated from the time constant as follows:

$$t_{1/2} = \ln(2) \tau_{1/2}$$

Proteome and secretome analysis by shotgun proteomics based mass spectrometry

Strains were grown and prepared as described above. After 2.5 h at 37°, cultures were normalized to an OD₆₀₀ of 0.5 and 2 ml were harvested by centrifugation at 9,391 g for 2 min. Cell were washed 3 times with 1x phosphate buffered saline (PBS) (8 g/l NaCl, 0.2 g/l KCl, 1.78 g/l Na₂HPO₄*2H₂O, 0.24 g/l KH₂PO₄, pH 7.4). After washing, the cell pellet was resuspended in 300 µl lysis buffer (2% sodium lauroyl sarcosinate (SLS), 100 mM ammonium bicarbonate (ABC)) and incubated at 90°C for 10 min. Protein concentrations were measured using a BCA assay (Thermo Scientific) and following reduction in 5 mM Tris(2-carboxyethyl) phosphin (TCEP, 15 min at 90 °C) and alkylation in 10 mM iodoacetamid (30 min, 20°C in the dark), 50 µg protein was used for tryptic digestion (1 µg, Serva) at 30°C overnight in presence of 0.5% SLS. After digestion, SLS was precipitated by adding a final concentration of 1.5% trifluoroacetic acid (TFA).

Desalting of cleared peptide samples was performed in C18 solid phase extraction (SPE) cartridges (Macherey-Nagel). First, cartridges were prepared by adding acetonitrile, followed by equilibration using 0.1% TFA and loading of peptide samples. Cartridges were washed with buffer containing 5% acetonitrile and 0.1% TFA, and finally eluted with 50% acetonitrile and 0.1% TFA into a fresh tube. Eluted peptides were dried and reconstituted in 0.1% TFA for LC-MS analysis.

Purified peptides were analyzed by liquid chromatography-mass spectrometry (MS) carried out on a Q-Exactive Plus instrument connected to an Ultimate 3000 rapid-separation liquid chromatography (RSLC) nano instrument and a nanospray flex ion source (all Thermo Scientific). Peptide separation was performed on a reverse-phase high-performance liquid chromatography (HPLC) column (75 μ m by 42 cm) packed in-house with C₁₈ resin (2.4 μ m; Dr. Maisch GmbH). The peptides were first loaded onto a C18 precolumn (preconcentration set-up) and then eluted in backflush mode with a gradient from 98 % solvent A (0.15 % formic acid) and 2 % solvent B (99.85 % acetonitrile, 0.15 % formic acid) to 25 % solvent B over 66 min, continued from 25 to 40 % of solvent B up to 90 min. The flow rate was set to 300 nl/min. The data acquisition mode for label-free quantification (LFQ) was set to obtain one high-resolution MS scan at a resolution of 70,000 (m/z 200) with scanning range from 375 to 1500 m/z followed by MS/MS scans of the 10 most intense ions at a resolution of 17,500. To increase the efficiency of MS/MS shots, the charged state screening modus was adjusted to exclude unassigned and singly charged ions. The dynamic exclusion duration was set to 10 s. The ion accumulation time was set to 50 ms (both MS and MS/MS). The automatic gain control (AGC) was set to 3×10^6 for MS survey scans and 1×10^5 for MS/MS scans.

Due to an instrument upgrade, the QExactive Plus system was replaced by an Exploris 480 mass spectrometer (Thermo Scientific). For total proteome analysis, purified peptides were analyzed using identical LC settings, and the Exploris MS data acquisition settings were the following: one high-resolution MS scan at a resolution of 60,000 (m/z 200) was obtained with scanning range from 350 to 1650 m/z followed by MS/MS scans of 2 sec cycle time at a resolution of 15,000. Charge state inclusion was set between 2 and 6. The dynamic exclusion duration was set to 14 s. The ion accumulation time was set to 25 ms for MS and AUTO for MS/MS. The automatic gain control (AGC) was set to 300% for MS survey scans and 200% for MS/MS scans.

LFQ analysis was performed using MaxQuant [89] in standard settings using a *Y. enterocolitica* protein database containing proteins of the closely related *Y. enterocolitica* strain W22703 [90] and of the pYVe227 virulence plasmid (GenBank entry AF102990.1). Statistical follow-up analysis of the

MaxQuant LFQ data was performed on an updated SafeQuant [91,92] R-script modified to routinely process MaxQuant “protein groups” outputs. The missing value imputation was adapted from Perseus [93] in default settings and implemented into the update SafeQuant script. Unless otherwise indicated, proteins with ≥ 3 detected peptides and a p value ≤ 0.01 of the sample versus the control using Student’s t-test were considered as enriched and displayed in the tables. Intensity values were shaded according to their value (blue for high intensity; transparent for low intensity) for easier visualization.

For secretome analysis and quantification of secreted effectors in the supernatant, 5 ml BHI cultures were grown under secreting conditions in 15 ml tubes as otherwise described before. After two hours of incubation at 37°C, the medium was changed from BHI to minimal medium supplemented with nalidixic acid (35 mg/ml), diaminopimelic acid (80 mg/ml) where required, glycerol (0.4%), MgCl₂ (20 mM) and EGTA (5 mM). Next, bacteria were again incubated at 37°C shaking for one hour. Samples were OD-normalized in a 2 ml volume, and bacteria were removed by centrifugation (10 min at 21,000 g , 4°C). In the next step, the supernatant was removed and precipitated with a final concentration of 10% TCA for 1–8 h at 4°C; proteins were collected by centrifugation for 15 min at 21,000 g at 4°C and washed once with 1 ml ice cold acetone. A detailed description of the experimental procedure is shown in [94]. The remaining pellet was resuspended in 300 μ l lysis buffer and incubated at 90°C for 10 min. Following reduction and alkylation (see above), proteins were digested using 1 μ g trypsin. All further steps including C18-SPE, LC-MS analysis and LFQ data analysis were identical to the ones described above. The LC separating gradient length was reduced to 45 min due to lower sample complexity.

Co-immunoprecipitation and shotgun proteomics

For co-immunoprecipitation, 100 ml of non-secreting culture medium were inoculated to an OD₆₀₀ of ~ 0.15 and treated as described above. After 2.5 h incubation at 37°C, the cultures were transferred to 50 ml tubes and centrifuged at 2,000 g for 15 min at 4°C. Pellets were washed with 10 ml of 1x PBS. The contents of two tubes were pooled and centrifuged at 2,000 g for 15 min at 4°C. The pellet was resuspended in 2 ml HNN lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 50mM NaF, sterile filtered; protease inhibitor (cOmplete Mini, EDTA free (Roche)) added before use), and incubated for 30 min on a turning wheel at 4°C. Afterwards the bacteria were sonicated with 12 pulses $\times$ 30 s in a Hielscher UP200ST ultrasonic homogenizer. The cells were again placed on a turning wheel for 30 min at 4°C and the sonification process and the subsequent incubation on a turning wheel were repeated. Unbroken cells were removed by centrifugation (20,000 g , 20 min, 4°C). For affinity

purification, 10 µl of bead slurry (GFP-Trap Magnetic Agarose, Chromotek) were added to the lysate and incubated shaking for 1 h at 4°C on a turning wheel. Beads were washed with 500 µl HNN lysis buffer + 0.1% NP40, followed by seven consecutive washing steps with 700 µl 100 mM ABC. 200 µl of elution buffer (100 mM ABC, 1 µg trypsin per sample) were added to the beads and incubated for 45 min on a thermomixer at 30°C at 1200 rpm. Beads were separated and the supernatant was collected. Beads were washed twice using 80 µl of elution buffer 2 (1 M urea, 100 mM ABC, 5 mM TCEP), combined with the first eluate fraction and incubated o/n at 30°C to complete proteolysis. To monitor the experimental steps, samples for Western blot analysis were taken throughout the whole procedure. After digest, 10 mM iodoacetamid were added and the samples were incubated for 30 min in the dark. For desalting, C18 solid phase extraction cartridges samples were acidified and the procedure was carried out as described above. The samples were analyzed on a QExactive Plus mass spectrometer using a resolving LC gradient of 40 min. The analytical settings and data analysis steps including MaxQuant and SafeQuant are described above. Unless otherwise indicated, proteins with ≥3 detected peptides and a *p* value ≤ 0.001 of the sample versus the control using Student's t-test were considered as enriched and displayed in the tables.

Sequence analysis

For the analysis of the genetic environment of T3SS pilotin proteins from model organisms, the following GenBank sequences were used: AF102990.1 (*Yersinia enterocolitica* plasmid pYVe227, complete sequence), NC_002516.2 (*Pseudomonas aeruginosa* PAO1, complete genome reference sequence), NC_003197.2 (*Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, complete genome reference sequence), NC_004851.1 (*Shigella flexneri* 2a str. 301 plasmid pCP301, complete sequence), AM286415.1 (*Yersinia enterocolitica* subsp. *enterocolitica* 8081, complete genome). Multiple sequence alignment of the *sctG* and *sctC* genes of the same systems was performed using T-COFFEE 11.0 [95].

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Author contributions

S.W. performed the majority of the experiments and data analysis and participated in study design and writing of the manuscript. M.F. performed experiments, implemented methods and generated key strains for this study. C.H., C.B. and K.L. assisted in experiments and strain generation. W.S., G.A. T.G. from the Proteomics facility provided this technology and assisted in data evaluation and statistical analysis. A.D. conceived and designed the study, performed data analysis and wrote the manuscript.

Competing interests

The authors declare no competing interests.

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