



HAL
open science

The Tat pathway exports multiple virulence proteins in the plant pathogen *Streptomyces scabies*

Madhumita V Joshi, Stefan G Mann, Haike Antelmann, David Widdick, Joanna K Fyans, Govind Chandra, Matthew I Hutchings, Ian Toth, Michael Hecker, Rosemary Loria, et al.

► To cite this version:

Madhumita V Joshi, Stefan G Mann, Haike Antelmann, David Widdick, Joanna K Fyans, et al.. The Tat pathway exports multiple virulence proteins in the plant pathogen *Streptomyces scabies*. *Molecular Microbiology*, 2010, 10.1111/j.1365-2958.2010.07206.x . hal-00552638

HAL Id: hal-00552638

<https://hal.science/hal-00552638>

Submitted on 6 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**The Tat pathway exports multiple virulence proteins in the
plant pathogen *Streptomyces scabies***

| | |
|-------------------------------|--|
| Journal: | <i>Molecular Microbiology</i> |
| Manuscript ID: | MMI-2010-09954.R1 |
| Manuscript Type: | Research Article |
| Date Submitted by the Author: | 01-May-2010 |
| Complete List of Authors: | <p>Joshi, Madhumita; Cornell University, Plant Pathology Mann, Stefan; John Innes Centre, Molecular Microbiology Antelmann, Haike; Institut für Mikrobiologie, University of Greifswald Widdick, David; University of Dundee, Molecular Microbiology Fyans, Joanna; University of Dundee, Molecular Microbiology Chandra, Govind; John Innes Centre, Molecular Microbiology Hutchings, Matthew; University of East Anglia, School of Biological Sciences Toth, Ian; Scottish Crop Research Institute, Plant Pathology Hecker, Michael; Institute for Microbiology, Department of Microbial Physiology Loria, Rosemary; Cornell University, Department of Plant Pathology Palmer, Tracy; University of Dundee, College of Life Sciences</p> |
| Key Words: | protein export, Tat pathway, <i>Streptomyces</i> , virulence, effector |
| | |

The Tat pathway exports multiple virulence proteins in the plant pathogen *Streptomyces scabies*

Madhumita V. Joshi^{1*}, Stefan G. Mann^{2*}, Haike Antelmann³, David A. Widdick^{4,5},
Joanna K. Fyans^{4,6}, Govind Chandra², Matthew I. Hutchings⁵, Ian Toth⁶, Michael
Hecker³, Rosemary Loria¹ and Tracy Palmer^{4†}

¹Department of Plant Pathology and Plant-Microbe Biology, Cornell University,
Ithaca, NY 14853, USA

²Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, UK

³Institute for Microbiology, Ernst-Moritz-Arndt-University of Greifswald, D-17487
Greifswald, Germany

⁴Division of Molecular Microbiology, College of Life Sciences, University of Dundee,
Dundee DD1 5EH, UK

⁵School of Biological Sciences, University of East Anglia, Norwich Research Park,
Norwich, NR4 7TJ

⁶Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

*These authors contributed equally to this work.

† For correspondence telephone +44 (0)1382 386464, fax +44 (0)1382 388216, e-
mail t.palmer@dundee.ac.uk

Summary

Streptomyces scabies is one of a group of organisms that causes the economically important disease potato scab. Analysis of the *S. scabies* genome sequence indicates that it is likely to secrete many proteins via the twin arginine protein transport (Tat) pathway, including several proteins whose coding sequences may have been acquired through horizontal gene transfer and share a common ancestor with proteins in other plant pathogens. Inactivation of the *S. scabies* Tat pathway resulted in pleiotropic phenotypes including slower growth rate and increased permeability of the cell envelope. Comparison of the extracellular proteome of the wild type and $\Delta tatC$ strains identified 73 predicted secretory proteins that were present in reduced amounts in the *tatC* mutant strain, and 47 Tat substrates were verified using a Tat reporter assay. The $\Delta tatC$ strain was almost completely avirulent on *Arabidopsis* seedlings and was delayed in attaching to the root tip relative to the wild type strain. Genes encoding 14 candidate Tat substrates were individually inactivated, and seven of these mutants were reduced in virulence compared to the wild type strain. We conclude that the Tat pathway secretes multiple proteins that are required for full virulence.

Introduction

Potato scab is a polyphyletic disease caused by organisms in the genus *Streptomyces*, the best studied of which is *Streptomyces scabies* (Loria *et al.*, 2006). Like other streptomycetes, *S. scabies* is a mycelial bacterium that undergoes complex morphological differentiation involving the formation of aerial hyphae and spores (see Flårdh and Buttner, 2009 for a review of the complex biology of *Streptomyces*). The hyphal form of *S. scabies* infects plants through expanding plant tissue, including roots and tubers (Loria *et al.*, 2006; 2008).

One of the major pathogenicity determinants of *S. scabies* is thaxtomin, a nitrated dipeptide toxin that is a potent inhibitor of cellulose synthesis. The toxin induces plant cell hypertrophy in expanding plant tissues and likely facilitates penetration of plant tissue by the pathogen (King *et al.*, 1989; Healey *et al.*, 2000; Scheible *et al.*, 2003; Bischoff *et al.*, 2009). The mechanism by which thaxtomin inhibits cellulose synthesis is undefined but it apparently interacts with a highly conserved target, as the toxin affects all higher plants; this is congruent with the fact that thaxtomin-producing streptomycetes have an extremely wide host range. The thaxtomin biosynthesis genes are conserved in the plant pathogenic species, including *S. turgidiscabies* and *S. acidiscabies*, and are regulated by TxtR, an AraC family transcriptional regulator that binds cellobiose as a co-inducer (Kers *et al.*, 2005; Joshi *et al.*, 2007a). The *S. scabies* 87-22 genome contains a biosynthetic cluster that is highly similar in structure and organization to the coronafacic acid clusters from the Gram negative plant pathogens *Pseudomonas syringae*, and *Pectobacterium atrosepticum*, (Bignell *et al.*, 2010). CFA is the polyketide component of coronatine which acts as a jasmonate mimic during plant interactions; mutational studies demonstrate that the cluster contributes to virulence in *S. scabies* 87-22 as it does in Gram negative pathogens. Unlike the thaxtomin biosynthetic cluster, the coronafacic acid-like cluster is not conserved in *S. turgidiscabies* or *S. acidiscabies*.

Secreted proteins are also critical determinants in the interaction between bacteria and eukaryotic hosts; virulence proteins are secreted either into the host environment or directly into host cells. Indeed, a secreted proteinaceous virulence factor, Nec1, lacks homologues outside of plant pathogenic streptomycetes, is required for colonisation of the roots and may function to suppress plant defence responses (Bukhalid and Loria 1997; Joshi et al. 2007b). Furthermore, the secreted protein TomA, is conserved among pathogenic streptomycetes (Kers *et al.*, 2005) and is homologous to products of saponinase-encoding genes which are important for host-pathogen interactions in some plant pathogenic fungi (Seipke and Loria, 2008).

The role of protein secretion in pathogenesis is particularly well characterised in the case of Gram-negative bacteria which have numerous systems dedicated to achieving the secretion of proteins across the complex double-membrane cell envelope (see e.g. Büttner and He, 2009; Christie *et al.*, 2005; Galán, 2009). In contrast, Gram-positive bacteria have a simpler cell envelope, and secretion generally requires passage of proteins across only a single membrane. Therefore it might be expected that the general protein transport machineries residing in the prokaryotic cytoplasmic membrane play more direct roles in the virulence of Gram-positive organisms. In support of this it has been shown that many Gram-positive bacteria have an accessory SecA protein, termed SecA2 that appears to contribute to bacterial virulence (e.g. Rigel and Braunstein, 2008).

The Tat system is, like Sec, a general protein export pathway that is found in the cytoplasmic membranes of some (although not all) bacteria and archaea. In Gram negative bacteria and in the Gram positive Actinobacteria, that include the streptomycetes, the Tat system is comprised of three essential components, TatA, TatB and TatC (Weiner *et al.*, 1998; Sargent *et al.*, 1998; 1999; Bogsch *et al.*, 1998; Schaerlaekens *et al.*, 2004; Hicks *et al.*, 2006). By contrast, the Tat machinery in the low G+C Gram positive bacteria (exemplified by *Bacillus subtilis*) and in the archaea

do not require a TatB protein and the Tat systems in these prokaryotes are made up only of TatA and TatC components (Jongbloed *et al.*, 2004; Dilks *et al.*, 2005).

Proteins are targeted to the Tat pathway by means of N-terminal signal peptides that superficially resemble Sec signal peptides, but that harbour a conserved S/T-R-R-x-F-L-K consensus motif, where the twin arginines are invariant and normally essential for efficient export by the Tat pathway (Berks, 1996; Stanley *et al.*, 2000). The main distinguishing feature of the Tat system is that it transports fully folded proteins across the cytoplasmic membrane. In bacteria such as *Escherichia coli* and *B. subtilis*, relatively few proteins are exported by the Tat pathway and, in *E. coli* at least, the majority of them contain complex redox cofactors which are assembled into the substrate protein prior to transport across the membrane (reviewed in Palmer *et al.*, 2005; Lee *et al.*, 2006).

Although in most organisms the Tat pathway is generally held to be a relatively minor route of protein export, there is evidence that in some halophilic archaea, and in particular in *Streptomyces* bacteria, Tat exports significant numbers of proteins. Thus, bioinformatic predictions on the genome sequence of *S. coelicolor* suggested that as many as 189 proteins may be substrates of the Tat pathway (Rose *et al.*, 2002; Dilks *et al.*, 2003; Bendtsen *et al.*, 2005). The validity of these prediction programmes were largely borne out by proteomic studies of *S. coelicolor* wild type (WT) and *tat* mutant strains coupled with testing of candidate Tat signal peptides in Tat-dependent reporter assays, where a total of 33 Tat substrate proteins have now been confirmed (Li *et al.*, 2005; Widdick *et al.*, 2006).

In spite of the fact that the Tat system is a general protein export system, Tat-secreted proteins contribute to virulence in some Gram-negative and Gram-positive bacteria (e.g. Ochsner *et al.*, 2002; Ding and Christie, 2003; Pradel *et al.*, 2003; Saint-Joanis *et al.*, 2006). Given the apparent importance of the Tat pathway in protein secretion in the streptomycetes, we reasoned that this pathway may play an essential role in the virulence of *S. scabies*. Analysis of the recently available *S.*

scabies 87-22 genome sequence (www.sanger.ac.uk/Projects/S_scabies/) with Tat signal peptide prediction programmes suggests that in excess of 100 proteins may be exported by the Tat pathway in this organism, including several that appear to share a common ancestor with homologues in other plant pathogens. Through proteomic studies coupled with reporter protein secretion assays we have verified 47 Tat substrates in this organism. Importantly, we show that a Δ *tatC* mutant of *S. scabies* is almost completely avirulent, and that the Tat pathway secretes multiple proteins that are required for full virulence.

For Peer Review

Results

Bioinformatic analysis of the *S. scabies* genome sequence indicates that it encodes many candidate Tat substrates.

Two generally available prediction programmes, TATFIND 1.4 and TatP have been developed to identify candidate Tat-targeting signal peptides (Rose *et al.*, 2002; Bendtsen *et al.*, 2005). When these programmes are applied to all of the ORFs encoded by the genome sequence of *S. scabies*, TATFIND 1.4 predicts 154 likely Tat substrates, whilst TatP predicts 177 (the list of these proteins is available at http://www.lifesci.dundee.ac.uk/groups/tracy_palmer/links.html). However, both of these programmes generate a degree of false positive and false negative predictions, for example when applied to the genome sequence of *E. coli* both programmes overpredict Tat substrates by 20-25% (Dilks *et al.*, 2003; Bendtsen *et al.*, 2005). In general, the sets of candidate Tat substrates predicted by the two programmes show partial but not complete overlap. However to date, where tested, all of those predicted to be Tat substrates by both TATFIND and TatP have been shown to have *bona fide* Tat targeting signals (Widdick *et al.*, 2006). This therefore gives confidence that the subset of proteins predicted to be Tat substrates by both programmes are highly likely to represent real substrates. For *S. scabies*, 82 proteins are predicted to be Tat substrates by each of the two programmes, and these are listed in Table S1.

It was noted from previous work with *S. coelicolor* that it is sometimes difficult to identify the correct N-terminus of a predicted protein and this can lead to mis-annotation of start codons (Widdick, Chandra and Palmer, unpublished). Therefore, all of the ORFs of *S. scabies* were re-analysed to take account of all potential start codons; these modified ORFs were also analysed by TATFIND 1.4 and TatP, leading to the identification of a further 21 potential Tat substrates which are also listed in Table S1. Additionally, it is known that some Tat signals have very long n-regions that preclude their identification by TATFIND 1.4 or TatP; these programmes have

maximum preferred lengths for signal peptide n-regions. Therefore all of the *S. scabies* ORFs were truncated *in silico* by 30 or 60 amino acids and reanalysed by TATFIND 1.4 and TatP resulting in the identification of an additional five candidate Tat substrates (Table S1). Finally, all of the ORFs that were predicted to have Tat signal peptides by only one of the two Tat signal peptide prediction programmes were sorted manually for those that were likely to be true Tat substrates on the basis of binding a complex cofactor, showing high homology to confirmed Tat substrates from other organisms, or by virtue of the fact that the twin arginine motif was highly conserved across bacterial homologues. This added a further 14 substrates to the manually curated list of likely Tat substrates (Table S1). An additional four proteins were added as a result of the outcome of the Tat-dependent reporter experiments described below and this curated list of likely Tat substrates in *S. scabies* (Table S1) therefore contains a total of 126 proteins.

Phenotype of a *S. scabies* Δ tatC deletion strain

In order to test the *in silico* predictions that there are many Tat substrates encoded in the genome of *S. scabies*, it was necessary to inactivate the Tat pathway. Inspection of the genome sequence of *S. scabies* reveals that, like other streptomycetes, it encodes the *tatA* (SCAB73591) and *tatC* (SCAB73601) genes in close proximity (separated by 51bp) and a probable *tatB* gene (SCAB31121) at a distant location. Since the *tatC* gene encodes an essential component of the Tat transporter (e.g. Bogsch *et al.*, 1998), we constructed a marked deletion of *tatC* as described in Experimental Procedures.

Inactivation of the Tat pathway in *S. coelicolor* and *S. lividans* is associated with a number of phenotypic changes including a dispersed growth in liquid culture (rather than the mycelial pellets that are seen for the WT strains), failure to sporulate on solid media containing sucrose and increased fragility of the hyphae that may reflect a cell wall defect (Schaerlaekens *et al.*, 2004; Widdick *et al.*, 2006). To ascertain

whether inactivation of the Tat pathway had similar pleiotropic effects on *S. scabies*, we initially assessed the growth rate of the *S. scabies* WT and isogenic $\Delta tatC$ strains. It should be noted that *Streptomyces* growth rates cannot be measured by spectroscopic analysis since the presence of mycelial clumps in liquid culture, coupled with the production of cellular debris by programmed cell death, distorts results obtained by spectroscopy (Miguélez *et al.*, 1999). Therefore to assess growth, total cytosolic protein was extracted from living cells and measured, as described in Experimental Procedures.

As shown in Figure 1A, when cultured in TSB medium, the $\Delta tatC$ mutant strain does not grow as well as the WT strain. Whilst both strains reach a peak of growth at around 28 hours post inoculation, the mutant strain showed only about half of the total protein content per ml of culture as the WT strain. Following the growth peak, both strains showed a subsequent decline in the total protein content that may be related to programmed cell death (Miguélez *et al.*, 1999). The slower growth rate of the $\Delta tatC$ strain was also apparent on solid growth media, for example the *tatC* mutant strain formed smaller colonies on YEME agar plates (Figure 1B). It is also striking to note in Figure 1B that the $\Delta tatC$ strain lacks the brown colouration of the medium and the hyphae that is associated with the WT strain. This may reflect a lack of production of melanin, which is catalyzed by tyrosinase, a cofactor-containing Tat substrate (Schaerlaekens *et al.*, 2001). Two probable tyrosinase enzymes are encoded in the *S. scabies* genome, SCAB85681/85691 and SCAB59231/59241, and the MelC1 components of both of these have candidate twin arginine signal peptides (see Table S1). It should be noted that although the phytotoxin thaxtomin is also pigmented, thaxtomin production was not affected by inactivating *tatC* (Figure S1). Complementation of the *S. scabies* $\Delta tatC$ strain with an integrative plasmid harbouring the *S. scabies* *tatAC* genes restored production of the brown pigment,

and also reversed the slow growth rates seen on solid media, indicating that these phenotypes are directly linked to the *tatC* mutation (Figure S2).

Inactivation of the Tat pathway is often associated with a decrease in the integrity of the bacterial cell envelope, and in some organisms at least this is linked to an inability to export Tat dependent proteins involved in cell wall remodelling (Ize *et al.*, 2003; Caldelari *et al.*, 2006). In *E. coli* and *Pseudomonas syringae* this has been observed as an increased sensitivity to the presence of the detergent SDS when the Tat pathway is inactivated. As shown in Figure 1C, deletion of the *S. scabies* *tatC* gene also results in an increased sensitivity to killing by SDS, which might also be suggestive of a Tat-linked cell envelope defect in this organism. A possible underlying defect in the *S. scabies* cell wall linked to inactivation of the Tat pathway was investigated by determining the sensitivity of the WT and Δ *tatC* strains to the cell wall-directed antibiotic vancomycin. As shown in Figure 1D, the Δ *tatC* strain was clearly significantly more sensitive to killing by vancomycin than the WT strain, and a similar result was also seen with a different cell wall-directed antibiotic, bacitracin (data not shown). These observations suggest that the cell wall of the *tatC* mutant strain differs from that of the WT.

Analysis of the exported proteome of the *S. scabies* WT and Δ *tatC* strains

We next chose to examine the Tat-dependent proteome of *S. scabies* by comparison of the extracellular proteins produced by the WT and the *tatC* mutant strains under different growth conditions. As noted previously, the complex lifecycle of streptomycetes results in significant lysis of the hyphae in liquid culture, and release of many cytoplasmic proteins into the growth medium. Therefore we followed the procedure of Widdick *et al.* (2006), which involves growing *Streptomyces* on solid media on top of cellophane discs and washing the biomass with lithium chloride to release cell wall-associated proteins. The strains were cultured on four standard *S.*

scabies growth media – oat bran medium (OBM), instant potato mash medium (IPM), soy-flour mannitol medium (SFM) and R5 medium (which is a more defined medium than the other three and lacks plant-derived material), and proteins from the cell wall washes were analysed by 2-dimensional gel electrophoresis. For each growth medium analysed, the proteomes of the WT and *tatC* mutant strain were compared using the Decodon Delta 2D software with the WT proteins false coloured in green and the *tatC* proteins in red to allow differences to be visualised. The results of the proteome comparison for each type of growth media are shown in Figure S3, and close ups of some abundant red protein spots that are detected in the WT but are absent or decreased in the Δ *tatC* strain in at least two different growth media are presented in Figure 2.

It is clear from inspecting the gels shown in Figure S3 and the panels in Figure 2 that many proteins are exported at lower amounts in the *tatC* mutant strain. The red appearing protein spots were identified using tryptic digestion and MALDI-ToF mass spectrometry. In total, 103 proteins were identified - these are indicated on Figures 2 and S3, and are listed in Table 1. The ratios of the protein amounts for all red spots that are more highly exported in the WT were quantified in relation to the Δ *tatC* strain and presented in Table S2. The sequence coverages and protein scores for all identified proteins are given in Table S3.

Among the 103 proteins we identified, 73 proteins were predicted to have N-terminal signal peptides. Of these, 14 were predicted to be Tat substrates by both TATFIND and TatP (and this was increased to 19 if alternative start sites were selected), and a further nine were predicted to be Tat substrates by one of the two programmes. Interestingly, these 28 predicted Tat substrates include several enzymes that are related to phosphate starvation conditions, such as alkaline phosphatases (SCAB77971, SCAB68191) glycerophosphoryl diesterase (SCAB74351), and 5'-nucleotidases (SCAB68841, SCAB49491) which are abundantly exported proteins in

WT cells (Figure 2). Furthermore, several glycosyl hydrolases (SCAB16771, SCAB17721), cellulases (SCAB25591) and mannosidases (SCAB16551) were exported strongly in WT cells but at significantly reduced levels in the *tatC* mutant strain.

In addition to these 28 predicted Tat substrates, there were 50 signal peptide-bearing proteins that were reduced in extracellular proteome of the *tatC* mutant but were not predicted to be Tat substrates and many of these did not have consecutive arginines in their signal peptides. It should be noted that the majority of exported proteins identified as being differentially missing from the extracellular proteome of the *S. coelicolor* Δ *tatC* strain were also not Tat substrates, and it was concluded that they were simply not produced in the Δ *tatC* strain because of the pleiotropic effects of the mutation (Widdick *et al.*, 2006). It is possible that some of these differences also relate to the different growth rates of the *S. scabies* WT and Δ *tatC* strains. Finally, 30 proteins were identified in the cell wall wash of the *S. scabies* WT strain that did not appear to have N-terminal signal peptides (Table 1). It is likely that most of these proteins are cytoplasmic and their presence in the cell wall is due to contamination, with the notable exception of tyrosinase (SCAB85681) which is a known Tat substrate which lacks a signal peptide and is transported through the Tat system by forming a complex with a signal peptide-bearing partner protein (Chen *et al.*, 1992; Leu *et al.*, 1992; Schaerlaekens *et al.*, 2001).

Verification of *S. scabies* Tat targeting signal peptides using the agarase reporter assay

Using a combination of bioinformatic and proteomic approaches described above we identified a large number of candidate Tat substrates in *S. scabies*. A common method for ascertaining whether a given protein is likely to be a Tat substrate is to fuse its signal peptide to a reporter protein and determine whether it is able to

mediate export by the Tat pathway. Recently Widdick *et al.* (2006; 2008) described the agarase reporter system as a convenient and reliable reporter to test for Tat dependent export. Agarase is a Tat exported extracellular enzyme produced by *S. coelicolor* whose extracellular activity can be readily detected by the formation of halos on agar plates after staining with iodine. Agarase cannot be exported in an active form by the Sec pathway, but has been shown to be exported by the *Streptomyces* Tat pathway when fused to Tat signal peptide sequences from a wide range of organisms including Gram negative bacteria, archaea and even eukaryotes (Widdick *et al.*, 2006; 2008). Since the size of the halo is related to the amount of agarase secreted, the assay is also semi-quantitative.

We therefore selected 35 proteins that had been identified from the extracellular proteome of the WT strain, including 13 from Table 1 that were predicted to be Tat substrates by both TATFIND 1.4 and TatP, three identified by TATFIND 1.4 only, two identified by TatP only, a further 16 that had recognisable signal peptides that were not predicted to be Tat targeting by either prediction programme, and one that as presented had no apparent signal peptide but if an upstream start site was used it had a good candidate Tat signal peptide. The signal peptides from each of these proteins were fused in-frame to the mature region of agarase. The constructs are designed such that each clone carries the *dagA* ribosome binding site (rbs), with identical spacing between the rbs and the start codon (Widdick *et al.*, 2006; 2008). The recombinant proteins were expressed in both *S. lividans* WT and Δ *tatC* strains, and scored for agarase activity. In total, 14 of the 35 signal peptides were able to mediate Tat-dependent export of agarase (Figure 3A) and these proteins are listed in Table 2.

Of the proteins that had signal peptides that could export agarase, ten of them were predicted to have Tat signal peptides by both programmes, and a further two by one or other programme. The remaining two (SCAB15581 and SCAB68191) did not have Tat signal peptides on the ORFs as called, but if plausible upstream start codons

were used both had very good signal peptides that were recognised as Tat-targeting by both TATFIND 1.4 and TatP. This observation strongly suggests that the start codons of these two proteins have been mis-annotated (www.sanger.ac.uk/Projects/S_scabies/).

Interestingly, for two of the signal peptides that were able to mediate export of agarase, no agarase activity was detected if alternative versions of these signal peptides were tested. For SCAB15581, if the n-region of the signal peptide was truncated by selecting an alternative start codon closer to the twin arginine motif, no agarase activity was detected. For SCAB68191, which does not have a twin arginine motif in the ORF as called, two alternative start codons were selected that both produced peptides containing a twin arginine motif but with different lengths of n-region. Again only the peptide with the longer n-region gave any detectable agarase activity. This indicates that the choice of start codon may be critical to whether a given signal peptide is able to mediate agarase export.

Surprisingly, three of the signal peptides in Table 2 that were predicted to be Tat-targeting by both TATFIND 1.4 and TatP did not mediate detectable export of agarase. The reason for this is not clear, however given the observation that choice of start site can significantly affect the extracellular agarase activity, it is possible that incorrect start sites were selected for these signal peptides. This may be particularly relevant for SCAB77971 which is a predicted PhoD family phosphatase which are known to have very long and variable signal peptide n-regions (Widdick *et al.*, 2006; 2008). Alternatively it is possible that these are either not Tat targeting signals or that they are genuine Tat-targeting signals, but that they do not direct the Tat-dependent export of agarase for some unknown reason.

In addition to testing signal peptides from proteins shown in the proteomic study to be predominantly present in the extracellular protein fraction of the WT strain, we also tested a further 38 signal peptides that were predicted to be Tat-targeting based on the bioinformatic analysis of the *S. scabies* genome sequence described above (and

listed in Table S1). These 38 proteins are made up of 28 proteins that were predicted to have Tat signal peptides based on the fact that they were recognised by TATFIND 1.4 and TatP, six that were recognised by TATFIND 1.4 and TatP following reassignment of the start codon, two that were recognised by both prediction programmes after *in silico* truncation of the N-terminal part of the ORF, one protein that was predicted to have a Tat signal peptide by TATFIND 1.4 only, and one that was predicted by TatP only.

As shown in Figure 3B, signal peptides derived from 33 of the proteins tested were able to mediate export of agarase and we therefore conclude that these proteins are Tat substrates. The proteins that bear these signals are also listed in Table 2. Of the signal peptides tested in this section, two variants of the SCAB00601 signal peptide were tested, of which only the clone that encoded a signal peptide with the longer n-region (pSM29b, see Table S5), gave any detectable activity. Likewise, three variants of the SCAB63071 signal peptide were tested. Of these, the agarase-producing clone that encoded the longest n-region (pSM35a) failed to give detectable agarase activity whilst the remaining two both gave detectable agarase export but at different levels. This indicates that the features of the cloned DNA sequence and/or the sequence of the signal peptide tested in this assay may have a profound effect on the outcome of the reporter assay.

Five of the signal peptides identified as Tat-targeting by bioinformatic means (SCAB08301, SCAB31461, SCAB34181, SCAB70581 and SCAB78851) did not promote the export of agarase. Again the reasons for this is not clear - it may be related to the fact that inappropriate start codons were selected for these signal peptides, or that they were genuine Tat signal peptides but were unable to mediate detectable export of agarase. Alternatively it is possible that despite the fact they were predicted to be Tat signal peptides by both TATFIND and TatP they are actually Sec targeting. In this context it is interesting to note that SCAB31461 is the *S. scabiei* homologue of *S. coelicolor* BldKB which has a KR rather than an RR motif in

its signal peptide and is also unable to mediate export of agarase (Widdick *et al.*, 2006). This would be consistent with the idea that this protein may be a Sec substrate in *Streptomyces*.

In total, the agarase reporter assay has identified 47 Tat-targeting sequences, four of which were predicted to be Tat targeting by only one of the two prediction programmes, and we conclude that it is highly likely that these proteins are Tat substrates in *S. scabies*.

The Tat secretion system contributes to the virulence of *S. scabies*

The demonstrated role of Tat secreted proteins in the virulence of other microbial pathogens (reviewed in De Buck *et al.*, 2008) and the existence of proteins in the *S. scabies* genome that have homologues in pathogenic microbes, suggested a likely role for Tat secretion in virulence. We therefore investigated the virulence of the $\Delta tatC$ mutant, relative to the WT strain, on the model plant *Arabidopsis*. *S. scabies* is a broad host range pathogen that causes root rot on both model plant species and agricultural crops (reviewed in Loria *et al.*, 2006). The root tips of *Arabidopsis* (Col-O) seedlings were inoculated with *S. scabies* spores from either the WT (WT; 87-22) or the $\Delta tatC$ mutant, and seedling growth was monitored weekly.

As shown in Figure 4, infection of seedlings by the WT strain caused root stunting and necrosis; secondary roots were killed soon after emergence from the taproot. In addition, the leaves and shoots were stunted, chlorotic, and necrotic; all of the plants inoculated with the WT strain were dead within 21 to 30 days post inoculation. By contrast, plants inoculated with the $\Delta tatC$ mutant strain grew vigorously and were similar to the uninoculated control in size and colour (Figure 4). Interestingly, the $\Delta tatC$ mutant grew extensively on the roots as yellow substrate mycelium, without noticeable sporulation, but did not appear to necrotise the colonised tissue. Plants

inoculated with the $\Delta tatC$ mutant did, however, differ from the uninoculated control in root morphology, particularly root branching pattern (Figure 4).

Given the dramatic virulence phenotype of the $\Delta tatC$ mutant, we carried out a time course study of Arabidopsis root colonisation using confocal scanning microscopy and EGFP-labelled *S. scabies* WT and $\Delta tatC$ strains. To facilitate observation of microbial growth, plants were grown hydroponically. The WT strain attached to the root tip and began to colonise plant tissues within 24 hours after spores were added to the hydroponic medium (Figure 5A). Within 48 hours post inoculation (hpi) the WT strain was aggressively colonising the root at the zone of cell differentiation (Figure 5B) and at lateral meristems (Figure 5C). Intercellular colonisation was evident at the root tip and at the point of secondary root emergence by 72 hpi (Figure 5D). By contrast, colonisation of root tissue by the $\Delta tatC$ mutant was greatly delayed and limited in scope. The mutant was not able to attach to the root tip during the first 48 hpi, however, there were loosely attached colonies at the root elongation zone (Figure 5E), and minimal colonisation and restricted growth at the differentiation and elongation zones (Figure 5F-G), and at lateral meristems (Figure 5H) after 72-96 hpi. Intercellular colonisation was delayed until 96-120 hpi (Figure 5I-J) and was limited in scope.

Individual Tat secreted proteins contribute to the virulence of *S. scabies*

Since the $\Delta tatC$ strain was essentially avirulent on Arabidopsis seedlings, we sought to address the contribution of individual candidate Tat substrates to host-pathogen interactions. Fifteen individual strains were constructed that were deleted for genes encoding the putative or confirmed Tat substrate proteins listed in Table 3. The strains were then assessed for virulence using Arabidopsis seedlings as a host. To this end, Arabidopsis seedlings were grown on MS medium and inoculated with spore suspensions of the WT or with mutant strains containing deletions in genes

encoding candidate Tat substrates using methods just described. Of the fifteen individual mutant strains tested, seven were reduced in virulence relative to the WT strain (Figure 6).

Gene deletions in *SCAB03871* and *SCAB10131* were among those coding for Tat substrates that have a reduced virulence phenotype in the *Arabidopsis* seedling assay. Interestingly, these genes encode paralogues with predicted glycosyl hydrolase and ricin-like domains. In spite of the similarity in their amino acid sequences, each of these proteins appears to make a large and unique contribution to virulence, based on the phenotypes of the individual mutants (Figure 6). Furthermore, proteins encoded by *SCAB03871* and *SCAB10131* have homologues in fungal plant pathogens such as *Chaetomium globosum* (CHGG_02005) and *Phaeosphaeria nodorum* (SNOG_01152), respectively.

SCAB06471 encodes a putative alpha-L-fucosidase; the cognate deletion mutant was compromised in virulence (Figure 6). Alpha-L-fucosidases are responsible for processing of fucosylated glycoconjugates which play a role in a wide variety of biological processes. Interestingly, *SCAB06471* has homologues in the fungal plant pathogens such as *Gibberella zeae* (FG11254.1) and *Magnaporthe grisea* (MGG_03257). Furthermore, all three of these proteins are putative glycosidases and the cognate mutants have similar virulence phenotypes; in all cases plant growth was substantially greater than those inoculated with the WT strain, root necrosis was lacking, and the mutant strains grew luxuriously on *Arabidopsis* roots (Figure 6). It is tempting to speculate that these proteins have a role in penetration of the plant cell wall.

The *SCAB77391* mutant strain was slightly reduced in virulence, relative to WT (Figure 6). The encoded protein is predicted to contain a galactose-binding domain. It is possible that this domain recognises specific carbohydrate moieties on the host cell surface; in that way the protein might function as a lectin which could enhance host binding or recognition. The limited virulence phenotype, however, suggests a

function that is either redundant in the genome or has only a minor role in host-pathogen interactions.

The *SCAB81841* mutant strain has a dramatic avirulence phenotype; plants inoculated with this mutant were comparable to the non-inoculated control, except for leaf chlorosis and a delay in flowering. The predicted function for the encoded protein is an alpha-L-rhamnosidase, which hydrolyzes the terminal non-reducing alpha-L-rhamnose residues in alpha-L-rhamnosides. Given the severe defect in virulence, it seems unlikely that this enzyme's primary function is to hydrolyze plant biomass. Some rhamnosides are biologically active, including antimicrobial saponins (Morrissey *et al.* 2000). Interestingly, the *SCAB81841* mutant grew poorly on *Arabidopsis* roots (Figure 6), consistent with a role in the degradation of an antimicrobial molecule.

The *SCAB81041* deletion mutant showed a moderate virulence phenotype. The cognate protein encodes a putative spermidine/putrescine transporter peptide-binding protein (Table 3).

SCAB80581 encodes a putative FAD-containing amine oxidase. Proteins in this family additionally contain a second domain that is responsible for specifically binding a substrate and catalyzing a particular enzymatic reaction. The deletion mutant has a moderate phenotype as shown in Figure 6.

Discussion

In this study we have characterised the Tat secretome of the plant pathogen, *S. scabies*. In keeping with analyses of other streptomycete genomes, *in silico* analysis using Tat substrate prediction programmes TATFIND 1.4 and TatP predicts many candidate Tat substrates are encoded by *S. scabies*. Using the agarase reporter assay, we show here that the signal peptides of 47 candidate *S. scabies* Tat substrates were able to mediate Tat dependent export, strongly suggesting that these represent *bona fide* Tat substrates. Interestingly, two of the Tat signal peptides identified in this study were not recognised by TATFIND 1.4 because they have hydrophobic amino acids in the +1 position of the twin arginine motif. Stanley *et al.* (2000) previously noted that the amino acid residue at this position is usually polar, but the work presented here shows that valine and leucine can both be tolerated at this position. Furthermore, additional highly likely Tat-dependent proteins listed in Table S1 and S2 also have signal peptides that are not recognised by TATFIND 1.4 due to the presence of His, Ile or Phe at the +1 position. Further work is required to ascertain whether these additional 'allowed' residues at the +1 position are specific to *Streptomyces* proteins, or whether they reflect a general tolerance for the nature of the amino acid at this position of Tat signal peptides. None-the-less, taken together our analysis supports the contention that there are well in excess of one hundred Tat substrates in *S. scabies*.

Consistent with the notion that the *S. scabies* Tat pathway is a major route of protein secretion, inactivation of the Tat pathway was associated with a number of phenotypes. In particular, growth rate of the $\Delta tatC$ strain was reduced relative to WT and the mutant displayed a cell wall defect, a phenotype which is also linked to Tat pathway inactivation in other bacteria (e.g. Ize *et al.*, 2003; Caledelari *et al.*, 2006; Widdick *et al.*, 2006). The $\Delta tatC$ strain was also slow to sporulate on some types of solid media. Interestingly, the signal peptide of the *S. scabies* homologue of the

extracellular signalling protein factor C (SCAB89661), that is involved in cellular differentiation in some species of *Streptomyces* (Birkó *et al.*, 1999) was shown to mediate Tat-dependent export of agarase (Figure 3B) indicating that this is a Tat substrate. The twin arginine signal peptides of streptomycete factor C proteins are highly conserved and the mature portion of the protein shows strong sequence identity to secreted proteins encoded by mycelial fungi, raising the possibility that this DNA coding this protein was acquired by lateral gene transfer (Chater *et al.*, 2010).

Analysis of the extracellular proteomes of the WT and $\Delta tatC$ strains showed that in total 73 predicted secreted proteins were present at decreased amounts or absent in the extracellular proteome of the $\Delta tatC$ mutant. Using the agarase reporter assay, 14 predicted Tat substrates detected by proteomics as being abundantly exported in more than one growth condition in WT cells and strongly reduced in the $\Delta tatC$ mutant were verified to have Tat-targeting signal peptides. However, the majority of proteins that were apparently absent from the $\Delta tatC$ cell wall wash fraction are unlikely to be Tat substrates, again underscoring the pleiotropic nature of the mutation.

Bacteria in symbiotic associations with eukaryotes rely on secreted molecules to manipulate host cell physiology. In the broadest definition, the small molecules and proteins that alter host-cell structure and function are referred to as “effectors” (Hogenhout *et al.*, 2009). In *S. scabiei* thaxtomin and a coronatine-like molecule meet the criteria of small molecule effectors. Thaxtomin acts as a cellulose synthesis inhibitor, through an undefined mechanism, and is believed to aid in penetration of plant tissue (Scheible *et al.*, 2003, Bischoff *et al.*, 2009). A coronatine-like molecule likely serves as a molecular mimic of the plant defence signaling molecule, jasmonic acid (Bignell *et al.*, 2010). The secreted proteins Nec1 and TomA are authentic and putative effectors, respectively, in *S. scabiei* but other effector proteins had not previously been identified.

Effector proteins interact with host cells in many ways and the host cell targets of most effectors are not known (Cunnac *et al.*, 2009); however, molecular mimicry and modification of host cell molecules are two broad categories of virulence mechanisms. Activity, abundance and localisation of eukaryotic proteins, including those involved in host defence, can be regulated through attachment of ubiquitin or ubiquitin-like molecules. Many pathogens produce proteins that mimic components of the host ubiquitination machinery (reviewed in Spallek *et al.*, 2009). Other defence and development signalling cascades are targeted by effector proteins, such as Rho GTPase regulators, cytoskeletal modulators, and host innate immunity (Shames *et al.*, 2009). Under the broad definition of protein effectors suggested by Hogenhout *et al.* (2009), secreted enzymes involved in degradation of host cell molecules, particularly plant cell wall components, are also considered to be effector proteins.

Microbial pathogens can undergo rapid evolution through the acquisition of pathogenicity islands (PAIs), which are regions in the genome encoding multiple virulence-associated genes (Lovell *et al.*, 2009). In Gram negative pathogens, PAIs often have a modular configuration and include genes encoding both the type III secretion apparatus and effector proteins that use that pathway (Lindeberg *et al.*, 2009). The type III secretion apparatus provides a very effective mechanism for introduction of effector proteins directly into host cells, but is lacking in Gram positive bacteria. Gram positive pathogens, such as *S. scabies* have PAIs (Kers *et al.* 2003) but must rely on general protein secretion systems, such as Tat and Sec, which deliver effector proteins outside of the bacterial cell; genes encoding effector proteins are not typically clustered with those encoding protein secretion machineries in these pathogens.

Translocation of putative virulence factors is Tat dependent in many microbial pathogens including those that contain type III secretion systems (reviewed in De Buck *et al.*, 2008). Given the importance of the Tat system in the genus *Streptomyces*, it was not surprising to find that inactivation of the Tat secretion

machinery in *S. scabies* resulted in an essentially avirulent phenotype (Figures 4 and 5). The *tatC* mutant strain was slow to colonise and invade rapidly expanding root tissue, relative to the WT strain, which likely was the result of several deficiencies. However, the avirulence phenotype was not due a deficiency in thaxtomin production (Figure S1). Regardless, the dramatic virulence phenotype of the Δ *tatC* mutant did suggest that a subset of Tat substrates have virulence functions. Furthermore, the combination of bioinformatic and proteomic analysis identified a large number of putative Tat substrates in *S. scabies*, of which more than one third are associated with stress responses or virulence in other bacterial or fungal pathogens. This group of putative Tat secreted virulence proteins includes putative lipoproteins, ABC transporters, phospholipases/phosphoesterases, beta-lactamase, and proteins involved in Fe homeostasis (see Tables 3 and S1).

To confirm that Tat substrates are involved in infection, we inactivated genes encoding 14 candidate Tat substrates, from which strains inactivated for production of seven confirmed Tat substrates showed a reduction in virulence (Figure 6). Since the growth rate of these mutant strains was comparable to the WT (shown in Figure S5 for the *SCAB03871*, *SCAB06471*, *SCAB10131* and *SCAB77391* knockout strains), it can be concluded that the Tat pathway secretes multiple virulence factors in *S. scabies*.

Inspection of the list of these authentic virulence proteins using bioinformatics reveals some interesting ecological and phylogenetic associations. Out of the seven Tat secreted proteins affecting virulence, *SCAB77391* and *SCAB81841* are conserved in *S. turgidiscabies*, but the encoding genes are absent from the genomes of non-pathogenic streptomycetes for which genome sequence data are available at this time (data not presented). *SCAB03871*, *SCAB06471* and *SCAB10131* all contain homologues in fungal plant pathogens and are all predicted to interact with glycans (Table 3). Surprisingly the amino acid sequences of the Tat secretion signals of these three proteins are highly conserved, possibly suggesting a common origin.

SCAB03871 and its paralogue (confirmed by reciprocal BLAST analysis) SCAB10131 have glycosyl hydrolase and ricin-like domains. The closest homologues to these proteins are a few closely related saprophytic actinomycetes, where the twin arginine signal peptide is always conserved. Outside of the actinomycetes, the closest homologues of these proteins are encoded by saprophytic and pathogenic fungi. In keeping with the fact that fungi generally lack the Tat system, the fungal homologues have typical eukaryotic signal peptides, rather than the longer and less hydrophobic Tat signal peptides. This is suggestive that the gene for one of these proteins was acquired by an ancestral actinomycete through horizontal transfer from a fungus and that a Tat-targeting sequence was acquired to allow secretion of the protein in the prokaryote. It is, however, not readily apparent why these proteins should be substrates for the Tat pathway rather than the Sec system – for example none of them are predicted to bind redox cofactors that would necessitate export in a folded conformation. Substrates for the Sec machinery are transported in an unfolded form and usually interact with cytoplasmic chaperones to maintain them in an unfolded, export competent state. It is possible that proteins coded by genes acquired by horizontal transfer are not recognised by host chaperones since they are non-native, and it therefore may be advantageous to export such proteins in a folded form.

Of the seven Tat secreted proteins affecting virulence, SCAB06471 is homologous to an alpha-L-fucosidase from *Gibberella zeae*, the cause of wheat headblight and *Magnaporthe oryzae*, the rice blast pathogen (Oh *et al.*, 2008). This enzyme hydrolyzes the alpha-1,6-linked fucose joined to the reducing-end N-acetylglucosamine of carbohydrate moieties in glycoproteins. SCAB80581 encodes a protein for amine oxidase, both are homologous to proteins in the nonpathogenic *Streptomyces pristinaespiralis* and pathogenic *Mycobacterium* spp. SCAB81041, a putative ABC transporter is a periplasmic binding protein and homologous to proteins

in *Streptomyces sviveus*, and in *Thermomicrobium roseum*, a Gram-negative, obligately thermophilic bacterium.

Seven putative effector proteins chosen based on bioinformatic analysis did not have a virulence phenotype. One of these was SCAB08951, which was highly similar (Table 3) to proteins encoded by the Gram negative soil bacterium *Acidovorax delafieldii*, the Gram negative plant pathogen *Dickeya dadantii* (which both have predicted Sec signal peptides) and the plant pathogenic oomycete *Phytophthora infestans* (which has a predicted eukaryotic signal peptide). Since effector proteins commonly have a role in host specificity (Lindeberg *et al.*, 2009), evaluation of these mutants on additional host plants, particularly potato, would be necessary.

Experimental Procedures

Strains, plasmids media and culture conditions.

Streptomyces scabies strain 87-22 and the cognate Δ *tatC* strain (see below) were routinely grown on either instant potato mash medium (IPM: which contained per litre of tap water 50g Smash^R instant potato mash and 12g agar), International *Streptomyces* Project (ISP) medium 2, or ISP medium 4 (BD Biosciences, San Jose, CA) at 28°C. For proteomic analysis, strains were cultured on either IPM, soy flour mannitol medium (SFM – Hobbs *et al.*, 1989), R5 medium (Thompson *et al.*, 1980) or oat bran broth (OBB) medium (Goyer *et al.*, 1998). For growth in liquid culture strains were cultured aerobically in tryptone soya broth (TSB – Kieser *et al.*, 2000). Phenotypic growth tests were carried out on DNA medium (Kieser *et al.*, 2000) and halo diameters were determined using the image processing software GIMP (GNU Image Manipulation Program - <http://www.gimp.org/>).

To test candidate signal peptides for Tat dependence in the agarase assay, DNA encoding the signal peptides of interest were cloned in frame (as *NdeI-BamHI/BglII* fragments) with the mature agarase sequence in the integrative plasmid pTDW46H (which is identical to pTDW46 except that the apramycin resistance specified by the vector has been replaced by hygromycin resistance from pIJ10700 using the REDIRECT method (Gust *et al.*, 2003) and the oligonucleotide primers *hyg_fwd* and *hyg_rev*. The oligonucleotide primer sequences used for signal peptide amplification are listed in Table S4, and the plasmids used in this study are listed in Table S5.

Agarase assays performed and quantified as described by Widdick *et al.* (2006; 2008).

Construction and complementation of the S. scabies Δ tatC strain.

For construction of the *S. scabies* Δ *tatC* strain, SM1, an approximately 1000 bp region directly upstream of the *tatC* gene (*SCAB73601*) was amplified by PCR using

the oligonucleotides utatcscabf1 and utatcscabr (see Table S4 for a list of oligonucleotide primers used in this study) and *S. scabies* chromosomal DNA as template, digested with *EcoRI* and *HindIII* and cloned into pBluescript (KS⁺) that had been similarly digested. An approximately 1000 bp region downstream of *tatC* was subsequently amplified using the oligonucleotides dtatcscabf and dtatcscabr1, digested with *EcoRI* and *XbaI* and cloned into pBluescript already containing the *tatC* upstream region that had been similarly digested. The apramycin resistance cassette from pIJ773 (Gust *et al.*, 2003) was amplified using oligonucleotides ecoriapraf and ecoriaparar, cleaved with *EcoRI* and cloned between the upstream and downstream regions of the *tatC* deletion allele assembled in pBluescript. To allow the detection of double-crossover strains by replica plating the ampicillin/carbenicillin resistance cassette of the carrier plasmid was replaced with a kanamycin resistance cassette which unlike *bla* is suitable for selection in *Streptomyces*. This was achieved using the REDIRECT method of Gust *et al.* (2003), and the kanamycin resistance cassette was amplified by PCR using the oligonucleotides KanRtrans2_fwd and KanRtrans2_rev (Table S4). The plasmid was transferred by mating into *S. scabies* 87-22 and single crossover recombinants were selected for on MS medium containing apramycin and kanamycin (Gust *et al.*, 2003). Double cross-over recombinants were subsequently selected by several rounds of growth on non-selective media followed by selection for colonies that were apramycin resistant and kanamycin sensitive. Loss of the *tatC* gene in strain SM1 was subsequently confirmed by PCR and by Southern blot analysis.

To test for complementation of the *S. scabies* Δ *tatC* strain, a synthetic construct covering the *S. scabies* *tatAC* genes (covering from 260bp upstream of *tatA* to 60bp downstream of *tatC*) in pUC57 was purchased from GenScript, NJ, USA. The *tatAC* containing region was excised by digestion with *XbaI* and cloned into similarly digested pSET-SOR-hyg (Sean O'Rourke, unpublished) to give plasmid pTDW185, which integrates site-specifically into the *Streptomyces* chromosome.

Deletion of genes encoding putative Tat substrates

Deletion mutants for 14 putative Tat secreted virulence proteins (Tables 3 and S1) were created using the PCR-based ReDirect gene disruption protocol (Gust *et al.*, 2003). In this case, a cosmid library of *S. scabies* strain 87-22 containing a kanamycin resistance marker (*neo*) was used. Cosmid clones containing the target genes were individually introduced into *E. coli* BW25113 carrying an arabinose-inducible λ Red-expressing plasmid pKD46 (AmpR) (Datsenko and Wanner, 2000). A gene replacement cassette [*aac(3)IV* (ApraR) + *oriTRK2*] from the plasmid pIJ773 (Gust *et al.*, 2003) was PCR-amplified using gene specific redirect primers (Table S4). The resulting PCR products were transformed into the cosmid-containing *E. coli* strain and selected for apramycin resistance. The mutant cosmids was then introduced into *S. scabies* 87-22 by intergeneric conjugation from *E. coli* ET12567 (carrying the helper plasmid pUZ8002). Exconjugants were screened for apramycin resistance ($100 \mu\text{g ml}^{-1}$) and kanamycin sensitivity ($50 \mu\text{g ml}^{-1}$), indicating a double-crossover allelic exchange in *S. scabies*; deletions were confirmed by PCR analysis.

Protein methods

Growth curves for *Streptomyces* strains were monitored by assaying total protein at regular intervals. To this end, 1×10^6 spores of each strain were inoculated into 100ml of TSB medium and the cultures were incubated with shaking at 30°C over 56 hours. Every two hours, 3 x 1ml samples were withdrawn, the cells pelleted and frozen at -20°C . The frozen samples were subsequently resuspended in 1 ml 1N NaOH, 0.1% SDS and boiled for 10 minutes to lyse the cells. The samples were then clarified by centrifugation and 500 μl of the resulting supernatants were diluted with an equal volume of sterile distilled water, and 25 μl of each sample was used for protein determination using the Biorad DC Protein Assay Kit.

For the preparation of extracellular proteins, autoclaved 75 mm cellophane discs were placed onto solid media plates (SFM, OBM, IPM or R5), inoculated with 10^6 spores of the *S. scabies* WT and $\Delta tatC$ strains and incubated for 48 hours at 30°C. The biomass was scraped from the cellophane discs and extracellular proteins were released following cell wall washing exactly as described by Widdick *et al.* (2006). Separation of TCA-precipitated cell wall-washes of *S. scabies* extracellular proteins by two-dimensional gel electrophoresis (2D-PAGE) was performed using the immobilised pH gradients (IPG) in the pH range 3-10 as described (Antelmann *et al.*, 2001). For quantification of the relative protein amounts of extracellular proteins that are decreased in the $\Delta tatC$ mutant proteome compared to the WT, 200µg protein were separated by 2D-PAGE and the resulting 2D gels were stained with Coomassie-Brilliant Blue as described (Antelmann *et al.*, 2001).

Quantitative image analysis was performed from the coomassie-stained 2D gels using the DECODON Delta 2D software (<http://www.decodon.com>). The 2D gel images from WT and the $\Delta tatC$ mutant cell wall proteomes were aligned using a warp transformation. Before spot detection and quantification was performed, a fused 2D gel of both images was created using the “union fusion” algorithm of Delta2D. Spot detection was performed in the fusion gel containing all spots present in both images according to the automatically suggested parameters for background subtraction, average spot size, and spot sensitivity. The resulting spot shapes were reviewed and manually edited in the fusion gel if necessary. This reviewed spot mask served as a spot detection consensus for all gel images which was applied to both images to guide the spot detection and quantification. This enables spot quantification in all gels at the same locations resulting in 100% matching and in a reliable analysis of complete expression profiles. Normalisation was performed by calculating the quantity of each single spot in percentage related to the total spot quantity per gel.

Proteins showing an induction of at least two-fold in the WT compared to the $\Delta tatC$ mutant strain are listed in Tables 1, S2 and S3.

For standard identification of the proteins from 2D gels, spot cutting, tryptic digestion of the proteins and spotting of the resulting peptides onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems) were performed using the Ettan Spot Handling Workstation (Amersham-Biosciences, Uppsala, Sweden) as described previously (Eymann *et al.*, 2004). The MALDI-TOF-TOF measurement of spotted peptide solutions was carried out on a Proteome-Analyzer 4800 (Applied Biosystems, Foster City, CA, USA) as described previously (Eymann *et al.*, 2004). The mascot search was performed against the available *S. scabies* database (www.sanger.ac.uk/Projects/S_scabies/).

Thaxtomin A extraction and quantification

S. scabies WT 87-22 and the $\Delta tatC$ mutant were grown in 6 × 5ml of oat bran broth (OBB) medium (Johnson *et al.*, 2007) in 6-well plates for 7 days at 25 ± 2°C with moderate shaking (~120 rpm). Mycelia were pelleted by centrifugation and discarded. Thaxtomin A was extracted from culture supernatants and was analyzed by HPLC as previously described (Johnson *et al.*, 2007).

Plant virulence assays

Arabidopsis thaliana (ecotype Columbia) surface-sterilised seeds were placed on Murashige and Skoog (MS) (Murashige and Skoog, 1962) agar medium with 2% sucrose. Plants were grown at 21 ± 2°C with a 16 h photoperiod for 7 days, then inoculated with *S. scabies* 87-22 (WT) or the cognate deletion mutants ($\Delta tatC$ mutant or one of the 14 strains harbouring deletions in genes encoding Tat substrates). In all cases, spore suspensions (1×10^6 cfu) were applied to seedling root tips. Disease symptoms were noted every week and images were taken 21–28 days post

inoculation (dpi). Each experiment was repeated three times with five replicates of 20-25 plants.

Root colonisation by the $\Delta tatC$ mutant strain

S. scabies WT 87-22 and the $\Delta tatC$ mutant strains each tagged with a gene encoding enhanced green fluorescent protein (EGFP) were created and used for colonisation studies with *A. thaliana*. Vector pIJ8641 (Sun *et al.*, 1999), carrying the *egfp* gene downstream of the constitutive *ermEp** promoter and an antibiotic-resistant marker were replicated in the methylation-deficient *E. coli* strain ET12567 prior to conjugation into *S. scabies* WT 87-22 and the $\Delta tatC$ mutant strain. *A. thaliana* seedlings were grown on liquid MS medium with 2% sucrose for 7 days and inoculated at the root tip with a spore suspension (1×10^6 cfu). Laser scanning confocal microscopy was used to visualise internal and external colonisation of Arabidopsis roots at 24 hr intervals. The roots of harvested plants were mounted in water immediately after harvesting and observed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), with a 10x and 63 x water immersion objectives as described (Joshi *et al.*, 2007a). EGFP was visualised using a 4-line argon laser with an excitation wavelength of 488 nm and an emission wavelength of 500 to 550 nm. DIC (differential interference contrast) images were collected simultaneously with the fluorescence images using the transmitted light detector and processed using Leica LAS-AF software (version 1.8.2).

Acknowledgements

We thank R. Morosoli for providing us with the *S. lividans tatC* strain. We also thank Kent Loeffler for photography of Arabidopsis plants. This work was supported by the CEU project LHSG-CT-2004-005257, the BBSRC through grant BB/F009224/1 and the MRC via a Senior Non-Clinical Fellowship award to TP. SGM was supported by a PhD studentship funded by the BBSRC and JKF by a joint SCRI/University of Dundee PhD studentship. JKF acknowledges the Society for General Microbiology for funding a research trip to Cornell. Partial support was also provided by the National Research Initiative of the United States Department of Agriculture Cooperative State Research, Education, and Extension Service, grant number 2008-35319-19202. We thank PCIC, Boyce Thompson Institute (funding sources NSF DBI-0618969 and Triad Foundation) for imaging facilities.

References

Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijl, J.M., and Hecker, M. (2001) A proteomic view on genome-based signal peptide predictions. *Genome Res* **11**: 1484-1502.

Bendtsen, J.D., Nielsen, H., Widdick, D., Palmer, T., and Brunak, S. (2005) Prediction of twin-arginine signal peptides. *BMC Bioinformatics* **6**: 167.

Berks, B.C. (1996) A common export pathway for proteins binding complex redox cofactors? *Mol Microbiol* **22**: 393-404.

Bignell, D.R., Seipke, R.F., Huguët-Tapia, J.C., Chambers, A.H., Parry, R.J., and Loria, R. (2010) *Streptomyces scabies* 87-22 contains a coronafacic acid-like biosynthetic cluster that contributes to plant-microbe interactions. *Mol Plant Microbe Interact* **23**: 161-175.

Birkó, Z., Sümegi, A., Vinnai, A., van Wezel, G., Szeszák, F., Vitális, S., *et al.* (1999) Characterization of the gene for factor C, an extracellular signal protein involved in morphological differentiation of *Streptomyces griseus*. *Microbiology* **145**: 2245-2253.

Bischoff, V., Cookson, S.J., Wu, S., and Scheible, W.R. (2009) Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J Exp Bot* **60**: 955-965.

Bogsch, E., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C., and Palmer, T. (1998) An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J Biol Chem* **273**: 18003-18006.

Büttner, D., and He, S.Y. (2009) Type III protein secretion in plant pathogenic bacteria. *Plant Physiol* **150**: 1656-1664.

Bukhalid, R.A., and Loria, R. (1997) Cloning and expression of a gene from *Streptomyces scabies* encoding a putative pathogenicity factor. *J Bacteriol* **179**: 7776-7783.

Bukhalid, R.A., Chung, S.Y., and Loria, R. (1998) *nec1*, a gene conferring a necrogenic phenotype, is conserved in plant-pathogenic *Streptomyces* spp. and linked to a transposase pseudogene. *Mol Plant Microbe Interact* **11**: 960-967.

Caldelari, I., Mann, S., Crooks, C., and Palmer, T. (2006) The Tat pathway of the plant pathogen *Pseudomonas syringae* is required for optimal virulence. *Mol Plant Microbe Interact* **19**: 200-212.

Chater, K.F., Biró, S., Lee, K.J., Palmer, T. and Schrempf, H. (2010) The evolution of *Streptomyces* has involved an amazingly complex extracellular biology. *FEMS Microbiol Rev* **34**: 171-198.

Chen, L.Y., Leu, W.M., Wang, K.T., and Lee, Y.H. (1992) Copper transfer and activation of the *Streptomyces* apotyrosinase are mediated through a complex formation between apotyrosinase and its trans-activator MelC1. *J Biol Chem* **267**: 20100-20107.

Christie, P.J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S., and Cascales E. (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu Rev Microbiol* **59**: 4514-85.

Cunnac, S., Lindeberg, M., and Collmer, A. (2009) *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. *Curr Opin Microbiol* **12**: 53-60.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**: 6640–6645.

De Buck, E., Lammertyn, E., and Anne, J. (2008) The importance of the twin-arginine translocation pathway for bacterial virulence. *Trends Microbiol* **16**: 442-453.

Dilks, K., Rose, R.W., Hartmann, E., and Pohlschröder, M. (2003) Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey. *J Bacteriol* **185**: 1478-1483.

Dilks, K., Giménez, M.I., and Pohlschröder, M. (2005) Genetic and biochemical analysis of the twin-arginine translocation pathway in halophilic archaea. *J Bacteriol* **187**: 8104-8113.

Ding, Z., and Christie, P. J. (2003) *Agrobacterium tumefaciens* twin-arginine-dependent translocation is important for virulence, flagellation, and chemotaxis but not type IV secretion. *J Bacteriol* **185**: 760-771.

Eymann, C., Dreisbach, A., Albrecht, D., Bernhardt, J., Becher, D., Gentner, S., *et al.* (2004) A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics* **4**: 2849-2876.

Flårdh, K., and Buttner, M.J. (2009) *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat Rev Microbiol* **7**: 36-49.

Galán, J.E. (2009) Common themes in the design and function of bacterial effectors. *Cell Host Microbe* **5**: 571-579.

Goyer, C., Vachon, J., and Beaulieu, C. (1998) Pathogenicity of *Streptomyces scabies* Mutants Altered in Thaxtomin A Production. *Phytopathology* **88**: 44244-5.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* **100**: 1541-1546.

Healy, F.G., Wach, M., Krasnoff, S.B., Gibson, D.M., and Loria, R. (2000) The *txtAB* genes of the plant pathogen *Streptomyces acidiscabies* encode a peptide synthetase required for phytotoxin thaxtomin A production and pathogenicity. *Mol Microbiol* **38**: 794-804.

Hobbs, G., Frazer, C.M., Gardner, D.C.J., Cullum J.A., and Oliver, S.G. (1989) Dispersed growth of *Streptomyces* in liquid culture. *Appl Microbiol Biotechnol* **31**: 272-277.

Hogenhout, S.A., Van der Hoorn, R.A., Terauchi, R., and Kamoun, S. (2009) Emerging concepts in effector biology of plant-associated organisms. *Mol Plant Microbe Interact* **22**: 115-122.

Hicks, M.G, Guymer, D., Buchanan, G., Widdick, D.A., Caldelari, I., Berks, B.C., and Palmer, T. (2006) Formation of functional Tat translocases from heterologous components. *BMC Microbiol* **6**: 64.

Ize, B., Stanley, N.R., Buchanan, G., and Palmer, T. (2003) Role of the *Escherichia coli* Tat pathway in outer membrane integrity. *Mol Microbiol* **48**: 1183-1193.

Johnson, E.G.; Joshi, M.V., Gibson, D.M. and Loria, R. (2007) Cello-oligosaccharides released from host plants induce pathogenicity in scab-causing *Streptomyces* species. *Physiol Mol Plant Path* **71**: 18-25.

Jongbloed, J.D., Grieger, U., Antelmann, H., Hecker, M., Nijland, R., Bron, S., and van Dijl, J.M. (2004) Two minimal Tat translocases in *Bacillus*. *Mol Microbiol*. **54**: 1319-1325.

Joshi, M.V., Bignell, D.R., Johnson, E.G., Sparks, J.P., Gibson, D.M., and Loria, R. (2007a) The AraC/XylS regulator TxtR modulates thaxtomin biosynthesis and virulence in *Streptomyces scabies*. *Mol Microbiol* **66**: 633-642.

Joshi, M., Rong, X., Moll, S., Kers, J., Franco, C., and Loria, R. (2007b) *Streptomyces turgidiscabies* secretes a novel virulence protein, Nec1, which facilitates infection. *Mol Plant Microbe Interact* **20**: 599-608.

Kers, J.A., Cameron, K.D., Joshi, M.V., Bukhalid, R.A., Morello, J.E., Wach, M.J., et al. (2005) A large, mobile pathogenicity island confers plant pathogenicity on *Streptomyces* species. *Mol Microbiol* **55**: 1025-1033.

Kieser, T., Bibb, M.J. , Buttner, M.J. , Chater, K.F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics* (The John Innes Foundation, Norfolk, U.K.).

King, R.R., Lawrence, C.H., Clark, M.C., and Calhoun, L.A. (1989) Isolation and characterization of phytotoxins associated with *Streptomyces scabies*. *J Chem Soc Commun* **13**: 849–850.

Lee, P.A., Tullman-Ercek, D., and Georgiou, G. (2006) The bacterial twin-arginine translocation pathway. *Annu Rev Microbiol* **60**: 373-395.

Leu, W.M., Chen, L.Y., Liaw, .L.L., and Lee Y.H. (1992) Secretion of the *Streptomyces* tyrosinase is mediated through its trans-activator protein, MelC1. *J Biol Chem* **267**: 20108-20113.

Li, H., Jacques, P.E., Ghinet, M.G., Brzezinski, R., and Morosoli, R. (2005) Determining the functionality of putative Tat-dependent signal peptides in *Streptomyces coelicolor* A3(2) by using two different reporter proteins. *Microbiology* **151**: 2189-2198.

Lindeberg, M., Cunnac, S., and Collmer, A. (2009) The evolution of *Pseudomonas syringae* host specificity and type III effector repertoires. *Mol Plant Pathol* **10**: 767-775.

Loria, R., Kers, J., and Joshi M. (2006) Evolution of plant pathogenicity in *Streptomyces*. *Annu Rev Phytopathol* **44**: 469-487.

Loria, R., Bignell, D.R., Moll, S., Huguet-Tapia, J.C., Joshi, M.V., Johnson, E.G., *et al.* (2008) Thaxtomin biosynthesis: the path to plant pathogenicity in the genus *Streptomyces*. *Antonie Van Leeuwenhoek* **94**: 3-10.

Lovell, H.C., Mansfield, J.W., Godfrey, S.A., Jackson, R.W., Hancock, J.T., and Arnold, D.L. (2009) Bacterial evolution by genomic island transfer occurs via DNA transformation *in planta*. *Curr Biol* **19**: 1586-1590.

Miguélez, E.M., Hardisson, C., and Manzanal, M.B. (1999) Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. *J Cell Biol* **145**: 515-525.

Morrissey, J.P., Wubben, J.P., and Osbourn, A.E. (2000) *Stagonospora avenae* secretes multiple enzymes that hydrolyze oat leaf saponins. *Mol Plant Microbe Interact* **13**: 1041-1052.

Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473-497.

Ochsner, U.A., Snyder, A., Vasil, A.I., and Vasil, M.L. (2002) Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. *Proc Natl Acad Sci U S A* **99**: 8312-8317.

Oh, Y., Donofrio, N., Pan, H., Coughlan, S., Brown, D. E., Meng, S., Mitchell, T., and Dean, R. A. (2008) Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biol.* **9**: R85.

Palmer, T., Sargent, F., and Berks, B.C. (2005) Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends Microbiol* **13**: 175-80.

Pradel, N., Ye, C., Livrelli, V., Xu, J., Joly, B., and Wu, L. F. (2003) Contribution of the twin arginine translocation system to the virulence of enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* **71**: 4908-4916.

Rigel, N.W., and Braunstein, M. (2008) A new twist on an old pathway—accessory Sec systems. *Mol Microbiol* **69**: 291-302.

Rose, R.W., Brüser, T., Kissinger, J.C., and Pohlschröder, M. (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol Microbiol.* 2002 **45**: 943-950.

Saint-Joanis, B., Demangel, C., Jackson, M., Brodin, P., Marsollier, L., Boshoff, H., and Cole, S.T. (2006) Inactivation of Rv2525c, a substrate of the twin arginine translocation (Tat) system of *Mycobacterium tuberculosis*, increases beta-lactam susceptibility and virulence. *J Bacteriol* **188**: 6669-6679.

Sargent, F., Bogsch, E., Stanley, N.R., Wexler, M., Robinson, C., Berks, B.C., and Palmer, T. (1998) Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J* **17**: 3640-3650.

Sargent, F., Stanley, N.R., Berks, B.C., and Palmer, T. (1999) Sec-independent protein translocation in *Escherichia coli*: a distinct and pivotal role for the TatB protein. *J Biol Chem* **274**: 36073-36083.

Schaerlaekens, K., Schierová, M., Lammertyn, E., Geukens, N., Anné, J., and Van Mellaert, L. (2001) Twin-arginine translocation pathway in *Streptomyces lividans*. *J Bacteriol* **183**: 6727-6732.

Schaerlaekens, K., Van Mellaert, L., Lammertyn, E., Geukens, N., and Anné, J. (2004) The importance of the Tat-dependent protein secretion pathway in *Streptomyces* as revealed by phenotypic changes in *tat* deletion mutants and genome analysis. *Microbiology* **150**: 21-31.

Scheible, W.R., Fry, B., Kochevenko, A., Schindelasch, D., Zimmerli, L., Somerville, S., *et al.* (2003) An Arabidopsis mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. *Plant Cell* **15**: 1781-1794.

Seipke, R.F., and Loria, R. (2008) *Streptomyces scabies* 87-22 possesses a functional tomatinase. *J Bacteriol* **190**: 7684-7692.

Shames, S.R., Auweter, S.D., and Finlay, B.B. (2009) Co-evolution and exploitation of host cell signaling pathways by bacterial pathogens. *Int J Biochem Cell Biol* **41**: 380-389.

Spallek, T., Robatzek, S., and Göhre, V. (2009) How microbes utilize host ubiquitination. *Cell Microbiol* **11**: 1425-1434.

Stanley, N.R., Palmer, T. and Berks, B.C. (2000) The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. *J Biol Chem* **275**: 11591-11596.

Sun, J., Kelemen, G. H., Fernandez-Abalos, J. M., and Bibb, M. J. (1999) Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**: 2221-2227.

Thompson, C.J, Ward, J.M., and Hopwood, D.A. (1980) DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. *Nature* **286**: 525-527.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., *et al.* (1998) A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* **93**: 93-101.

Widdick, D.A., Eijlander, R. T., van Dijl, J.-M., Kuipers, O., and Palmer, T. (2008) A facile reporter system for the experimental identification of twin arginine (Tat) signal peptides from all Kingdoms of life. *J Mol Biol* **375**: 595-603.

Widdick, D.A., Dilks, K., Chandra, G., Bottrill, A., Naldrett, M., Pohlschroder, M., and Palmer, T (2006) The twin-arginine translocation pathway is a major route of protein export in *Streptomyces coelicolor*. *Proc Natl Acad Sci USA* **103**: 17927-17932.

Figure 1. Inactivation of the Tat pathway in *S. scabies* results in pleiotropic growth phenotypes. A. 100ml of TSB medium was inoculated with either *S. scabies* WT or $\Delta tatC$ strains, at a concentration of 100,000 spores per ml and incubated at 30°C with shaking. At the indicated time points, 3 x 1ml samples were removed from each culture, the hyphae pelleted and total protein content was determined using the Biorad DC Protein Assay. The error bars represent the standard error of the mean of samples taken at indicated time points, where n = 3. B. Spores of the indicated strains were streaked out from a fresh solid medium culture with an inoculation loop onto YEME medium and incubated for 7 days at 30°C. C. Spores dilutions of each strain were plated onto YEME medium and onto the same medium containing 0.01% SDS. The plates were incubated at 30°C for seven days. D. Approximately 10^7 spores of each strain were spread on 144cm² DNA medium. Antibiotic discs with of 6mm diameter were imbued with one of the following different amounts of vancomycin in μ g as indicated and plates were incubated at 30°C for 7 days.

Figure 2. Close-up of protein spots that are predominantly present in the WT extracellular proteome in all four types of growth media. The *S. scabies* WT and $\Delta tatC$ strains were grown on all four of the following growth media; IPM, OBM, R5 and SFM, and extracellular proteins were harvested from cell wall washes, TCA precipitated and subjected to 2D PAGE as described in Experimental Procedures. Depicted are enlarged sections of the dual channel images of the extracellular proteomes of *S. scabies* WT (red colour) in comparison to the *tatC* mutant strain (green colour). The red-appearing proteins are labelled by the SCAB number and include 28 proteins that are strongly exported in more than one growth medium in the WT and decreased or absent in the $\Delta tatC$ mutant. The ratios for all identified protein exported at lower amounts by the $\Delta tatC$ mutant are given in Table S2.

Figure 3. Export of agarase mediated by *S. scabiei* signal peptides. The x-axis shows a range of signal peptides from *S. scabiei* from either (A) cell wall-associated proteins that were differentially absent from the $\Delta tatC$ strain (selected from the list in Table 2) or (B) that were predicted to be Tat signal peptides by bioinformatic analysis (listed in Table S1). The y-axis gives a measure of agarase export from each fusion protein, compared with agarase bearing its native signal peptide (DagA) cloned in the same manner (set at 100%). The error bars represent the standard error of the mean, where $n = 3$. Each construct carries the native agarase promoter and ribosome binding site. (A) Signal peptides from the following proteins listed in Table 1 were also tested and found to be negative in this assay: SCAB07651, SCAB13551, SCAB16711 (two variants), SCAB16721, SCAB17571, SCAB27931 (two variants), SCAB36371, SCAB37611, SCAB43901, SCAB44691, SCAB47131 (two variants), SCAB59651, SCAB63891 (two variants), SCAB64081, SCAB69691, SCAB72441 (two variants), SCAB77971 (two variants), SCAB78431, SCAB84971, SCAB90091. (B). Two variants of the SCAB63071 signal peptide were able to mediate export of agarase to differing degrees - both are shown in the Figure. Signal peptides from the following proteins listed in Table S1 were also tested and found to be negative in this assay: SCAB08301, SCAB31461, SCAB34181, SCAB70581 and SCAB78851. The exact amino acid sequences of each of the sequences tested in these assays can be found in Table S6.

Figure 4. Reduced disease severity of Arabidopsis seedlings inoculated with the $\Delta tatC$ mutant strain. Arabidopsis seedlings (7 days old) were inoculated with spores (corresponding to 1×10^6 CFU) of the *S. scabiei* WT (87.22) and $\Delta tatC$ mutant ($\Delta tatC$) strains, keeping un-inoculated seedlings as controls. The plants were grown for a further 21-28 days, and the images are taken at 28 days post inoculation.

Figure 5. Micrographs of Arabidopsis roots colonised by EGFP-labelled *S. scabies* strains. Colonisation and disease progression was monitored on hydroponically grown Arabidopsis roots after inoculation with EGFP labelled *S. scabies* strains. (A-D) Inoculation with the WT strain 87-22, and (E-J) inoculation with the Δ *tatC* mutant strain. Colonies of the WT strain are shown (A) 24 hours post inoculation (hpi) showing cells at and near the root tip (red arrow); (B and C) 48 hpi showing colonisation of (B) the root at the zone of cell differentiation and (C) at a lateral meristem; and (D) 72 hpi at a lateral root meristem - intercellular growth by is indicated by the red arrows. Colonies of the *tatC* mutant strain are shown (E) at the root tip zone during the first 24-48 hpi; the arrow indicates loosely attached mycelium at the elongation zone; (F-H) after 72-96 hpi; (F) at the differentiation zone, (G) at elongation zones and (H) at a lateral meristem; and (I and J) at 96-120 hpi. (K and L) Un-inoculated control seedlings showing (K) normal root growth, and (L) loose cells surrounding the root cap region. Images were taken using a Leica TCS SP5 confocal microscope. Size bars represent 50 μ m.

Figure 6. Tat substrates are essential for the virulence of *S. Scabies*.

Deletion mutants of putative Tat secreted virulence proteins are constructed using the ReDirect gene disruption protocol and compared to WT 87-22 for disease severity. Mutants SCAB77391, SCAB80581, SCAB81041, SCAB81841, SCAB03871, SCAB06471, and SCAB10131 show suppression of disease severity in terms of root necrosis and aerial growth.

| Protein | Growth medium detected | Putative function | Agarase test [†] |
|---------------------------|------------------------|---|---------------------------|
| Pass TATFIND 1.4 and TatP | | | |
| SCAB08951 | R5 | ABC-type Fe ³⁺ transport system, periplasmic component | Pass |
| SCAB15571 | IPM, OBM, R5 | putative secreted phosphoesterase | Pass |
| SCAB16551 | OBM, SFM | putative mannosidase | ND |
| SCAB16651 | IPM | putative secreted protein | Pass |
| SCAB25591 | IPM, OBM, R5, SFM | putative secreted cellulase | Pass |
| SCAB38731 | OBM | putative secreted beta-lactamase | Pass |
| SCAB47131 | IPM, OBM | peptidase family M23/M37 protein | Fail ^a |
| SCAB59671 | R5 | putative hydrolase | Pass |
| SCAB63891 | IPM, SFM | putative secreted transport-associated protein | Fail ^a |
| SCAB68841 | IPM, OBM, R5, SFM | putative 5' nucleotidase | Pass |
| SCAB74351 | IPM, OBM, R5, SFM | glycerophosphoryl diester phosphodiesterase | Pass |
| SCAB75721 | IPM, OBM, R5, SFM | putative secreted protein | Pass |
| SCAB77971 | IPM, OBM, R5, SFM | putative secreted phosphatase (fragment) | Fail ^a |
| SCAB81841 | OBM | putative secreted hydrolase | Pass |
| Pass TATFIND 1.4 only | | | |
| SCAB18501 | IPM, OBM, R5, SFM | alpha-N-acetylglucosaminidase | Pass |
| SCAB27931 | SFM | putative neutral zinc metalloprotease | Fail ^a |
| SCAB37611 | IPM, OBM, R5, SFM | putative secreted aminopeptidase | Fail |
| SCAB57891* | OBM, R5 | conserved hypothetical protein | ND |
| SCAB82451 | SFM | putative secreted protein | ND |

| | | | |
|---|-------------------|--|-------------------|
| Pass TatP only | | | |
| SCAB08221 | IPM, OBM, R5, SFM | Conserved hypothetical protein | Pass |
| SCAB64081 | IPM, SFM | putative secreted protein | Fail |
| SCAB78761 | OBM | putative secreted protein | ND |
| SCAB84861 | R5 | putative secreted amidase | ND |
| Pass Signal P but not TATFIND 1.4 or TatP | | | |
| SCAB01191 | IPM | putative secreted protein | ND |
| SCAB04761 | R5 | putative secreted protein | ND |
| SCAB05351 | R5 | substrate-binding component of ABC transporter | Fail |
| SCAB07651 | IPM | neutral zinc metalloprotease | Fail |
| SCAB08871 | IPM, OBM | secreted endoglucanase | ND |
| SCAB13321 | R5 | S15 non-peptidase homologue family | ND |
| SCAB13551 | SFM | conserved hypothetical protein | Fail |
| SCAB14911 | SFM | putative secreted protein | ND |
| SCAB16431 | OBM | putative possible cellulase CELA1 | ND |
| SCAB16711 | IPM, OBM, R5, SFM | putative secreted glycosyl hydrolase | Fail ^a |
| SCAB16721 | IPM, OBM, R5, SFM | putative secreted glycosyl hydrolase | Fail |
| SCAB17001 | IPM, SFM | secreted cellulase | ND |
| SCAB17571 | IPM | putative secreted peptidase | Fail |
| SCAB18081 | IPM, SFM | gamma-glutamyltranspeptidase | ND |
| SCAB18661 | SFM | putative serine protease | ND |
| SCAB19481 | IPM | RTX-family exoprotein | ND |
| SCAB19491 | SFM | putative probable exported protein | ND |
| SCAB19941 | SFM | cyclase | ND |

| | | | |
|------------|-------------------|---|-------------------|
| SCAB24731 | OBM | putative secreted aminopeptidase | ND |
| SCAB24891 | SFM | glutamate binding protein | ND |
| SCAB26841 | SFM | putative serine protease | ND |
| SCAB27771 | OBM | putative secreted protein | ND |
| SCAB31531 | IPM, R5 | putative BldKB-like transport system extracellular solute-binding protein | ND |
| SCAB33981 | SFM | hypothetical protein 2SCK36.08 | |
| SCAB36371 | IPM | putative xylanase/cellulase | Fail |
| SCAB41181 | SFM | conserved hypothetical protein | ND |
| SCAB43901 | IPM | putative secreted hydrolase | Fail |
| SCAB44001 | SFM | putative secreted protein | ND |
| SCAB44161 | R5, SFM | hydrolase | ND |
| SCAB44541 | IPM, OBM | putative secreted protein | ND |
| SCAB44691 | IPM, OBM, SFM | putative secreted protein | Fail |
| SCAB45141 | SFM | D-alanyl-D-alanine carboxypeptidase | ND |
| SCAB47841 | R5, SFM | lipoprotein | ND |
| SCAB49491 | IPM, OBM, R5, SFM | putative secreted 5'-nucleotidase | ND |
| SCAB56441 | IPM | putative secreted protease | ND |
| SCAB58251 | SFM | putative secreted tripeptidyl aminopeptidase | ND |
| SCAB59651 | SFM | putative secreted serine protease | Fail |
| SCAB66031 | SFM | putative glycosyl hydrolase | ND |
| SCAB68191* | IPM, OBM, R5, SFM | putative secreted alkaline phosphatase | Pass ^a |
| SCAB68931 | SFM | branched chain amino acid binding protein | ND |
| SCAB69691 | IPM | zinc-binding carboxypeptidase | Fail |
| SCAB72441 | IPM, SFM | putative membrane protein | Fail ^a |

| | | | |
|-------------------|-------------------|-------------------------------------|-------------------|
| SCAB72781 | IPM | penicillin acylase | ND |
| SCAB74081 | IPM, OBM, R5 | putative secreted protein | ND |
| SCAB78431 | SFM | secreted tripeptidylaminopeptidase | Fail |
| SCAB80071 | SFM | putative secreted protein | ND |
| SCAB84971* | IPM | conserved hypothetical protein | Fail |
| SCAB85291 | IPM | conserved hypothetical protein | ND |
| SCAB90091 | IPM, OBM, SFM | secreted cellulase | fail |
| SCAB90101 | SFM | secreted cellulase | ND |
| No signal peptide | | | |
| SCAB15581* | IPM, OBM, R5, SFM | conserved hypothetical protein | Pass ^a |
| SCAB17551 | IPM | conserved hypothetical protein | ND |
| SCAB20121 | R5, SFM | putative germacradienol synthase | ND |
| SCAB21191 | IPM, SFM | hypothetical protein | ND |
| SCAB25251 | OBM | guanosine pentaphosphate synthetase | ND |
| SCAB26011 | OBM | elongation factor Ts | ND |
| SCAB31831 | R5 | cytochrome P-450 hydroxylase | ND |
| SCAB36931 | R5 | 50S ribosomal protein L4 | ND |
| SCAB37201 | OBM | 50S ribosomal protein L7/L12 | ND |
| SCAB39491 | OBM, R5, SFM | Clp-family ATP-binding protease | ND |
| SCAB39811 | R5 | conserved hypothetical protein | ND |
| SCAB41891 | R5 | adenylosuccinate synthetase | ND |
| SCAB44821* | IPM, OBM, R5, SFM | conserved hypothetical protein | ND |
| SCAB45751 | R5 | DNA gyrase subunit A | ND |
| SCAB50441 | R5 | chaperonin 2 | ND |
| SCAB51341 | OBM, R5 | citrate synthase | ND |
| SCAB51541 | SFM | calcium binding protein | ND |
| SCAB54151 | OBM | ribosomal L25p family protein | ND |
| SCAB55881 | IPM | transcriptional regulator | ND |

| | | | |
|-------------|-----|-------------------------------------|----|
| SCAB57721 | OBM | cellobiose hydrolase | ND |
| SCAB58791 | R5 | citrate synthase | ND |
| SCAB59311 | R5 | ABC transporter ATP-binding protein | ND |
| SCAB59871 | SFM | GntR family DNA-binding regulator | ND |
| SCAB62141 | SFM | pyruvate phosphate dikinase | ND |
| SCAB64141 | SFM | acyl carrier protein | ND |
| SCAB64311 | SFM | conserved hypothetical protein | ND |
| SCAB67061 | SFM | dihydrolipoamide dehydrogenase | ND |
| SCAB69391 | R5 | conserved hypothetical protein | ND |
| SCAB71391 | OBM | conserved hypothetical protein | ND |
| SCAB85681** | R5 | putative tyrosinase MelC2 | ND |

Table 1. Proteins that are predominantly found in the extracellular fraction of the *S. scabies* wild type strain following 2D gel analysis.

[†]Signal peptide sequences (listed in Table S5) of the indicated proteins were fused to the mature region of agarase and their ability to export agarase in a Tat-dependent manner was determined as described in the text. ND – not determined.

*The N-termini of these proteins are recognised as Tat signal peptides by both TATFIND 1.4 and TatP if alternative start sites are selected.

**This protein lacks an N-terminal signal peptide but is a Tat substrate by virtue of interacting with a Tat signal peptide-bearing partner protein, MelC1 (Leu *et al.*, 1992; Schaerlaekens *et al.*, 2001)

[‡]Two variants of each of these signal peptides were tested (see Table S5)

| Signal peptide tested | Putative function |
|--------------------------------------|---|
| Protein detected by proteomics | |
| SCAB08221 | Putative phosphoesterase |
| SCAB08951 | ABC-type Fe ³⁺ transport system, periplasmic component |
| SCAB15571 | putative secreted phosphoesterase |
| SCAB15581 | Possible histidinol phosphatase (PHP family) |
| SCAB16651 | Putative L-xylulose-5-phosphate 3-epimerase |
| SCAB18501 | alpha-N-acetyl glucosaminidase |
| SCAB25591 | endo-1,4-beta-glucanase |
| SCAB38731 | beta-lactamase |
| SCAB59671 | cyclic 3',5'-adenosine monophosphate phosphodiesterase |
| SCAB68191 | phospholipase D precursor |
| SCAB68841 | 2',3'-cyclic-nucleotide 2'-phosphodiesterase |
| SCAB74351 | glycerophosphoryl diester phosphodiesterase |
| SCAB75721 | A sub-family of peptidase family C39 |
| SCAB81841 | alpha-L-rhamnosidase |
| Proteins predicted by bioinformatics | |
| SCAB00601 | Hypothetical protein |
| SCAB03871 | glycosyl hydrolase domain followed by ricin domain |
| SCAB04961 | endo-xylanase |
| SCAB06471 | putative alpha-L-fucosidase |
| SCAB09381 | hypothetical protein |
| SCAB09591 | Alpha-L-fucosidase |
| SCAB10131 | glycosyl hydrolase domain followed by ricin domain |
| SCAB13731 | phospholipase C |
| SCAB15711 | putative endo-1,3-beta-glucanase |
| SCAB16951 | hypothetical protein |

| | |
|-----------|--|
| SCAB19551 | rhamnogalacturonase B precursor |
| SCAB25621 | lipoprotein |
| SCAB37061 | putative secreted protein |
| SCAB46711 | protocatechuate dioxygenase |
| SCAB48941 | ATP-dependent nuclease subunit B-like |
| SCAB57331 | No homologues in database |
| SCAB57661 | putative multiple sugar ABC transporter solute-binding protein |
| SCAB63071 | putative gluconolactonase |
| SCAB66251 | iron-dependent peroxidase |
| SCAB73161 | Gluconolactonase |
| SCAB74641 | lipoprotein |
| SCAB74681 | hypothetical protein |
| SCAB77391 | Hypothetical Protein |
| SCAB77401 | glycosyl hydrolase |
| SCAB77511 | Beta-galactosidase |
| SCAB78741 | putative secreted protein |
| SCAB79011 | lipase/acylhydrolase, putative |
| SCAB79571 | large secreted protein |
| SCAB80581 | amine oxidase |
| SCAB81041 | putative ABC transporter periplasmic binding protein |
| SCAB81901 | peptide ABC transporter peptide-binding protein |
| SCAB88501 | rhamnogalacturonase B precursor |
| SCAB89661 | putative factor C protein precursor |

Table 2. Proteins from *S. scabies* with signal peptides that are able to mediate the Tat-dependent export of agarase. The amino sequences of each signal peptide tested are given in Table S5.

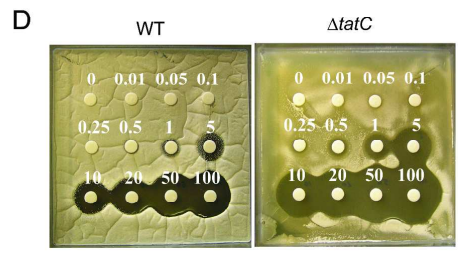
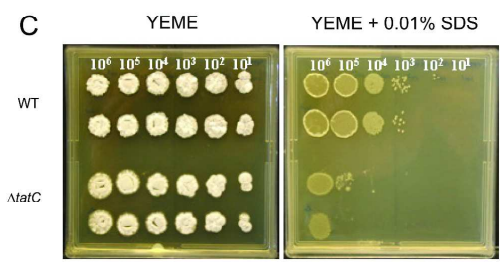
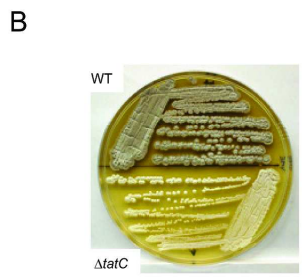
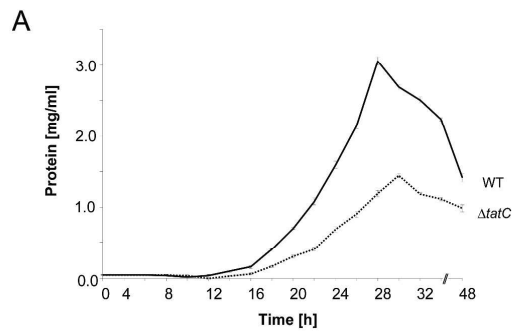
| Gene ID | Putative function | Homologs with highest similarity (Identity /Similarity) | locus_tag | Agarase Test ¹ | Virulence phenotype ² |
|-----------|--|---|--|---------------------------|----------------------------------|
| SCAB03871 | glycosyl hydrolase domain followed by ricin domain | <i>Streptomyces sviceus</i> (89/93) <i>Micromonospora aurantiaca</i> (76/85) <i>Actinosynnema mirum</i> (71/81) | SSEG_02060 MicauDRAFT_3913 Amir_3107 | Yes | Yes |
| SCAB06471 | putative alpha-L-fucosidase | <i>Streptomyces sviceus</i> (82/90) <i>Streptomyces viridochromogenes</i> (83/89) <i>Streptomyces hygroscopicus</i> (57/70) | SSEG_02413 SvirD4_010100003282 ShygA5_010100010179 | Yes | Yes |
| SCAB08951 | ABC-type Fe ³⁺ transport system, periplasmic component | <i>Acidovorax delafieldii</i> (60/74) <i>Phytophthora infestans</i> (51/67) <i>Pectobacterium carotovorum</i> (48/63) | AcdeIDRAFT_2816 PITG_03258 PC1_1331 | Yes | No |
| SCAB10131 | glycosyl hydrolase domain followed by ricin domain | <i>Streptomyces sviceus</i> (84/92) <i>Micromonospora</i> sp. L5 (75/83) <i>Actinosynnema mirum</i> (70/80) | SSEG_02060 ML5DRAFT_2741 Amir_3107 | Yes | Yes |
| SCAB18791 | Hypothetical secreted protein | <i>Bacteroides cellulosilyticus</i> (39/57) <i>Pectobacterium carotovorum</i> (34/50) <i>Ralstonia solanacearum</i> (34/49) | BACCELL_04049 PC1_0414 RSIPO_04319 | ND | No |
| SCAB58511 | Hypothetical protein. | <i>Pseudomonas putida</i> (27/45) <i>Nitrobacter winogradskyi</i> (27/43) <i>Clostridium botulinum</i> A3 (25/42) | PP_2006 Nwi_0886 CLK_A0070 | ND | No |
| SCAB58531 | Hypothetical protein | <i>Nitrobacter winogradskyi</i> (29/45) <i>Clostridium botulinum</i> Ba4 (26/43) <i>Pseudomonas putida</i> (29/45) | Nwi_0886 CLJ_0010 PP_2006 | ND | No |
| SCAB70581 | Hydrolase of the alpha/beta superfamily | <i>Streptomyces sviceus</i> (76/82) <i>Streptomyces griseoflavus</i> (76/83) <i>Streptomyces ghanaensis</i> (76/83) | SSEG_08785 SgriT_010100028100 SghaA1_010100027639 | ND | No |
| SCAB76661 | Rare lipoprotein A | <i>Streptomyces sviceus</i> (54/65) <i>Micromonospora</i> sp. (66/79) <i>Streptomyces viridochromogenes</i> (72/82) | SSEG_04607 MCAG_02629 SvirD4_010100009886 | ND | No |
| SCAB77391 | Conserved hypothetical protein containing a galactose-binding domain | <i>Bacteroides cellulosilyticus</i> (36/53) <i>Pectobacterium carotovorum</i> (30/47) <i>Ralstonia solanacearum</i> (29/44) | BACCELL_04049 PC1_0414 RRSL_03557 | Yes | Yes |
| SCAB77401 | Putative glycosyl hydrolase | <i>Bacteroides ovatus</i> (45/63) <i>Dickeya dadantii</i> (36/52) <i>Ralstonia solanacearum</i> (36/52) | BACOVA_01686 Dd586_1768 RSMK02989 | Yes | No |
| SCAB80581 | Putative FAD-containing amine oxidase | <i>Streptomyces pristinaespiralis</i> (78/87) <i>Streptomyces</i> sp. (76/85) <i>Nocardia farcinica</i> (54/65) | SSDG_02025 StreC_010100030349 nfa3040 | Yes | Yes |

| | | | | | |
|-----------|--|--|--|-----|-----|
| SCAB81041 | putative spermidine/putrescine transporter peptide-binding protein | <i>Streptomyces sviveus</i> (58/72) <i>Streptomyces hygrosopicus</i> (56/72) <i>Sphaerobacter thermophilus</i> (36/53) | SSEG_09980 ShygA5_010100044540 Sthe_3297 | Yes | Yes |
| SCAB81841 | alpha-L-rhamnosidase | <i>Clostridium leptum</i> (35/54) <i>Victivallis vadensis</i> (37/51) <i>Lactobacillus rhamnosus</i> (33/48) | CLOLEP_02977 Vvad_PD1953 LRH_00532 | Yes | Yes |

Table 3. Potential Tat-dependent virulence factors encoded in the genome of *S. scabies*.

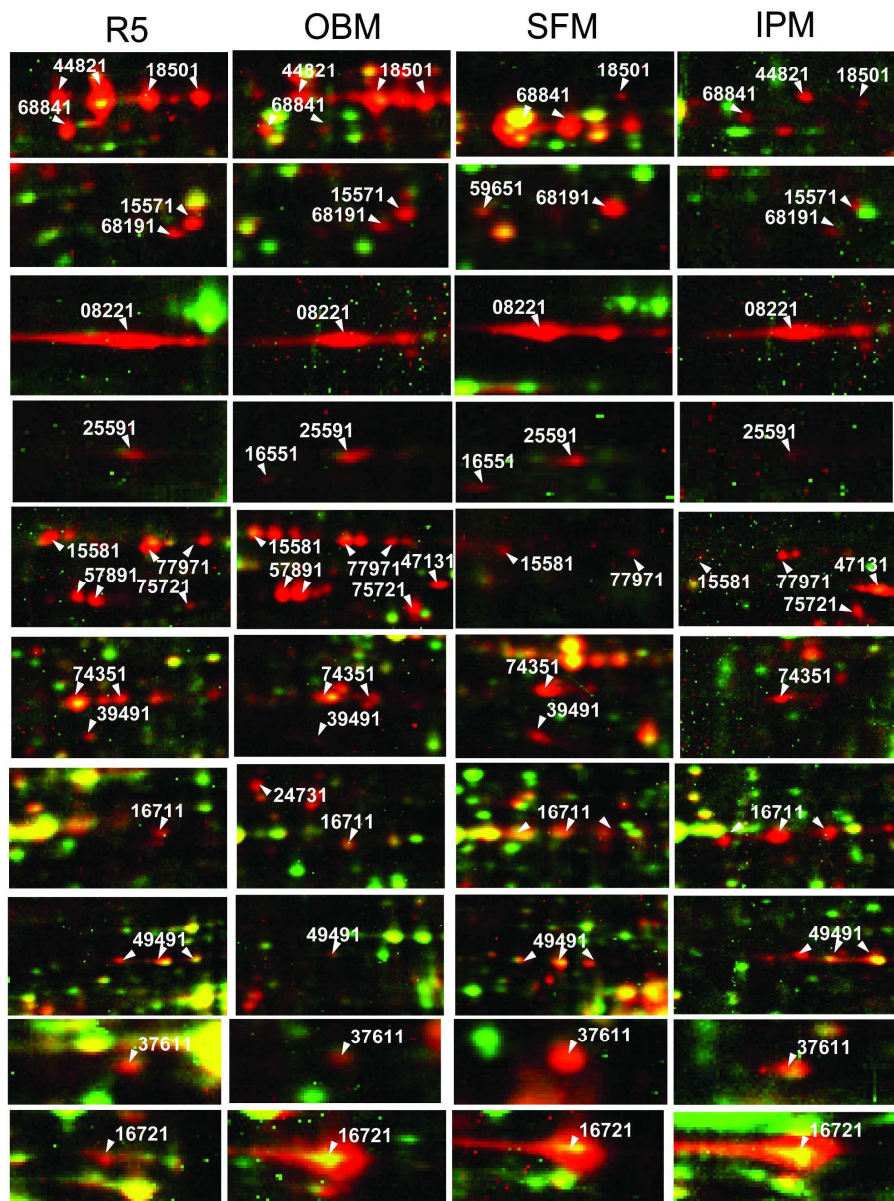
¹Signal peptide sequences (listed in Table S5) of the indicated proteins were fused to the mature region of agarase and their ability to export agarase in a Tat-dependent manner was determined as described in the text. ND – not determined.

²Virulence phenotype of knock-out strains tested using the *Arabidopsis thaliana* infection model.

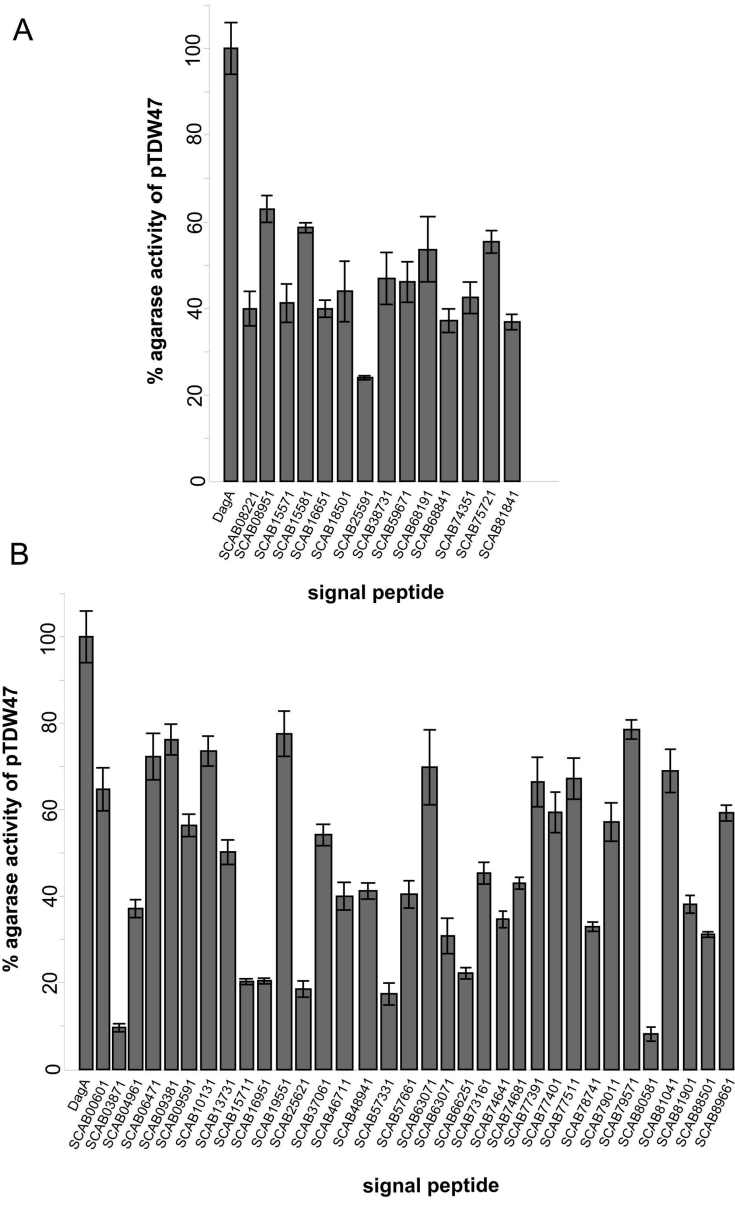


168x114mm (600 x 600 DPI)

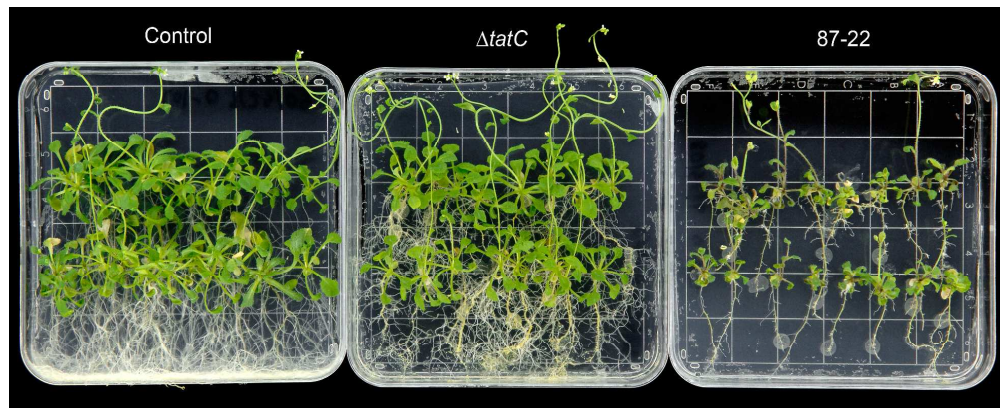
Review



168x223mm (600 x 600 DPI)

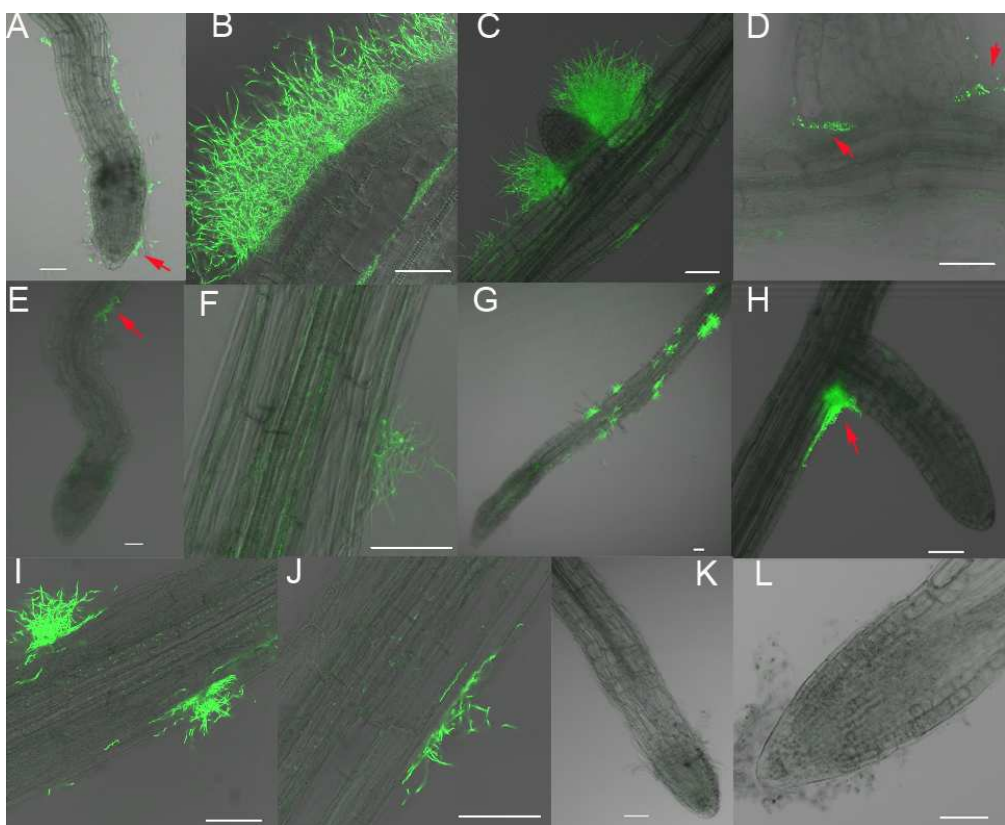


80x127mm (600 x 600 DPI)



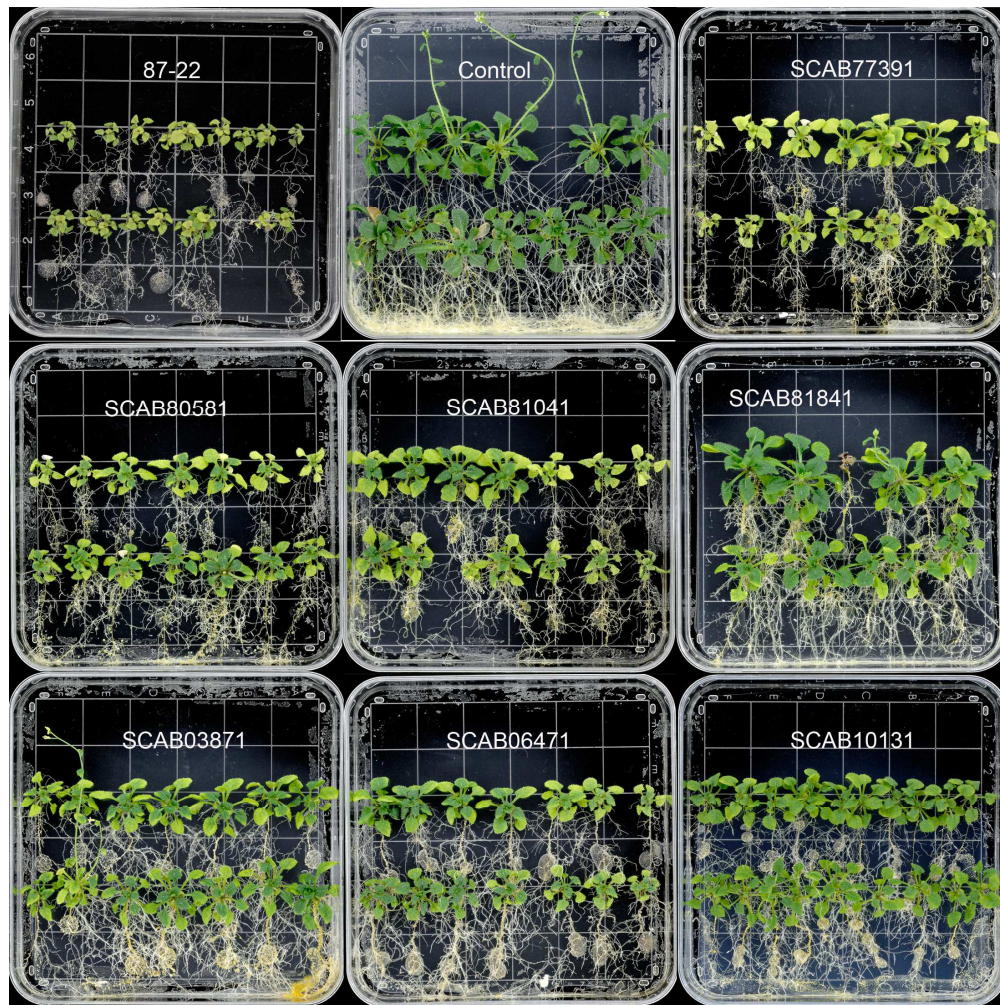
167x67mm (600 x 600 DPI)

Peer Review

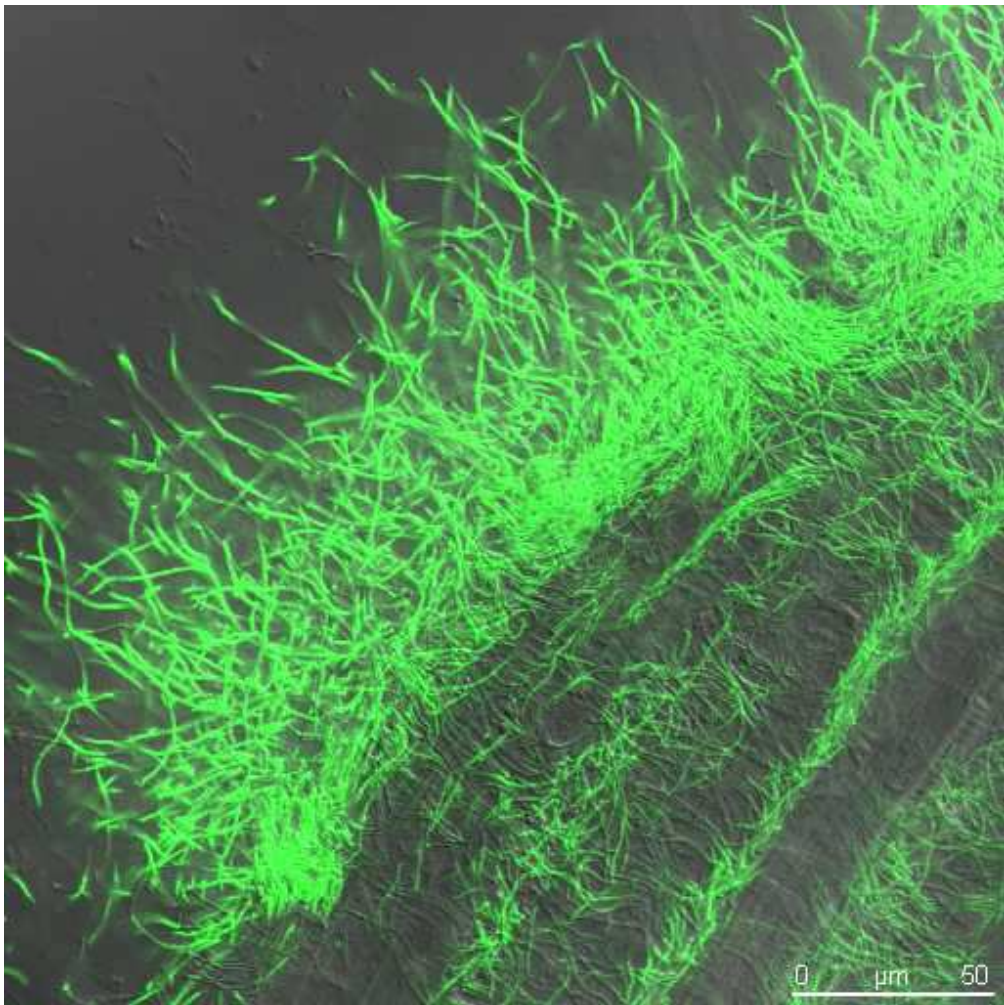


40x33mm (600 x 600 DPI)

view



93x93mm (600 x 600 DPI)



135x135mm (96 x 96 DPI)

Confocal fluorescence micrograph showing EGFP-labelled hyphae of *Streptomyces scabies* 87-22 colonising an *Arabidopsis thaliana* Col-0 root.

For Peer Review