Identification of genetic and environmental factors stimulating excision from *Streptomyces scabiei* chromosome of the toxicogenic region responsible for pathogenicity

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SUMMARY

The genes conferring pathogenicity in *Streptomyces turgidiscabies*, a pathogen causing common scab of potato, are grouped together on a pathogenicity island (PAI), which has been found to be mobile and appears to transfer and disseminate like an integrative and conjugative element (ICE). However, in *Streptomyces scabiei*, another common scab-inducing species, the pathogenicity genes are clustered in two regions: the toxicogenic region (TR) and the colonization region. The *S. scabiei* 87.22 genome was analysed to investigate the potential mobility of the TR. Attachment sites (*att*), short homologous sequences that delineate ICEs, were identified at both extremities of the TR. An internal *att* site was also found, suggesting that the TR has a composite structure (TR1 and TR2). Thaxtomin biosynthetic genes, essential for pathogenicity, were found in TR1, whereas candidate genes with known functions in recombination, replication and conjugal transfer were found in TR2. Excision of the TR1 or TR2 subregions alone, or of the entire TR region, was observed, although the excision frequency of TR was low. However, the excision frequency was considerably increased in the presence of either mitomycin C or *Streptomyces coelicolor* cells. A composite TR structure was not observed in all *S. scabiei* and *Streptomyces acidiscabies* strains tested. Of the ten strains analysed, seven lacked TR2 and no TR excision event could be detected in these strains, thus suggesting the implication of TR2 in the mobilization of *S. scabiei* TR.

Keywords: common scab, ICE, lantibiotic, pathogenicity island, potato, SOS response, *Streptomyces scabiei*.

INTRODUCTION

Bacteria of the genus *Streptomyces* are filamentous Gram-positive bacteria widely distributed in nature, but mainly found in soil. *Streptomyces scabiei* is one of the few *Streptomyces* species known to cause plant disease. It is responsible for common scab,

a disease affecting potato tuber quality by creating corky lesions, which causes significant annual losses to potato growers (Hill and Lazarovits, 2005). *Streptomyces scabiei* was the first species reported to cause this disease in 1890 (Thaxter, 1892). However, other *Streptomyces* species causing common scab emerged in the late 1990s, notably *S. turgidiscabies* in Japan and *S. acidiscabies* in the north-east USA (Bignell *et al.*, 2010).

Horizontal gene transfer is a major driving force in bacterial evolution. It allows for the rapid acquisition of novel features, such as pathogenicity, resistance to antibiotics or adaptation to niche environments (Juhas *et al.*, 2009). Conjugation is a widespread mechanism of horizontal gene transfer in which DNA translocates across the membranes of two cells, forming a mating pair. Streptomycetes, characterized by their complex morphological development, undergo conjugation through the intertwining of growing bacterial tip hyphae, which leads to the intermycelial transfer of DNA (Possoy *et al.*, 2001). It has been suggested that the emergence of new species causing common scab has resulted from horizontal gene transfer of pathogenicity elements from pathogenic to non-pathogenic *Streptomyces*. The pathogenic determinants of *S. turgidiscabies* are located on a pathogenicity island (PAI) that has been shown to be mobilizable to the non-pathogenic species *S. coelicolor* and *S. diastatochromogenes* under *in vitro* conditions (Kers *et al.*, 2005).

It could therefore be hypothesized that the pathogenic determinants of potato scab-inducing *Streptomyces* disseminate like integrative and conjugative elements (ICEs). These elements integrate and replicate in the host chromosome, and are therefore vertically inherited. ICEs site-specifically excise from the host chromosome to form extrachromosomal circular elements, which can then be transferred by conjugation to a recipient cell, where they integrate into the chromosome by a tyrosine or serine recombinase encoded by an *int* gene (Bordeleau *et al.*, 2012). Short homologous sequences, called attachment sites (*att*), on the circular elements (*attP*) and on the chromosome (*attB*) are used for site-specific recombination. The transfer of the circular molecule of DNA is initiated by a relaxase that introduces a single-strand nick on the DNA at the origin of transfer (*oriT*). The relaxosome complex is then recruited by a coupling protein to the mating site and transfers the DNA to the recipient cell. In the recipient cell, a relaxase catalyses the circulari-

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zation of the transferred ICE DNA and second strand synthesis. The double-strand circular DNA can then be inserted into the recipient cell chromosome by an integrase (Wozniak and Waldor, 2010). A new class of integrase gene, including the *intSt* gene, has recently been found to be responsible for PAI mobility in *S. turgidiscabies* (Huguet-Tapia *et al.*, 2014). However, this gene has no homologue in *S. scabiei* or *S. acidiscabies*.

The 674-kb PAI of *S. turgidiscabies* carries several genes, including pathogenic determinants, such as the thaxtomin biosynthetic genes responsible for the production of toxins essential to pathogenicity, and the *nec1* and *tomA* genes that potentially increase colonization by reducing the plant's defence response (Bignell *et al.*, 2010). The genomic organization of pathogenic determinants differs between *S. turgidiscabies* strain Car8 and *S. scabiei* strain 87.22. Genes conferring pathogenicity in *S. scabiei* strain 87.22 are not adjacent, but instead are located in two distinct regions of the chromosome. The thaxtomin biosynthetic gene cluster is found in the toxicogenic region (TR), whereas the virulence genes *nec1* and *tomA* are located 4.9 Mb away in the colonization region (CR) (Lerat *et al.*, 2009b).

The aim of this study was to elucidate the genetic organization of the TR and its ability to excise in various *S. scabiei* and *S. acidiscabies* strains. The TR of *S. scabies* strains 87.22, ATCC 49173 and Lip-17 was found to be able to excise from the chromosome in three distinct forms: as TR1, TR2 or the entire TR. In contrast, the thaxtomin biosynthetic genes of other pathogenic *Streptomyces* strains, lacking the TR2 element, were unable to excise from the chromosome. TR excision in *S. scabiei* 87.22 was found to be promoted by environmental conditions, including exposure to a DNA-damaging agent, known to induce the SOS response, or the presence of recipient cells.

RESULTS

Genomic analysis of *S. scabiei* PAI

Lerat *et al.* (2009b) reported that the pathogenicity determinants of *S. scabiei* 87.22 are associated with two regions of the chromosome, designated TR and CR. A bioinformatic analysis was carried

out to further characterize the TR. In a first step to determine whether the *S. scabies* TR can excise from the chromosome, an analysis with YASS software (Noé and Kucherov, 2005) was performed to find candidate attachment sites (*att*) by searching for short repeated sequences within which site-specific recombination, mediated by a tyrosine recombinase (integrase), takes place. In three sites, nearly identical repeats were found, which probably are *attL* (TAGACGTTGAAGCGGAAC), *attPi* (the internal *att* site, TACACATTGAAGCGGAAC) and *attR* (TAGACATTGAAGCGGAAC). According to their positions on the chromosome, the *attL* and *attR* sequences were identified as TR borders (Fig. 1). The regions between *attL* and *attPi*, and between *attPi* and *attR*, are hereafter defined as TR1 (toxicogenic region 1) and TR2 (toxicogenic region 2), respectively (Fig. 1). The whole TR element is integrated at the 3' end of an *aviX1* gene (SCAB_33441), encoding a putative ATP/GTP-binding protein.

The hidden Markov model used to analyse the predicted proteome of *S. scabiei* TR revealed that TR1 contained 14 open reading frames (ORFs), including the entire thaxtomin biosynthesis cluster, as well as additional genes encoding for hypothetical proteins (Figs 1 and S1, see Supporting Information). No candidate genes for recombination or conjugation were found in this region. TR2 contains 152 ORFs, including a lantibiotic biosynthesis cluster (SCAB_31961, *lanK*; SCAB_31981, *lanR*; SCAB_32031, *lanB*; SCAB_32041, *lanC*; SCAB_32051, *lanA*) (Figs 1 and S1). A candidate gene for a recombination directionality factor (SCAB_31881) was found upstream of the integrase gene (SCAB_31871) and the *attPi* site. Candidate genes for conjugation were also present in this region (Table 1). Genes encoding proteins typically found in Gram-positive type IV secretion systems were found in the TR element (Goessweiner-Mohr *et al.*, 2013). A locus with a replication-partition motif (SCAB_32241) was also found within this region. A schematic representation of the entire TR is presented in Fig. 1.

The TR region can excise from the chromosome of *S. scabiei* 87.22

ICEs can excise from the chromosome to form extrachromosomal circular intermediates prior to conjugative transfer. A nested poly-

Fig. 1 Schematic genetic organization of the *Streptomyces scabiei* strain 87.22 toxicogenic region (TR) located between 3 597 603 and 3 617 691 bp. A more detailed map of the TR can be found in Fig. S1. The location and orientation of the open reading frames (ORFs) are represented by arrowed boxes. ORFs were deduced from functional analysis and BLAST comparison. Arrows represent thaxtomin and lantibiotic biosynthesis genes (red), recombination genes (dark blue), the candidate replication gene (light blue), conjugation genes (green) and candidate regulatory genes (yellow). HSP, hydrophobic spread protein; IMP, inner membrane protein; RDF, recombination directionality factor.

Table 1 Open reading frames (ORFs) of the toxicogenic region coding for conjugation function.

SCAB_number	Size (amino acids)	Position from <i>attL</i>	Predicted function	Most significant Pfam match	Domain position
SCAB_32301	96	60 260–60 547	Relaxase	MobC (PF05713)	40–64
SCAB_32311	580	60 544–62 283	Relaxase	Relaxase (PF03432)	75–274
SCAB_32341	274	64 554–65 375	Hydrophobic spread protein	SPDY (PF03771)	78–140, 198–257
SCAB_32361	120	65 762–66 121	Hydrophobic spread protein	SPDY (PF03771)	48–113
SCAB_32791	459	106 428–107 804	Inner membrane protein VirB6	TrbL (PF04610)	117–283
SCAB_32961	533	120 401–121 999	ATPase, provides energy	NACHT (PF05729), AAA (PF00004), FtsK_SpoIIIE (PF01580)	200–422
SCAB_33121	254	136 299–137 060	Hydrophobic spread protein	SPDY (PF03771)	59–120, 140–209
SCAB_33122	92	137 926–138 201	Hydrophobic spread protein	SPDY (PF03771)	21–92
SCAB_33161	599	138 697–140 493	Coupling protein	T4SS-DNA_transf (PF02534), TraG-D-C (PF12696.2)	172–563, 421–539
SCAB_33181	299	141 620–142 516	Cell wall hydrolase	Abhydrolase_6 (PF12697), VirJ (PF06057.6)	28–135
SCAB_33281	360	153 463–154 542	Hydrophobic spread protein	SPDY (PF03771)	26–327, 164–226

merase chain reaction (PCR) approach was used to amplify the *attP* and *attB* sites generated by the excision of TR elements from the chromosome. For these qualitative assays, PCR primers (Fig. 2A) were designed to specifically detect the circular forms of TR1 (*attP1*), TR2 (*attP2*) and the whole TR (*attP3*) or the corresponding repaired chromosomal junctions formed on excision (*attL1*, *attR1* and *attB*, respectively). Excisions of TR1 alone, TR2 alone or the accretion of the two elements, TR, were observed (Fig. 2B) in 48-h-old tryptic soy broth cultures. PCR products were sequenced to confirm the events.

TR2 is not conserved among common scab-causing *Streptomyces* strains

In addition to strain 87.22, eight other *S. scabiei* strains (ATCC 49173, EF-35, Warba-6, CG1, CG11, Lip-17, CFBP 4518 and 89-01-04) and one *S. acidiscabies* strain (ATCC 49003) were tested for excision of the TR element. The excision of TR, TR1 and TR2 was observed only in *S. scabiei* strains ATCC 49173 (wild-type) and Lip-17 (data not shown), although the *txtA* gene was detected by PCR in all strains (Table 2). The *int* (SCAB_31871) and *lanC* (SCAB_32041) genes, which are associated with TR2, could not be detected by PCR in strains in which TR, TR1 and TR2 excision events were not observed (Table 2). Southern blot analysis confirmed that *int* and *lanC* genes were absent from the genomes of these strains (data not shown). The absence of these genes suggests that these strains lack the TR2 region and that TR1 cannot excise in the absence of TR2.

In strains lacking TR2, PCR amplification of the *attR* site was carried out using a forward primer hybridizing with the end of TR1 (IT1_F, Table 3) and a reverse primer hybridizing with the *aviX1* gene (IT2_R1). Successful amplification of the *attR* site was detected with *S. scabiei* strains EF-35, CG1, CG11, Warba-6 and 89-01-04, and with *S. acidiscabies* strain ATCC 49003 (data not shown). Sequencing of these amplicons (Fig. S2, see Supporting Information) revealed that TR1 is inserted in the *aviX1* gene, as observed in *S. scabiei* 87.22 for the TR. Conversely, no amplifica-

Table 2 Polymerase chain reaction (PCR) detection of genes associated with the toxicogenic region (TR) in various common scab-causing strains.

Strain	Origin	<i>txtA</i> (TR1)	<i>int</i> (TR2)	<i>lanC</i> (TR2)
<i>Streptomyces scabiei</i>				
87.22	USA	+	+	+
ATCC 49173	USA	+	+	+
Lip-17	Israel	+	+	+
CG1	Canada	+	–	–
CG11	Canada	+	–	–
EF-35	Canada	+	–	–
Warba-6	Canada	+	–	–
CFBP 4518	France	+	–	–
89-01-04	USA	+	–	–
<i>Streptomyces acidiscabies</i>				
ATCC 49003	USA	+	–	–

+, presence; –, absence.

tion of the *attR* site was detected with *S. scabiei* CFBP 4518, thus revealing a possible different insertion site for TR1 in this strain or polymorphism of the sites in which the primers annealed.

TR excision in *S. scabiei* 87.22 is promoted by mitomycin C and by the presence of potential recipient cells

To measure the frequency of TR excision, a quantitative PCR assay was developed to measure the percentage of cells in an *S. scabiei* culture that contained repaired chromosomal junctions (*attB* sites, i.e. *attL1*, *attR1* and *attB*) remaining after excision of TR1, TR2 or the whole TR, respectively. The excision rate was calculated as the copy number of *attB* sites per *aviX1* copy (adjacent chromosomal locus) as described in Experimental Procedures. After 48 h of growth, excision of the TR in minimal medium was low, as *attB* sites were detected in approximately 0.001%–0.003% of cells, regardless of the excision event (excision of TR1, TR2 or TR).

Excision rates were also compared under the following different environmental conditions: presence of thaxtomin; presence of compounds that induce (cellobiose and suberin; Francis *et al.*, 2015; Lerat *et al.*, 2010) or repress (scopoletin; Lerat *et al.*, 2009a)

Fig. 2 (A) Excision of the toxicogenic region. Black arrows represent the primers used to amplify *att* sites formed on excision of circular forms and repaired chromosomal junctions. Three distinct forms were observed: excision of TR1 containing the thaxtomin biosynthesis operon (blue), excision of TR2 containing recombination and conjugation modules and a lantibiotic biosynthesis cluster (green), and the combination of TR1 and TR2 (TR). (B) PCR products resulting from the excision of TR1, TR2 and TR from the chromosome. *attL1*, *attR1* and *attB* represent the chromosomal junctions formed on excision of TR1, TR2 and TR, respectively. *attP1*, *attP2* and *attP3* represent the *att* sites on the circular form of the element on excision of TR1, TR2 and TR, respectively.

the expression of the thaxtomin biosynthetic genes; presence of an inducer of the SOS response (mitomycin C); and presence of recipient cells (*S. coelicolor*). The excision frequency was also analysed at different time points on an *S. scabiei* culture to investigate the effect of cell density, related to quorum sensing. Bacteria grown in minimal medium were therefore collected 12, 24, 48 and 72 h after inoculation.

The effect of the SOS response on the excision of TR1, TR2 and TR was examined by adding a subinhibitory concentration of mitomycin C to the minimal medium growth culture. The addition of mitomycin C induced a significant increase in the excision rates for both TR and TR2 ($P < 0.05$, least-significant difference test), with the excision frequency increasing up to 25-fold for TR2 and 10-fold for the entire TR. The excision frequency of TR1 was not significantly increased by mitomycin C treatment (Fig. 3). Excision was also significantly induced by the presence of potential recipient cells, by eight-fold for TR2 and six-fold for TR (Fig. 3). None of the other environmental conditions tested, nor the age of the culture, had a significant effect on TR excision (data not shown).

DISCUSSION

The *S. scabiei* 87.22 genome was analysed to investigate the potential mobility of the TR region. Candidate genes for recombination, replication and conjugal transfer, as well as *att* sites, were found in the TR, which is integrated at the 3' end of an *aviX1* gene (SCAB_33441). This *aviX1* gene encodes a putative ATP/GTP-binding protein, homologues of which are present in at least 50 *Streptomyces* species (BLASTP, >85% identity). This position is therefore a good location for site-specific integration in *Streptomyces* species, although it is unusual, given that over 50% of genomic islands (GIs) are inserted into tRNA/tmRNA genes (Song *et al.*, 2012). Nearly identical *att* sites were found in the *aviX1* homologues of all *Streptomyces* species whose genomes have been completely sequenced and are available in GenBank (Harrison and Studholme, 2014), as well as in other actinobacterial species (Fig. S3, see Supporting Information). The level of polymorphism at the *att* site among species is similar to the level observed among the three repeats identified in *S. scabiei* 87.22 (*attL*, *attPi*

Table 3 Primers used in this study.

Primer name	Sequence 5'–3'
IT2_R1	AAGCCGTTCTGTACGTCTTCAAC
IT2_R2	GACCGACGAGGACTTCAAGAACGA
GTP_R1	CAACAAGTTCCTCGCAACATCCG
GTP_R2	CCAAGGACACATCGAGACGATCA
GTP_R2_SphI	ACGCATCCAAGGACACATCGAGACGATCA
qtr1F	AGACGGCCTTTCGTGCAACGTATT
qtr1R	TGCTGATTCTGTGCTGACTTTGCC
qtr2F	TGCATGTTGCCAGATCAGTGACG
qtr2R	ATGGAGGGCAAGGACTACGTGAT
qtrR	CGCATGGAGGGCAAGGACTACGTGA
qgtpF	GGCGCTGCTCGTTCTTGAAGTCCT
qgtpR	CTGCTCACCACCAAGCCGTTCTGT
txtA_F	GGTACTCGTGCCACATCTGCATCT
txtA_R	AACGAACTCAGCCGCTATCGCTAC
Int20_F	GTAGCGAAGGCGAGAGTCTCACTG
Int20_R	GAGCCGACGAACAAGTACTACCCG
lantCF	TGTCCTGACACGCATTTCATC
lantCR	GTCATGGTCTTGGCGAATCT
R1	CGACGCTGAGATCCTGACCG
R2	CGACCTCCACTACGACCAGC
R2_XbaI	TCTAGACGACCTCCACTACGACCAGC
R2_HindIII	ACAACGCGACCTCCACTACGACCAGC
IT2_R2_EcoRI	GAATTCGACCGAGGACTTCAAGA
TNi1	GGTGATCTGCGTGAGGACTG
TNi2	CCCGAACCTGATCTTGATCTGAGG
TNi3	CGGACGCGACAGGTTACCC
TNi4	GTACGTCGTCTCAGCCATGACC
TNi4_KpnI	GGTACCGTACGTCGTCTCAGCCATGACC
TNi5	GCCACAAGTCGATCAAGACCAG
TNi5_BamHI	GGATCCGCCACAAGTCGATCAAGACCAG
TNi6	CCTCGACTGCTCTGGCGAAC
TNi7	CGAAGATCGAGAAGCTCAGGAAGG
TNi8	GCGAGTGAAGTCATGTGCGG
IT1_F	TGCATGTTGCCAGATCAGTGACG

Fig. 3 Excision frequency ($\pm$ standard deviation) of TR, TR1 and TR2, determined by measuring repaired chromosomal junctions formed on excision at the *attB* sites, in *Streptomyces scabiei* 87.22 grown in control minimal medium (MM, white bars) and supplemented with subinhibitory concentrations of mitomycin C (SOS, light hatched bars), or in the presence of *Streptomyces coelicolor* (SCO, dark hatched bars). Values with the same letters are not statistically significantly different (least-significant difference test, $P < 0.05$).

and *attR*), suggesting that the *att* site could allow the integration of TR in various actinobacteria.

Based on the protein sequences of the TR2 ORFs coding for potential conjugative functions, the mating pair formation for the TR element transfer relies on a type IV secretion system. This system is widespread in Gram-negative bacteria, but less frequent in actinobacteria (Bordeleau *et al.*, 2012). In Gram-positive bacteria, the mating pair formation system is often simpler because of the single-membrane (monoderm) cell envelope. The *spd* genes are related to pock formation, which allows the intermycelial transfer of plasmids between adjacent cells (Ghinet *et al.*, 2011). A locus with a replication-partition motif (SCAB_32241) was also present in the TR, suggesting that this region undergoes autonomous replication after excision from the chromosome. Autonomous replication is known to participate in the stability and propagation of ICEs (Thomas *et al.*, 2013).

In *S. scabiei* strain 87.22, the TR has a composite structure, including three *att* sites, and we have demonstrated that the TR can excise from the chromosome in three distinct forms: TR1 alone, TR2 alone and the entire composite TR. However, several *S. scabiei* strains and *S. acidiscabies* strain ATCC 49003 lacked a TR2 element. As this region encodes the recombination and conjugation modules, strains harbouring only TR1 would therefore have the main pathogenic determinants, the thaxtomin biosynthetic genes, stabilized in their genomes. In most strains lacking TR2, including *S. acidiscabies*, TR1 was also shown to be inserted in *aviX1*, suggesting that TR2 has been lost in some populations. The acquisition or retention of TR2 might nevertheless confer a competitive advantage to the pathogen under certain conditions, as this region harbours a bacteriocin lanthionine biosynthetic gene cluster. Lanthionine-containing peptides with antimicrobial functions (Sahl and Bierbaum, 1998) are commonly produced by Gram-positive bacteria (Riley and Gordon, 1999). The presence of such a cluster has been observed in other ICEs (Horn *et al.*, 1991), including an ICE harboured by the actinobacterium *Frankia*, which lives in symbiosis with plant roots (Ghinet *et al.*, 2011).

The only tested strain for which *aviX1* could not be unambiguously identified as the integration site of the TR was CFBP 4518. Thus, as seen with the *S. turgidiscabies* PAI, the thaxtomin biosynthetic genes of CFBP 4518 may be located at a different chromosomal position, especially as a DNA hybridization study has shown that the CFBP 4518 genome shares only 72% homology with the genome of the *S. scabiei* type strain (Bouchek-Mechiche *et al.*, 2000).

Since 1990, approximately a dozen novel common scab-inducing *Streptomyces* species have been described (Loria *et al.*, 2008). These phenotypically and genetically distinct pathogens produce thaxtomins, suggesting that they have acquired genes found on TR1 via horizontal transfer. Although several actinobacteria possess the *aviX1* gene, a potential TR1 integra-

tion locus, acquisition of the thaxtomin biosynthetic genes does not necessarily lead to pathogenicity. Kers *et al.* (2005) showed that conjugation between a pathogenic strain of *S. turgidiscabies* and two non-pathogenic streptomycetes, *S. diastatochromogenes* and *S. coelicolor*, resulted in the acquisition of the thaxtomin biosynthetic genes by both species; however only *S. diastatochromogenes* acquired a pathogenic phenotype.

Similar to the *S. scabiei* TR, the PAI of *S. turgidiscabies*, which is integrated in *bacA*, a bacitracin resistance gene, harbours a composite structure with three possible excision events (Huguet-Tapia *et al.*, 2014). These authors suggested that modular excision of genomic islands in plant-pathogenic streptomycetes could be a significant source of genomic plasticity. However, the *att* sites, the integrase gene and the overall gene composition of the PAI differ between *S. scabiei* and *S. turgidiscabies*. The pathogenicity genes associated with TR1 and CR in *S. scabiei* are present in the *S. turgidiscabies* PAI, but the genes associated with *S. scabiei* TR2 are not present. The *S. turgidiscabies* PAI also contains genes lacking in the *S. scabiei* genome, notably the cytokinin biosynthetic pathway *fas* operon. These observations suggest a complex evolutionary pathway for the acquisition of pathogenic determinants in *Streptomyces* species.

Other ICEs with accretion-type structures have been described previously. Several GIs flanked by *att* sites are devoid of recombination and conjugation modules. They can be mobilized in *cis* by an adjacent ICE (co-integrated) that harbours recombination and conjugation modules. The high pathogenicity island (HPI) is often physically linked to another ICE and mobilized in *cis* with ICE (Paauw *et al.*, 2010). ICEst3 of *Streptococcus thermophilus* can transfer another element, CIMEl3catR3 (with no recombination and conjugation modules), as the accretion ICE (ICEst3CIMEl3catR3), as well as the two elements independently (Bellanger *et al.*, 2011). Multiple integrations of mobile elements at the same site could increase the plasticity of genomes (Hochhut *et al.*, 2000).

Excision of the TR or of its subunit elements (TR1 and TR2) appeared to be a relatively rare event in minimal medium, occurring in approximately 0.001% of cells. However, ICE excision is usually regulated by complex regulation mechanisms that can be activated or repressed by various environmental stimuli, the growth phase or the presence of recipient cells. Unlike *Streptococcus thermophilus* ICEst1 and ICEst3 (Carraro *et al.*, 2011) or *Pseudomonas* sp. ICEclc (Wozniak and Waldor, 2010), the age of the culture did not influence the excision frequency of *S. scabiei* TR under the conditions tested.

In *Bacillus subtilis*, the excision frequency of ICEBs1 was increased by the presence of cells lacking the element (Auchtung *et al.*, 2005). Similarly, the TR excision rate was increased when *S. scabiei* 87.22 was grown in the presence of a non-pathogenic bacterium, *S. coelicolor*. This suggests that the *S. scabiei* TR could,

like the *S. turgidiscabies* PAI, be transferred to non-pathogenic streptomycetes (Kers *et al.*, 2005).

Stress conditions that damage DNA seem to be a stimulus for ICE dissemination in several bacteria. DNA-damaging agents, such as mitomycin C, increase the transfer of the *Vibrio cholerae* SXT element by up to 100-fold (Beaber *et al.*, 2004). In *B. subtilis*, ICEBs1 excision and transfer are also induced by mitomycin C (Auchtung *et al.*, 2005). *Streptomyces scabiei* TR excision frequency was also increased by mitomycin C. Interestingly, sequence analysis of TR2 revealed the presence of a transcriptional regulator with a LexA-DNA-binding motif (SCAB_31881). LexA is the repressor protein of the DNA repair SOS response regulon (Little and Mount, 1982). Therefore, the involvement of this gene in excision regulation deserves further investigation. Although excision of the TR2 module was increased in the presence of mitomycin C, TR1 excision was not influenced by the SOS response. Similar results have been observed for the accretion of ICEst3 of *Streptococcus thermophilus*, which can transfer as an accretion ICE or as the two elements independently (Bellanger *et al.*, 2011).

Many soil environmental conditions might induce the SOS response and consequently increase the excision rate of TR2. For instance, several types of antibiotic have been shown to trigger the bacterial SOS response (Fajardo and Martinez, 2008; Heo *et al.*, 2014). The fact that TR2 excision, but not TR1 excision, was induced by both the SOS response and the presence of actinobacterial recipient cells suggests that excision events represent the TR2 response to microbial competition, especially as TR2 carries genes conferring resistance to lanthionine. Compounds that promote or inhibit thaxtomin biosynthesis in *S. scabiei* showed no effect on TR excision. Even suberin, a major constituent of potato periderm which induces the overproduction of stress-related proteins, including LexA (Lauzier *et al.*, 2008), did not promote TR excision. As the excision of TR appears to depend on TR2 genes, the mobilization of pathogenicity determinants might therefore respond to environmental stimuli for the purpose of disseminating the lantibiotic genes, rather than in response to plant signals controlling plant-microbe interactions. However, the molecular mechanisms linked to the excision and subsequent TR transfer to non-pathogenic bacteria need to be explored further.

EXPERIMENTAL PROCEDURES

Streptomyces scabiei 87.22 genome analysis

The *S. scabiei* 87.22 genome sequence was extracted from GenBank (<http://www.ncbi.nlm.nih.gov>). ORFs of the TR were determined using GeneMark.hmm (prokaryotic). Protein domains were analysed using the Pfam protein families database, based on the Profile Hidden Markov model (Punta *et al.*, 2012). ORFs were analysed with BLASTP from

the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) to identify protein domain positions.

Bacterial strains, plasmids and growth media

The strains and plasmids used in this study are listed in Table 4. The pUC Quant plasmid containing *attB* sites was constructed as follows. *attB* fragments were amplified using primers introducing restriction sites at both ends of the PCR products. The primers used for the amplification were R2_XbaI + Tni5_BamHI for *attL1*, Tni4_KpnI + IT2_R2_EcoRI for *attR1* and R2_HindIII + GTP_R2_SphI for *attB* (Table 3). The amplified fragments were digested with appropriate restriction enzymes, ligated into a pUC19 plasmid (New England Biolabs, Pickering, ON, Canada) and digested with the same enzymes. Ligations were performed according to the manufacturer's instructions with T4 DNA ligase (New England Biolabs) for the three PCR fragments, one fragment at a time. The plasmids were transformed into *Escherichia coli* DH5 α and purified using the GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). The final plasmid harbouring the three *attB* sites (pUC_Quant) was cloned into *E. coli* DH5 α .

Escherichia coli strains were routinely grown in lysogeny broth (LB; Sambrook *et al.*, 1989) in a rotary shaker (250 rpm) at 37 °C. *Streptomyces* strains were maintained on mannitol soya flour agar medium (Kieser *et al.*, 2000) or yeast malt extract agar (YMEA = ISP-2 medium; Shirling and Gottlieb, 1966) medium. Tryptic soy broth (BD, Mississauga, ON, Canada) was used for qualitative excision assay, whereas minimal medium (Kieser *et al.*, 2000), supplemented with 1% starch, was used for the quantitative excision assay. These minimal media were supplemented with cellobiose (0.5%), suberin (0.1%), scopoletin (100 μ g/mL) or thaxtomin (200 μ g/mL). The following antibiotics were used when required: apramycin (100 μ g/mL), chloramphenicol (25 μ g/mL), spectinomycin (100 μ g/mL), streptomycin (10 μ g/mL) and mitomycin C (500 ng/mL). For the excision assays, *Streptomyces* spores (10^7) were used to inoculate 50 mL of culture medium in 250-mL flasks,

and bacteria were grown in a rotary shaker (250 rpm) at 30 °C for 48 h. A combination of 10^7 spores of *S. scabiei* 87.22 and 10^7 spores of *S. coelicolor* M145 was used for the excision assay carried out in the presence of potential recipient cells.

Excision assay

DNA used for the experiments was extracted from *Streptomyces* cultures using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), following the manufacturer's instructions, with a sonication step (20 s at 40% amplitude, Vibra-Cell 130 W, Sonics, Newtown, CT, USA) after the lysozyme digestion step. Qualitative nested PCR assays were performed with 50 ng of DNA using primers R1 + Tni6 and R2 + Tni5 for *attL1* (final PCR product, 553 bp); Tni3 + IT2_R1 and Tni4 + IT2_R2 for *attR1* (715 bp), R1 + GTP_R1 and R2 + GTP_R2 for *attB* (1016 bp), Tni2 + Tni3 and Tni1 + Tni4 for *attP1* (559 bp), Tni6 + Tni7 and Tni5 + Tni8 for *attP2* (499 bp), and Tni2 + Tni7 and Tni1 + Tni8 for *attP3* (526 bp). Each reaction (20 μ L) contained primers at a final concentration of 1 μ M, 200 μ M deoxynucleoside triphosphates (dNTPs), 1 $\times$ Taq Buffer (New England Biolabs) and 1.00 unit of Taq DNA polymerase (New England Biolabs). PCRs were amplified for 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 55–60 °C and 1 min at 68 °C. PCR products were visualized after electrophoresis on a 1% agarose gel. PCR products were sequenced (Sanger sequencing at Genome Quebec Innovation Center, Montreal, QC, Canada) and compared with the predicted excision/circularization sequences.

Quantitative PCRs (20 μ L) were performed on approximately 10 ng of DNA. The assay to measure the percentage of cells in a culture containing the repaired chromosomal junctions formed on excision was performed as described by Burrus and Waldor (2004). DNA normalization was performed by amplifying the 3' end of the *aviX1* gene (SCAB_33441) located near the *attR* site (present in the *attB* PCR fragment). Primer pairs qtr1F + qtr1R, qtr2F + qtr2R, qtr1F + qtrR and qgtpF + qgtpR were used

Table 4 Bacterial strains and plasmids used in this study.

Strains and plasmids	Description	Reference
<i>Streptomyces scabiei</i>		
87.22		Bukhalid <i>et al.</i> (1998)
EF-35		Faucher <i>et al.</i> (1992)
ATCC 49173		Lambert and Loria (1989a)
Lip-17		Komeil <i>et al.</i> (2013)
CG1		Beauséjour <i>et al.</i> (1999)
CG11		Lauzier <i>et al.</i> (2002)
Warba-6		Goyer (2005)
CFBP 4518		Bouchek-Mechiche <i>et al.</i> (2000)
89-01-04		This study
<i>Streptomyces acidiscabies</i>		
ATCC 49003		Lambert and Loria (1989b)
<i>Streptomyces coelicolor</i>		
M145	<i>Streptomyces coelicolor</i> A3(2) SCP1SCP2	Kieser <i>et al.</i> (2000)
<i>Escherichia coli</i>		
DH5 α	F-80 Φ dlacZDM15D(lacZYA ⁻ argF)U169deoRrecA1endA1hsdR17(rk ⁻ mk ⁺)supE44 λ -thi-1gyrA96 relA	GIBCO-BRL (Gaithersburg, MD, USA)
DH5 α pQuant	<i>Escherichia coli</i> DH5 α with pQuant vector	This study
Plasmids		
pUC19	Plasmid cloning vector in <i>E. coli</i>	New England Biolabs (Pickering, ON, Canada)
pQuant	pUC19 with a single copy of <i>attBTR</i> , <i>attBTR1</i> , <i>attBTR2</i> , <i>SCAB_33441</i>	This study

to amplify the *attL1* (108 bp), *attP2* (104 bp), *attB* (90 bp) and *aviX1* (86 bp) fragments, respectively (Table 3). Each reaction (20 µL) contained 10 µL of 2 × iQaq Universal SYBR® Supermix (Bio-Rad, Hercules, CA, USA) and primers at a final concentration of 1 µM. Reactions were incubated for 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 55–60 °C, using the Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA). Three reactions were performed for each sample. Excision frequencies were calculated according to Burrus and Waldor (2004), using *E. coli* pUC_Quant (see above) DNA to create standard curves. The reported frequencies of excision are the mean of biological triplicates.

Detection of genes associated with the TR in various *Streptomyces* strains

The presence of TR genes (*txtA*, *int* and *lanC*) was determined by amplification as described above using the primer pairs *txtA_F* + *txtA_R*, *Int20_F* + *Int20_R* and *lantCF* + *lantCR*, respectively (Table 3). When no amplification was detected, the absence of the gene in the genome was confirmed by Southern blot. For this experiment, total genomic DNA (6 µg) was digested with *Bam*HI-HE restriction enzyme and migrated on a 1.1% agarose gel (100 V). DNA fragments were transferred on a Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK). Probes for the target genes *txtA*, *int* and *lanC* were obtained by amplifying genomic DNA from *S. scabiei* 87.22 with the primers described above. The resulting PCR products were then labelled using the DIG High Prime DNA Labelling and Detection Starter kit I (Roche, Laval, QC, Canada). Hybridization and detection of the digoxigenin (DIG)-labelled hybrids were carried out as recommended by the manufacturer using a hybridization temperature of 45 °C.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Detailed map of the genetic organization of the toxicogenic region (TR) in *Streptomyces scabiei* 87.22.

Fig. S2 Sequence alignment of polymerase chain reaction (PCR) products obtained after amplification with primers IT1_F and IT2_R1 in *Streptomyces scabiei* strains lacking TR2 (toxicogenic region 2). The *att* sites are highlighted in yellow; the end of TR1 (toxicogenic region 1) and the *aviX1* gene are upstream and downstream, respectively, of the *att* sites.

Fig. S3 Sequence alignment of the *attL* site of *Streptomyces scabiei* 87.22 with *att* sites identified in randomly chosen *Streptomyces* and other actinobacterial species with sequenced genomes available in GenBank. Homologies with the *S. scabiei* *attL* site are represented by shaded letters.