Emergence of Novel Pathogenic *Streptomyces* Species by Site-Specific Accretion and *cis*-Mobilization of Pathogenicity Islands

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The main pathogenicity factor of *Streptomyces* species associated with the potato common scab disease is a nitrated dike-topiperazine called thaxtomin A (ThxA). In *Streptomyces scabiei* (syn. *S. scabies*), which is thought to be the most ancient pathogenic *Streptomyces* species, the ThxA biosynthetic cluster is located within a mobile genomic island called the toxicogenic region (TR). Three attachment (*att*) sites further separate TR into two subregions (TR1 and TR2). TR1 contains the ThxA biosynthetic cluster and is conserved among several pathogenic *Streptomyces* species. However, TR2, an integrative and conjugative element, is missing in most pathogenic species. In our previous study, we demonstrated the mobilization of the whole TR element or TR2 alone between *S. scabiei* and nonpathogenic *Streptomyces* species. TR1 alone did not mobilize in these experiments. These data suggest that TR2 is required for the mobilization of TR1. Here, we show that TR2 can self mobilize to pathogenic *Streptomyces* species harboring only TR1 and integrate into the *att* site of TR1, leading to the tandem accretion of resident TR1 and incoming TR2. The incoming TR2 can further mobilize resident TR1 *cis* and transfer to a new recipient cell. Our study demonstrated that TR1 is a non-autonomous *cis*-mobilizable element and that it can hijack TR2 recombination and conjugation machinery to excise, transfer, and integrate, leading to the dissemination of pathogenicity genes and emergence of novel pathogenic species. Additionally, comparative genomic analysis of 23 pathogenic *Streptomyces* isolates from ten species revealed that the composite pathogenicity island (PAI) formed by TR1 and TR2 is dynamic and various compositions of the island exist within the population of newly emerged pathogenic species, indicating the structural instability of this composite PAI.

Although *Streptomyces* spp. are viewed predominantly as free-living terrestrial soil-inhabiting bacteria, some species are symbionts with fungi, insects, plants, and animals (Seipke et al. 2012) and some strains reside in marine soils (Fiedler et al. 2005). A few *Streptomyces* species are plant pathogens and cause diseases of underground crop structures, such as roots and tubers; the most economically important disease caused by *Streptomyces* species is potato scab, characterized by raised, pitted, or superficial lesions formed on the tuber surface (Loria et al. 2006). The

scab-causing species *Streptomyces scabiei* (syn. *S. scabies*) also infects the pericarp of the peanut fruit, causing peanut pod wart disease; however, this little-known disease has only been described in Israel (Kritzman et al. 1996) and South Africa (de Klerk et al. 1997).

Though *S. scabiei* is thought to be the most ancient scab-causing *Streptomyces* species (Bukhalid et al. 2002), there are a number of other species that are also the causal agents of scab lesions on actively growing tubers. The best-characterized scab-causing species are *S. scabiei*, *S. acidiscabies*, and *S. turgidiscabies* (Labeda 2016). An additional pathogenic species, *S. ipomoeae*, incites the soil rot disease on sweet potato (*Ipomoea batatas* [L.] Lam.) (Tomihama et al. 2016). Besides these four well-studied pathogenic species, seven other potato scab-causing *Streptomyces* species have also been described, namely *S. europaeiscabiei*, *S. stelliscabiei*, *S. luridiscabiei*, *S. puniscabiei*, *S. niveiscabiei*, *S. reticuliscabiei*, and *S. caviscabies* (Bouchek-Mechiche et al. 2000; Goyer et al. 1996; Park et al. 2003a).

The primary pathogenicity determinant of scab-causing *Streptomyces* species is a family of dipeptide phytotoxins known as thaxtomins (Loria et al. 2006). A total of 11 family members have been isolated; these thaxtomin analogs are differentiated by the presence or absence of either hydroxyl, N-methyl groups, or both (Bignell et al. 2010a). In *S. scabiei*, *S. acidiscabies*, and *S. turgidiscabies*, thaxtomin A (ThxA) is the predominant family member (Healy et al. 2000; King et al. 1989; Lawrence et al. 1990). However, the major thaxtomin analog produced by *S. ipomoeae* is the nonhydroxylated analog called thaxtomin C (King et al. 1994). *S. europaeiscabiei*, *S. stelliscabiei*, *S. niveiscabiei*, and *S. caviscabies* also produce thaxtomins (Goyer et al. 1996; Loria et al. 2006; Park et al. 2003a). However, thaxtomin production by *S. luridiscabiei* and *S. puniscabiei*, two species only reported in Korea (Park et al. 2003a), has not been demonstrated (Park et al. 2003b).

In *S. turgidiscabies* Car8, ThxA biosynthetic genes and other virulence genes, including *nec1*, a tomatinase gene (*tomA*), and a plant fasciation (*fas*) operon, reside on a mobile 674-kb pathogenicity island (PAI) (Huguet-Tapia et al. 2011; Kers et al. 2005). In contrast, virulence factors in *S. scabiei* and *S. acidiscabies* reside on two remote chromosomal regions, namely the toxicogenic region (TR) and the colonization region (CR) (Lerat et al. 2009). CR contains *nec1* and *tomA* (Lerat et al. 2009). In *S. scabiei*, TR is further separated into two subregions (TR1 and TR2) by an internal and two flanking attachment (*att*) sites (Chapleau et al. 2016). TR1 harbors the ThxA biosynthetic gene cluster and TR2 possesses a putative integrative and conjugative element (ICE) (Chapleau et al. 2016). ICEs are self-transmissible elements that encode the machinery for excision, conjugation, and integration as well as regulatory systems to regulate their excision and

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and one supplementary table are published online.

conjugative transfer (Burrus and Waldor 2004; Osborn and Böltner 2002; Salyers et al. 1995). Chapleau et al. (2016) demonstrated the excision of TR from *S. scabiei* in three distinct forms: as TR1 alone, as TR2 alone, and as the entire TR element; reintegration of the TR elements was not reported. Recently, we demonstrated the site-specific integration of TR2 alone or the entire TR from *S. scabiei* into the chromosome of the soil saprophyte *Streptomyces diastatochromogenes* ATCC 12309; acquisition of the entire TR but not TR2 alone conferred a pathogenic phenotype on *S. diastatochromogenes* (Zhang et al. 2016). In this study, independent integration by TR1 was not observed, suggesting that TR1 may be a *cis*-mobilizable element (CIME). CIMEs are genomic islands that lack proteins involved in recombination and conjugation but are flanked by *att* sites (Bellanger et al. 2014). We also observed that TR2, which is a putative ICE, only exists in *S. scabiei* and a novel pathogenic species (*Streptomyces* sp. strain 96-12, GenBank accession number GCA_001550315.1) but is absent in other pathogenic species, such as *S. acidiscabies* and *S. europaeiscabiei* (Zhang et al. 2016). These data suggest that *S. scabiei* is likely to be the source of the entire TR region and that its mobilization contributes to the emergence of novel pathogenic species (Zhang et al. 2016).

Our previous study found that *S. acidiscabies* 84-104, an emergent pathogen (Huguet-Tapia and Loria 2012), has a TR1 region that is almost identical to the TR1 region in *S. scabiei*; however, TR2 is absent from *S. acidiscabies* 84-104 (Zhang et al. 2016). We hypothesized that *S. acidiscabies* first acquired the entire TR element but, subsequently, lost TR2. In the current study, we sequenced the genomes of an additional four *S. acidiscabies* isolates from different locations to explore the diversity of TR elements within the population of *S. acidiscabies*. This study benefited from the very recent availability of genome sequences for plant-pathogenic streptomycetes (Huguet-Tapia et al. 2016; Labeda 2016; Tomihama et al. 2016). These genomes allowed us to characterize the variation in the sequences of PAIs within and among pathogenic *Streptomyces* species and to investigate the origin and evolution of pathogenicity in the *Streptomyces* genus. Here, we demonstrate that TR2 can transfer itself to a pathogenic isolate harboring only TR1 and integrate into one of the two *att* sites flanking TR1, leading to the tandem accretion

of resident TR1 and incoming TR2. Furthermore, the resulting composite genomic island can excise and be transferred to a new host. These data demonstrated that TR1 is a CIME and the site-specific accretion and *cis*-mobilization of TR1 together with the related ICE (TR2) have a key role in the dissemination of pathogenicity and virulence genes and emergence of novel pathogenic species in *Streptomyces* genus.

RESULTS

Pathogenicity and ThxA production of four *S. acidiscabies* isolates.

Two *S. acidiscabies* isolates from the United States (85-06, FL01) and two isolates from Japan (98-48, 98-49) were selected for genome sequencing (Table 1). Three of these four *S. acidiscabies* isolates and three well-studied pathogenic isolates (*S. acidiscabies* 84-104, *S. turgidiscabies* Car8, and *S. scabiei* 87-22) caused the expected disease symptoms on radish seedlings (Fig. 1A). Non-inoculated seedlings and seedlings inoculated with the non-pathogenic *S. diastatochromogenes* grew normally. In contrast, *S. acidiscabies* 98-48 had a phenotype intermediate to the healthy seedlings and those inoculated with the other pathogenic strains.

We compared ThxA production by the four *S. acidiscabies* strains (85-06, FL01, 98-48, and 98-49) with three well-studied pathogenic isolates, namely, *S. acidiscabies* 84-104, *S. scabiei* 87-22, and *S. turgidiscabies* Car8 in oat bran broth (OBB) (Fig. 1B), a complex medium used to induce ThxA biosynthesis (Wach et al. 2007). Four of the five *S. acidiscabies* isolates (84-104, 85-06, 98-49, and FL01), *S. turgidiscabies* Car8, and *S. scabiei* 87-22 produced ThxA as expected. However, *S. acidiscabies* 98-48 did not produce a detectable level of ThxA in OBB, which can explain why this strain cannot induce the expected pathogenic phenotype on radish seedlings (Fig. 1A). We also found that all of the four ThxA-producing *S. acidiscabies* isolates (84-104, 85-06, 98-49, and FL01) produced a significantly greater quantity of ThxA than *S. scabiei*.

Phylogenomic and genome similarity analysis of pathogenic and related nonpathogenic species.

Previously, we demonstrated the power of core genome-based phylogenetic analysis to infer evolutionary relationships

Table 1. Genome summary statistics of plant-pathogenic *Streptomyces* spp.

Species	Strain name	Genome status	Contig no.	Contig N50 (kb)	Genome size (Mb)	Geographic location	GenBank assembly accession no.	Source or reference
<i>S. acidiscabies</i>	84-104	Draft	244	113.4	11.0	U.S.A.: upstate New York	GCA_000242715.2	Huguet-Tapia and Loria 2012
<i>S. acidiscabies</i>	85-06	Draft	1073	20.0	11.0	U.S.A.: West Virginia	LYDU000000000	This study
<i>S. acidiscabies</i>	98-48	Draft	983	20.3	10.6	Japan: Saga	LYDS000000000	This study
<i>S. acidiscabies</i>	98-49	Draft	1211	16.5	10.2	Japan: Saga	LYDT000000000	This study
<i>S. acidiscabies</i>	FL01	Draft	993	19.3	10.6	U.S.A.: Florida	LYDV000000000	This study
<i>S. acidiscabies</i>	a10	Draft	279	82.2	10.7	Japan: Kagoshima, Nagashima	GCA_001485105.1	Tomihama et al. 2016
<i>S. acidiscabies</i>	NCPPB 4445	Draft	263	88.0	10.0	Unknown	GCA_001189015.1	University of Exeter
<i>S. europaeiscabiei</i>	NRRL B-24443	Draft	1056	16.4	10.0	France: Le Rhue	GCA_000988945.1	Labeda 2016
<i>S. europaeiscabiei</i>	NCPPB 4086	Draft	295	97.2	10.5	Canada: Ontario	GCA_000738695.1	University of Exeter
<i>S. stelliscabiei</i>	NRRL B-24447	Draft	991	19.0	10.0	France	GCA_001008135.1	USDA ARS NCAUR ^a
<i>S. stelliscabiei</i>	P3825	Draft	705	32.8	10.4	Unknown	GCA_001189035.1	University of Exeter
<i>S. turgidiscabies</i>	Car8	Draft	692	27.4	10.8	Japan	GCA_000331005.1	Huguet-Tapia et al. 2011
<i>S. turgidiscabies</i>	T45	Draft	101	264.4	10.6	Japan: Kagoshima, Kushira	GCA_001485145.1	Tomihama et al. 2016
<i>S. ipomoeae</i>	91-03	Draft	687	26.4	10.4	U.S.A.	GCA_000317595.1	Huguet-Tapia et al. 2016
<i>S. scabiei</i>	87-22	Complete	1071	10.1	10.7	U.S.A.: Wisconsin	GCA_000091305.1	Bignell et al. 2010a and b
<i>S. scabiei</i>	NRRL B-16523	Draft	1091	15.1	9.8	U.S.A.: central New York	GCA_001005405.1	Labeda 2016
<i>S. scabiei</i>	S58	Draft	149	193.2	10.0	Japan: Kagoshima, Nagashima	GCA_001485125.1	Tomihama et al. 2016
<i>S. luridiscabiei</i>	NRRL B-24455	Draft	821	18.0	7.9	South Korea: Jeju	GCA_001418625.1	Labeda 2016
<i>S. puniscabiei</i>	NRRL B-24456	Draft	688	25.4	8.8	South Korea: Jeju	GCA_001419685.1	Labeda 2016
<i>S. niveiscabiei</i>	NRRL B-24457	Draft	1568	10.1	9.5	South Korea: Jeju	GCA_001419795.1	Labeda 2016
<i>S. niveiscabiei</i>	ST1015	Draft	1126	14.6	9.8	Uruguay	LXWC000000000	Universidad de la República
<i>S. niveiscabiei</i>	ST1020	Draft	1010	17.8	9.9	Uruguay	LXWD000000000	Universidad de la República
<i>Streptomyces</i> sp.	96-12	Draft	1875	8.7	10.1	Egypt	GCA_001550315.1	Zhang et al. 2016

^a USDA-ARS-NCAUR = United States Department of Agriculture-Agricultural Research Service-National Center for Agricultural Utilization Research.

among *Streptomyces* species, and the power of in silico DNA-DNA hybridization (isDDH) and the average nucleotide identity (ANI) to identify novel pathogenic species (Zhang et al. 2016). However, our previous study did not include less-studied pathogenic *Streptomyces* species, namely *S. luridiscabiei*, *S. puniscabiei*, and *S. niveiscabiei*, whose genomes have been sequenced recently (Labeda 2016). To obtain a better understanding of evolutionary relationships of pathogenic *Streptomyces* species, we included these less-studied pathogenic species into the phylogenomic and systematic analysis.

A core genome-based maximum likelihood phylogeny of selected pathogenic isolates was constructed by 1,104 single-copy ortholog groups that are shared among all tested pathogenic *Streptomyces* isolates as well as isolates from their closely related nonpathogenic *Streptomyces* species (*S. bottropensis* and *S. galbus*) and two outgroups (Fig. 2). The phylogenetic tree was distributed into two major clades. The first clade contains *S. scabiei*, *S. stelliscabiei*, *S. bottropensis* ATCC 25435, *S. europaeiscabiei*, *Streptomyces* sp. strain 96-12, *S. galbus* KCCM 41354, *S. ipomoeae*, and *S. turgidiscabiei*; the second clade includes *S. acidiscabiei*, *S. niveiscabiei*, and *S. puniscabiei*. *S. luridiscabiei*, however, cannot be placed into either of these two groups. Although *S. bottropensis* ATCC 25435 and *S. galbus* KCCM 41354 are

nonpathogenic species, they are more closely related to *S. scabiei*, *S. stelliscabiei* and *S. europaeiscabiei* than *S. acidiscabiei* and *S. niveiscabiei*. This indicates that these pathogenic species acquired pathogenicity genes after their ancestor diverged into different species. However, an alternative hypothesis is that *S. bottropensis* and *S. galbus* lost their pathogenicity determinants after divergence.

ANI and isDDH are two of the most widely accepted in silico tools used to determine strain relationships, and 95 to 96% ANI and 70% isDDH values were applied as the threshold for species delineation (Auch et al. 2010a; Goris et al. 2007). In our analysis, we found that isolates in the same phylogenetic clade also showed high ANI and isDDH values (Supplementary Fig. S1). Although *S. niveiscabiei* and *S. acidiscabiei* are closely related in the phylogenetic tree (Fig. 2), the low ANI (approximately 93%) and isDDH (52 to 54%) values unambiguously indicate that the current classification of these strains into two species is appropriate.

Acquisition of the ThxA biosynthetic genes through recent horizontal gene transfer (HGT) events.

To infer the evolution history of ThxA biosynthetic genes, we constructed a phylogenetic tree of pathogenic isolates, using

Fig. 1. Virulence assays and thaxtomin A (ThxA) production of pathogenic *Streptomyces* isolates. **A**, Surface-sterilized radish seedlings (six per treatment) were inoculated with mycelia from tryptic soy broth-grown cultures of *Streptomyces* strains. Seedlings treated with *S. scabiei* 87-22, the nonpathogen *S. diastatochromogenes* ATCC 12309, or with water were used as controls. Plants were incubated at room temperature under a 12-h photoperiod for 4 days. Representative plants for each treatment are shown. **B**, Production of ThxA by plant-pathogenic *Streptomyces* isolates. Isolates were grown in triplicate in oat bran broth medium for 6 days at 28°C. The average thaxtomin production of *S. scabiei* 87-22 that is normalized using the dried cell weight is set to 100%. The average percent production for each strain relative to *S. scabiei* 87-22 and normalized using dried cell weights is shown. The error bars represent the standard deviation from the mean. Letters represent results of a one-way analysis of variance with Tukey's honestly significantly different (HSD) test; bars not sharing letters are significantly different at $P < 0.05$.

their *txtA*, *txtB*, *txtC*, *txtD*, *txtE*, *txtH*, and *txtR* genes (Supplementary Fig. S2). In the ThxA tree, five *S. acidiscabies* isolates (a10, 84-104, 85-06, 98-49, and FL01), two *S. scabiei* isolates (87-22 and NRRL B-16523), *S. niveiscabiei* NRRL B-24457, and *Streptomyces* sp. strain 96-12 were closely grouped, indicating the high similarity of their ThxA clusters. In contrast, *S. acidiscabies*, *S. niveiscabiei*, *Streptomyces* sp. strain 96-12, and *S. scabiei* were distantly related in the core-genome tree (Fig. 2). The incongruence (Fig. 3) between the core-genome phylogeny (Fig. 2) and the ThxA phylogeny supports the notion that a recent HGT of ThxA clusters occurred in these strains.

TR1 can excise from the chromosome of pathogenic *Streptomyces* strains lacking TR2.

The excision of TR regions from the chromosome of *S. scabiei* 87-22 was detected by nested polymerase chain reaction (PCR) assays. However, in strains lacking TR2, the TR1 element did not excise (Chapleau et al. 2016). In our analysis, we found that the regions flanking TR elements used for primer design in the study by Chapleau et al. (2016) were not conserved

in different pathogenic species. We hypothesized that this is the reason why they did not detect the excision of TR1 elements in strains lacking TR2. To re-analyze excision of TR1, we designed nested PCR primers to detect the circular form of TR1 and the corresponding repaired chromosomal junctions formed upon excision in *S. acidiscabies* 84-104, which contains only TR1. Excision of TR1 was observed in *S. acidiscabies* 84-104 (Fig. 4A and B), indicating TR1 can excise in strains lacking the TR2 region. Surprisingly, the sequence of the PCR products showed that the excision of TR1 in *S. acidiscabies* 84-104 did not occur at the previously identified *att* sites (TTGAAGCGGAAC) (Chapleau et al. 2016; Zhang et al. 2016) but via a different repeated sequence (TTTGCCAGATCAG) (Fig. 4C). This result demonstrates that the excision mechanism of *S. acidiscabies* 84-104 TR1 may be different from that of the *S. scabiei* TR region.

PAI variation exists within pathogenic *Streptomyces* species.

Understanding the evolution of *Streptomyces* PAIs is important to our understanding of the emergence of pathogenic

Fig. 2. Phylogeny of pathogenic *Streptomyces* species and their related nonpathogenic *Streptomyces* species, based on the concatenation of 1,104 single-copy gene clusters with the maximum likelihood algorithm RAXML.

Streptomyces species in agricultural systems. The best strategy we have to approach this goal is to identify and compare the PAIs from pathogens utilizing whole genome sequences (Zhang et al. 2016). In our previous study, we have shown that TR is the most common PAI in scab-causing *Streptomyces* species, except in *S. turgidiscabies* Car8 (Zhang et al. 2016). Here, we expand on previous analyses and include newly available genome sequences, including those reported here (Fig. 5).

We had previously reported that TR2, which is an ICE, only existed in *S. scabiei* and *Streptomyces* sp. strain 96-12 but not in other pathogenic species, such as *S. acidiscabies* and *S. europaeiscabies* (Zhang et al. 2016). In this study, we identified the complete TR element (TR1 and TR2) in the newly sequenced genome of *S. acidiscabies* a10 (Tomihama et al. 2016). However, *S. acidiscabies* 98-48 and the newly released sequence of *S. acidiscabies* NCPPB 4445 lack both TR1 and TR2; this is consistent with the lack of pathogenicity and thaxtomin production by *S. acidiscabies* 98-48 (Fig. 1). Therefore, within the *S. acidiscabies* species, three distinct compositions of the TR element exist: i) TR1 alone, ii) the entire composite TR (TR1+TR2), and iii) neither TR1 nor TR2 (Fig. 5). The genomes of *S. niveiscabies* isolates also vary in their TR composition. *S. niveiscabies* NRRL B-24457, isolated from South Korea, contains TR1, while *S. niveiscabies* ST1015 and *S. niveiscabies* ST1020, two strains recently isolated from pitted potato scab lesions in Uruguay (M. Julia-Pianzola *personal communication*), do not contain either TR1 or TR2.

Analysis of the genomes of two *S. stelliscabies* isolates (NRRL B-24447 and P3825) suggests that these two isolates contain both TR1 and TR2. In these *S. stelliscabies* assemblies, the TR1 regions were complete but the TR2 regions were separated into several contigs. For example, in *S. stelliscabies* P3825, TR1 and part of TR2 reside on a 111.4-kb contig (Genbank accession number JPPZ01000626.1), and the other part of TR2 resides on a 102.4-kb contig (Genbank accession number JPPZ01000624.1). Similar to *S. scabiei*, the TR2 region of *S. stelliscabies* P3825 was also inserted into the *att* site at the 3' end of *aviX1* gene. Chapleau et al. (2016) described the genes in TR2 that may be involved in conjugation and integration. *S. stelliscabies* P3825 possesses all of these genes, except the gene encoding the ATPase (SCAB_32961). Whether the lack of this ATPase gene affects the mobilization of

S. stelliscabies TR requires further investigation. In addition, comparison of the TR regions in *S. scabiei* and *S. stelliscabies* revealed significant genetic variation (data not shown), suggesting that the TR regions of *S. stelliscabies* and *S. scabiei* have evolved independently for some time.

S. turgidiscabies Car8 is an emergent pathogen first reported in Japan (Miyajima et al. 1998; Takeuchi et al. 1996). *S. turgidiscabies* Car8 does not contain the TR region but it contains a large mobile PAI of 674 kb (Huguet-Tapia et al. 2011; Kers et al. 2005). Recently, the genome of another Japanese isolate, *S. turgidiscabies* S58, was sequenced (Tomihama et al. 2016). Although the *S. turgidiscabies* S58 assembly is not complete, we identified several contigs of the *S. turgidiscabies* S58 assembly that share synteny with the 674-kb PAI of *S. turgidiscabies* Car8. These data indicate that the two *S. turgidiscabies* isolates appear to contain the same 674-kb PAI, which is

Fig. 4. Excision of the toxicogenic region (TR) in *Streptomyces acidiscabies* 84-104. **A**, Illustration of the excision of TR1 from the chromosome of *S. acidiscabies* 84-104. Arrows represent primers used to amplify the attachment site (*attP1*) formed upon the excision of a circular module of TR1 and repaired chromosomal junction (*attB1*). **B**, Nested polymerase chain reaction products of *attP1* and *attB1*. **C**, TR1 is excised from the chromosome of *S. acidiscabies* 84-104 by site-specific recombination between two 13-bp direct repeats (*attL1* and *attR1*).

Fig. 3. Comparison of evolutionary relationships of phytopathogenic *Streptomyces* species derived from the core-genome tree (left) and the thaxtomin A (ThxA) tree (right).

consistent with the previous conception of a high homologous PAI in *S. turgidiscabies* isolates from Hokkaido Island, Japan (Bukhalid et al. 1998; Miyajima et al. 1998).

Site-specific accretion between resident TR1 and incoming TR2.

Recently, we demonstrated that TR2 had a high integration specificity, as it was always integrated into the *att* site at the 3' end of *aviX1*, which encodes a putative ATP/GTP binding protein (Zhang et al. 2016). Since *S. scabiei* TR1 is not mobile by itself (Zhang et al. 2016), we hypothesized that *cis*-mobilization of *Streptomyces* TR1-TR2 is via site-specific accretion. To test this hypothesis, we used strains from previous studies in mating experiments: i) *S. acidiscabies* 84. 303. 1 (kanamycin resistance [*kan^R*]) (Healy et al. 2000), containing only TR1 with a deletion mutation in *txtA* and *txtB* genes (hereinafter referred to as *S. acidiscabies* Δ *txtAB*), and ii) the *S. diastatochromogenes* transconjugant strain (hygromycin B resistance [*hyg^R*]) that acquired TR2 from *S. scabiei* with a deletion mutation in lantibiotic biosynthesis genes *lanA* (SCAB_32021) and *lanB* (SCAB_32031) (hereinafter referred to as *S. diastatochromogenes* Δ *lanAB*) (Zhang et al. 2016). *S. diastatochromogenes* Δ *lanAB* (*hyg^R*) served as the putative TR2 donor, while *S. acidiscabies* Δ *txtAB* (*kan^R*) with the resident TR1 acted as the putative recipient strain (Figs. 6A and 7). Following selection with kanamycin and hygromycin B, we

obtained *S. acidiscabies* transconjugants containing both TR1 and TR2 (hereinafter referred to as *S. acidiscabies* Δ *txtAB* Δ *lanAB*). Using PCR primers a+b, c+d, e+f, and g+h (Supplementary Table S1; Supplementary Fig. S3), we found that TR2 was always inserted into the TR1 attachment site (*attR*) located at the 3' end of the *S. acidiscabies* *aviX1* gene rather than the attachment site (*attL*) on the other side of TR1 (Fig. 7). Furthermore, in all 15 *S. acidiscabies* Δ *txtAB* Δ *lanAB* transconjugants tested, the orientation and structure of TR1 and TR2 is the same as TR1 and TR2 in *S. scabiei* (Fig. 5). These data indicate that, although the resident TR1 has two attachment sites, the incoming TR2 preferably integrates into one attachment site (*attR*) (Fig. 7), leading to the tandem accretion of two elements.

Conjugative mobilization of resident TR1 in *cis* by incoming TR2.

We then investigated the conjugative transfer of the composite element of resident (*S. acidiscabiei*) TR1-incoming (*S. scabiei*) TR2 (Fig. 6B). *S. acidiscabies* Δ *txtAB* Δ *lanAB* served as the putative TR donor, while the *S. diastatochromogenes* transconjugant (thiostrepton resistance) from a previous study (Kers et al. 2005), containing the 674-kb PAI of *S. turgidiscabies* Car8 with a deletion mutation in *nec1* (hereinafter referred to as *S. diastatochromogenes* Δ *nec1*) acted as the putative recipient strain. Following selection with hygromycin B and thiostrepton,

Fig. 5. Schematic genetic organization of toxicogenic regions (TRs) from different pathogenic *Streptomyces* isolates. Arrowed boxes represent the location and orientation of the open reading frames of thaxtomin A biosynthetic genes, integrase and recombination directionality factor (RDF), lantibiotic biosynthetic genes, conjugative genes, and *aviX1*. The sequences of attachment (*att*) sites at the junctions for TR1, TR2, and TR are also shown.

we obtained three types of transconjugants: i) *S. diastatochromogenes* strains containing TR2 and the *S. turgidiscabies* PAI, resulting from the mobilization of TR2 from *S. acidiscabies* to *S. diastatochromogenes*, ii) *S. diastatochromogenes* strains containing TR1, TR2, and the *S. turgidiscabies* PAI, resulting from the mobilization of TR1 and TR2 together from *S. acidiscabies* to *S. diastatochromogenes*, and iii) *S. acidiscabies* strains containing TR1, TR2, and *S. turgidiscabies* PAI, resulting from the mobilization of *S. turgidiscabies* PAI from *S. diastatochromogenes* to *S. acidiscabies*. This mating experiment showed that, even though TR2 is absent in some pathogenic isolates, such as *S. acidiscabies* 84-104, these isolates can acquire TR2 from other isolates, leading to the site-specific accretion and *cis*-mobilization of resident TR1 and incoming TR2. In addition, these three types of transconjugants provide evidence for the exchange of the two currently known PAI types (*S. turgidiscabies* PAI and *S. scabiei* TR) between species.

TR1 is fixed in pathogenic strains lacking TR2.

We have demonstrated that *S. scabiei* TR1 is not mobile by itself and its transfer depends on TR2 (Zhang et al. 2016); however, comparative genomic analysis showed that many pathogenic isolates contain only TR1 but not TR2 (Fig. 5). We also observed the excision of *S. acidiscabies* TR1 in the absence of TR2 via repeat sequences different from *S. scabiei* (Fig. 4). It is possible that pathogenic strains that contain only TR1 use alternative mechanisms to disseminate TR1 to nonpathogenic species, which would be consistent with the observation that many pathogenic isolates only contain TR1. To test whether TR1 from those pathogenic strains that lack TR2 can be mobilized by alternative mechanisms, we carried out a third mating experiment, using *S. acidiscabies* $\Delta txtAB$ with *S. diastatochromogenes* $\Delta nec1$ (Fig. 6C). Following selection with thiostrepton and kanamycin and confirmation with primers a+b, k+l, and m+n, we only obtained *S. acidiscabies* transconjugants containing TR1 and *S. turgidiscabies* PAI, which result from the mobilization of *S. turgidiscabies* PAI from *S. diastatochromogenes* to *S. acidiscabies*. Failure to detect the mobilization of *S. acidiscabies* TR1 to *S. diastatochromogenes* suggests that, without TR2, TR1 cannot be transferred. It therefore appears that strains containing TR1 but not TR2 are unlikely to result from the excision of TR1.

DISCUSSION

The capacity of bacteria to modulate their genome structure affects adaptation to changing environmental conditions and,

therefore, impacts their evolution. The present study would appear to be the most comprehensive investigation of PAI diversity and evolution of pathogenicity in the genus *Streptomyces*, including 23 pathogenic isolates from nine known pathogenic species and a novel pathogenic species (*Streptomyces* sp. strain 96-12). We demonstrated that the entire TR region is a composite element that likely originated from the accretion of a putative ICE (TR2) and a CIME (TR1). Their site-specific accretion leads to the *cis*-mobilization and conjugative transfer of ThxA

Fig. 7. Illustration of the site-specific accretion and *cis*-mobilization of TR1 and TR2. *Streptomyces scabiei* TR2 was site-specifically integrated into the attachment site at the 3' end of the *S. acidiscabies* *aviX1* gene. The composite toxicogenic region (TR) element can excise in three distinct forms: TR1, TR2, and the entire TR. TR2 alone or the whole TR can integrate into the *S. diastatochromogenes* chromosome. The *cis*-mobilization of TR1 and TR2 can lead to the emergence of novel pathogenic species.

Fig. 6. Schematic representation of the mating experiments for investigation of the site-specific accretion and *cis*-mobilization of TR1 and TR2. **A**, The mating experiment between *Streptomyces acidiscabies* $\Delta txtAB$ and *S. diastatochromogenes* $\Delta lanAB$, used to investigate site-specific accretion between TR1 and TR2. **B**, The mating experiment between *S. acidiscabies* $\Delta txtAB \Delta lanAB$ and *S. diastatochromogenes* $\Delta nec1$ used to investigate the *cis*-mobilization of TR1 and TR2. **C**, The mating experiment between *S. diastatochromogenes* $\Delta nec1$ and *S. acidiscabies* $\Delta txtAB$ used to investigate whether TR1 can be transferred in strains lacking TR2. PAIst = pathogenicity island of *S. turgidiscabies* Car8.

biosynthetic genes and can be responsible for the emergence of novel *Streptomyces* pathogenic species in agricultural systems. Since the *Streptomyces* genus is ubiquitous in soils, our study suggests that the spread of ThxA biosynthesis genes will continue to result in the emergence of new pathogenic *Streptomyces* species.

Recent sequencing efforts have provided more than 30 genomes of pathogenic *Streptomyces* isolates (Labeda 2016; Tomihama et al. 2016; Zhang et al. 2016). The comparative genomic analysis presented here allows us to characterize the pathogenicity factors in different pathogenic isolates. A striking finding is that PAI variation exists not only among different species but also among strains from the same species (Fig. 5). Seven *S. acidiscabies* isolates carried three different PAI structures, namely, TR1 alone (84-104, 85-06, 98-49, and FL01), the entire TR element (a10), and no TR (98-48 and NCPPB 4445). Interestingly, PAI structures of the three Japanese *S. acidiscabies* isolates (98-48, 98-49, and a10) differed from each other. Similarly, three *S. niveiscabiei* carried two different PAI structures. The isolate from South Korea (NRRL B-24457) carried the TR1 alone, but two isolates from Uruguay (ST1015 and ST1020) do not contain the TR element. If we sequence additional *S. niveiscabiei* genomes, we are likely to detect some isolates that carry the third TR version (the entire TR element). The variability in the structure of the TR element in newly emerged pathogenic species, such as *S. acidiscabies* and *S. niveiscabiei*, likely results from the instability of the composite TR element. Excision of an ICE could cause the loss of this ICE from the host; if ICE excision happens before chromosome replication or cell division, this element probably would not be passed down to progeny (Wozniak and Waldor 2010). Previous studies have shown that genomic islands can be lost from the bacterial population in the absence of selective pressure (Bellanger et al. 2011; Lechner et al. 2009). However, this leads to an important question regarding the lack of TR diversity in *S. scabiei* in the more than ten geographically distributed *S. scabiei* isolates that have been sequenced. It is possible that TR encodes functions that provide a significant selective advantage for *S. scabiei*.

Previously, the *cis*-mobilization of two CIMEs by related ICEs has been reported, i.e., the transfer of CIME_{L3catR3} by ICE_{St3} in *Streptococcus thermophilus* (Bellanger et al. 2011) and the transfer of CIME_{Nem_tRNA^{Lys}} by ICE_{515_tRNA^{Lys}} in *Streptococcus agalactiae* (Puymège et al. 2013). Our results indicate the *cis*-mobilization of TR1 by TR2 qualifies as another example of this mechanism. Additionally, studies of other genomic islands suggest that CIMEs probably stem from ICEs by deletion of genes involved in conjugation and integration (Pavlovic et al. 2004). It is possible that, in *Streptomyces* TR1, the CIME harboring the ThxA biosynthetic cluster has evolved by a similar mechanism. One hypothesis is that an ICE integrated into the 3' end of *aviX1* could have lost its integration and conjugation genes (ICE1) while retaining two attachment sites (*attL* and *attR*), resulting in a CIME (TR1) (Fig. 8, step 1). A related ICE (ICE2/TR2) could then have been acquired by conjugation (Fig. 8, step 2) and integrated by site-specific recombination between its *attI* site and the *attR* site of TR1 (Fig. 8, step 3), resulting in the composite structure *attL*-CIME (TR1)-*attPi*-ICE2 (TR2)-*attR*-*aviX1*. The whole structure could then have been excised by recombination among three attachment sites in three different forms (Fig. 8, step 4), i.e., as TR1, as TR2, or as the entire TR element. Then, TR2 or the entire TR could have been integrated into the attachment site at the 3' end of the *aviX1* gene of new hosts (Fig. 8, step 5). Acquisition of the entire TR element confers the pathogenic phenotype on new hosts, leading to the emergence of novel pathogenic species (Zhang et al. 2016). Due to the instability of

the composite TR element, TR2 itself or the whole TR element may get lost and lead to diverse types of TR structures in the population of newly emerged pathogenic species (Fig. 8, step 6).

S. luridiscabiei, *S. puniscabiei*, and *S. niveiscabiei* were reported to cause potato common scab in South Korea (Park et al. 2003a). Among these three species, only *S. niveiscabiei* produced thaxtomins (Park et al. 2003a). Interestingly, *S. niveiscabiei* strains that were isolated from pitted scab lesions in Uruguay in 2010 do not produce thaxtomins but are pathogenic (M. Julia-Pianzola *personal communication*). Comparative genomic analysis showed the isDDH values between *S. niveiscabiei* isolates from South Korea and Uruguay were only 87%, suggesting that these isolates possibly are not derived from a common source. Indeed, although *S. niveiscabiei* has been discovered only in South Korea and Uruguay, this does not mean that other potato-growing regions are free of this species. Our core genome-based phylogenetic analysis revealed the close relationship between *S. niveiscabiei* and *S. acidiscabies*. Owing to the poor characterization of this less-studied species, *S. niveiscabiei* isolates

Fig. 8. Schematic representation of a hypothesis for the evolution of TR1 and TR2 by deletion, site-specific accretion, and *cis*-mobilization. An integrative and conjugative element (ICE) integrated into the 3' end of *aviX1* could have lost its integration and conjugation genes while retaining two attachment (*att*) sites (*attL* and *attR*), resulting in a *cis*-mobilizable element (CIME) (TR1) (step 1). In a subsequent event, a related ICE (TR2) could be acquired by conjugation (step 2) and site-specifically integrated between its *attI* site and the *attR* site of TR1 (step 3), resulting in the composite structure *attL*-CIME (TR1)-*attPi*-ICE2 (TR2)-*attR*-*aviX1*. The whole structure could be excised by recombination among three attachment sites in three different forms (step 4), i.e., as TR1, as TR2, or as the entire TR element. Then, TR2 or the entire TR could have been integrated into the attachment site at the 3' end of the *aviX1* gene of new recipient strains (step 5). Due to the instability of the composite TR structure, TR2 or the whole TR could get lost (step 6), leading to the diversity of the TR structure within bacterial populations.

could easily be misidentified as *S. acidiscabies*. *S. niveiscabiei* isolates from Uruguay lack the ThxA cluster, while isolates from South Korea harbor a ThxA cluster that is almost identical to the one in *S. scabiei* 87-22, despite large differences in their whole-genome sequences. These results suggest that, similar to *S. acidiscabies*, *S. niveiscabiei* is probably also an emergent pathogenic species and that the isolate from South Korea acquired the ThxA cluster via a recent HGT event.

Some *S. turgidiscabies* isolates from the potato fields of Finland infected by both *S. scabiei* and *S. turgidiscabies* were reported to contain a PAI with a structure similar to the TR element of *S. scabiei* 87-22 rather than to the PAI of *S. turgidiscabies* Car8, suggesting the exchange of PAI regions between two species (Aittamaa et al. 2010). Here, we demonstrated the exchange of the Car8-type PAI and TR-type PAI between *S. acidiscabies* and *S. diastatochromogenes* transconjugants. Such genetic exchange is critical to the evolution of pathogenicity of bacterial pathogens; the acquisition of new genes can enable pathogens to inhabit a new niche rapidly (Holden et al. 2009).

The mobility of the 674-kb PAI of *S. turgidiscabies* Car8 has been demonstrated (Kers et al. 2005), bringing up the possibility of the appearance of this large island in new pathogenic species. However, all newly identified pathogenic species contain the TR-type PAI or its derivative but not the *S. turgidiscabies* Car8-type PAI. It may be that the 674-kb Car8-type PAI cannot be stably transferred or maintained. This possibility is supported by an earlier report that considerable variability existed in the composition of PAI genes of *S. turgidiscabies* isolates in Finland (Aittamaa et al. 2010). Some isolates lack the *fas* operon, while other isolates lack all known virulence genes. However, in the present study, two Japanese *S. turgidiscabies* isolates (Car8 and T45) contained identical PAIs. These two isolates probably originated from the same source. Further genome sequencing projects should include additional *S. turgidiscabies* strains that contain diverse PAIs to investigate the evolution of mosaic regions of the Car8-type PAI.

Until recently, all *Streptomyces* species causing potato scab disease were thought to produce thaxtomins. Therefore, PCR primers that can amplify ThxA genes have been proposed for the detection of common scab pathogens (Flores-González et al. 2008; Lerat et al. 2009). However, a growing body of evidence suggests that other secondary metabolites may be pathogenicity factors. A *Streptomyces* isolate was reported to cause pitted scab lesions by producing an 18-membered macrolide named borrelidin (Cao et al. 2012). Recently, Fyans et al. (2016) isolated a *Streptomyces* strain from Newfoundland, Canada that has a severe pathogenic phenotype on several plant hosts. Symptoms were associated with an unidentified phytotoxin. *S. reticuliscabiei*, *S. luridiscabiei*, and *S. puniscabiei* do not produce thaxtomins, but they are pathogenic species (Aittamaa et al. 2010; Park et al. 2003a). *S. reticuliscabiei* induces netted scab lesions on potato tubers (Aittamaa et al. 2010), while the latter two species cause typical potato scab lesions (Park et al. 2003a). Furthermore, two Uruguay *S. niveiscabiei* isolates (ST1015 and ST1020) that lack thaxtomin production cause deep-pitted lesions on potato tubers (M. Julia-Pianzzola *personal communication*). Taken together, these results suggest a new paradigm for pathogenicity in *Streptomyces*. The secondary metabolite ThxA does appear to be the most common pathogenicity factor in *Streptomyces* species, but other secondary metabolites may serve this role in some plant-pathogenic strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Streptomyces strains were cultured at 28°C on International *Streptomyces* Project medium 4 (ISP4) agar medium or in tryptic soy broth (TSB) (BD Biosciences). All liquid cultures were

shaken at 250 rpm. Genomic DNA was extracted from TSB-grown cultures using the MasterPure gram-positive DNA purification kit (Epicentre), based on the manufacturer's instructions.

Virulence bioassays.

To assess the virulence phenotype of different strains, an in vitro radish seedling bioassay was performed as described previously (Bignell et al. 2010b), except that 'German Giant' seeds (Burpee) were used in place of the 'Burpee White' variety. For each strain tested, mycelial suspensions were used to inoculate six newly germinated radish seedlings of similar size. The assay was performed three times, each of these experiments included three biological replicates.

Analysis of ThxA production.

Plant-pathogenic *Streptomyces* strains were cultured in TSB for 48 to 72 h. Mycelia were then pelleted by centrifugation, were washed twice with sterile water, and were resuspended in sterile water, to obtain an optical density at 600 nm of 1.0. The mycelial suspension solution (400 µl) was then inoculated into 3 × 50 ml of OBB medium (Johnson et al. 2007) in 250-ml flasks. After incubation for 6 days at 28°C with shaking (250 rpm), ThxA was purified from the culture supernatants and was analyzed by high-pressure liquid chromatography, as described previously (Johnson et al. 2007). All experiments were repeated at least three times using different biological replicates of the *Streptomyces* strains, with three technical repeats per strain. The pellets of the culture samples were dried and weighed as a measure of bacterial growth. The data for *S. scabiei* 87-22 was normalized to 100%. The data for all pathogenic isolates were square-root transformed to improve homogeneity of variance. Transformed data were subjected to one-way analysis of variance using SAS 9.4 (SAS Institute) and means were compared based on Tukey's honestly significantly different test at $P < 0.05$.

Genome sequencing and assembly.

We sequenced the genomes of *S. acidiscabies* isolates using the Illumina-HiSeq 2500 paired-end technology. The SPAdes version 3.5.0 assembly algorithm (Bankevich et al. 2012) was used to perform genome assemblies. Prokka version 1.12-beta was used for gene-finding and annotation (Seemann 2014).

Ortholog retrieval and single-copy cluster search.

Streptacidiphilus albus JL83 and *Kitasatospora setae* NBRC 14216 were selected as outgroups to build the rooted phylogenetic tree. Protein sequences from 25 *Streptomyces* genomes and two outgroups were clustered into ortholog groups using OrthoMCL v1.4 (Li et al. 2003). OrthoMCL uses BLASTP similarity for clustering and was run using the default settings. Ortholog groups with a single copy per genome were selected as potential phylogenetic markers.

Gene alignment and filtering criteria.

To minimize the inclusion of alignments of low quality in the analysis, various filtering criteria were used as described previously (Zhang et al. 2016).

Maximum likelihood phylogeny estimation.

The gene alignment of nucleic acid sequences was used for maximum likelihood inference on the RAXML webserver (Stamatakis et al. 2008) with the GTR+G substitution model, including an estimation of invariable sites. For inferring tree robustness, 1,000 bootstrap replicates were computed.

ANI and isDDH estimation.

The ANI analyses of whole-genome data were performed using the method proposed by Richter and Rosselló-Móra

(2009). Pair-wise ANI values were obtained from nucmer (Delcher et al. 2002) with the script calculate_an.py.

The isDDH was estimated using the genome-to-genome distance calculator (GGDC) (Auch et al. 2010b; Meier-Kolthoff et al. 2013). The contig files were uploaded to the GGDC 2.0 webserver, where isDDH calculations were performed using Formula 2 alone. We used the point estimates plus the 95% model-based confidence intervals as the isDDH estimates.

Selection of transconjugant strains.

Streptomyces strains were first grown on ISP4 plates, and spores were harvested after 5 to 7 days. Conjugal mating was carried out by mixing spores of donor and recipient strains and coculturing at 28°C on soya flour mannitol (SFM) plates (Kieser et al. 2000). After 48 h, 5 ml of soft nutrient agar containing antibiotics was spread onto the plates to select transconjugants. Final concentrations of antibiotics were (per milliliter) 100 µg of hygromycin B, 50 µg of kanamycin, or 15 µg of thiostrepton, singly or in combination. Qualitative PCR assays were used to confirm the transfer and integration of TR elements and PAI of *S. turgidiscabies* Car8.

Excision assay.

S. acidiscabies 84-104 was first streaked on ISP4 agar medium and was incubated at 28°C for 6 days. Three single colonies were picked and were transferred into TSB (BD Biosciences). All liquid cultures were shaken at 250 rpm for 4 days. Genomic DNA was extracted from TSB-grown cultures using the MasterPure gram-positive DNA purification kit (Epicentre), based on the manufacturer's instructions. Qualitative nested PCR assayed were performed with 50 ng genomic DNA, using primers TNi2 + TNi3 and TNi1 + TNi4 (Chapleau et al. 2016) for *attP1* and primers AcidF1 + AcidR1 and AcidF2 + AcidR2 for *attB1*. Each PCR reaction (25 µl) was performed using 2× Apex Red Taq DNA polymerase master mix (Genesee Scientific) and primers at a final concentration of 0.4 µM. DNA amplification was performed for 4 min at 98°C, followed by 30 cycles of 30 s at 98°C, 30 s at 57°C, and 1 min at 72°C, with a post-PCR extension at 72°C for 7 min and cooling to 4°C. In the nested PCR, 0.5 µl of PCR product from the first-step PCR was used as the template for the second-step PCR. All PCR products were also sequenced to confirm the excision.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Genome-to-Genome Distance Calculator (GGDC) 2.0 webserver:
<http://ggdc.dsmz.de/distcalc2.php>
- CIPRES Science Gateway RAxML webserver:
<https://www.phylo.org/portal2/login!input.action>
- Widdowsript calculate_ani.py:
https://github.com/widdowquinn/scripts/blob/master/bioinformatics/calculate_ani.py