Streptomyces turgidiscabies Car8 contains a modular pathogenicity island that shares virulence genes with other actinobacterial plant pathogens

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ABSTRACT

Streptomyces turgidiscabies Car8 is an actinobacterium that causes the economically important disease potato scab. Pathogenesis in this species is associated with a mobile pathogenicity island (PAISt) that site specifically inserts into the bacA gene in Streptomyces spp. Here we provide the 674,223 bp sequence of PAISt, which consists of two non-overlapping modules of 105,364 and 568,859 bp. These modules are delimited by three copies of an 8 bp palindromic sequence (TTCATGAA), that also is the integration site (att) of the element. Putative tyrosine recombinase (IntSt) and excisionase (XisSt) proteins are encoded just upstream of att-R. PAISt has regions of synteny to pathogenic, symbiotic and saprophytic actinomycetes. The 105,364 bp PAISt module is identical to a genomic island in Streptomyces scabies 87–22, while the 568,859 bp module contains only a short region of synteny to that genome. However, both modules contain previously characterized and candidate virulence genes.

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1. Introduction

Acquisition of accessory genes via horizontal gene transfer (HGT) is one of the driving forces in bacterial evolution. Genes are often mobilized in syntenic blocks termed genomic islands (GEIs). GEIs are distinct regions of DNA that have, or once had, functional mobility genes. This category of elements includes many types of mobile elements: integrative and conjugative elements (ICEs), conjugative transposons, and some prophages (reviewed in Juhas et al. (2009)). The term GEI refers to relatively large regions (>20 kb) that differ among microbial strains and share features derived from their evolutionary history, including variation in nucleotide bias, insertion in tRNA genes, flanking direct repeats, and functional or cryptic mobility genes. Importantly, GEIs contribute to genome plasticity and strain differentiation by providing new functions such as antibiotic production, antibiotic resistance, catabolic activities, or virulence to the recipient strain. The role of HGT in the evolution of plant and animal virulence in microbes has been known for some time and GEIs that confer virulence are known as pathogenicity islands (PAIs). Though the concept of PAIs was introduced more than 10 years ago (Groisman and Ochman, 1996), examples of PAIs in plant pathogens are almost entirely limited to Gram-negative bacteria.

The first example of a PAI in a Gram-positive plant pathogen was from the root and tuber pathogen Streptomyces turgidiscabies (Kers et al., 2005), PAISt. This emergent pathogen was first discovered on the island of Hokkaido, Japan,

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where it causes disease in commercial potato fields (Miyajima et al., 1998; Takeuchi et al., 1996). DNA sequence of a small part of PAISt revealed that it carries genes encoding at least four virulence factors: the biosynthetic pathway for the phytotoxin thaxtomin (Txt proteins), a secreted saponinase (TomA), a secreted necrogenic protein (Nec1), and a cytokinin biosynthetic pathway (Fas proteins) (Kers et al., 2005). Surprisingly, this study also demonstrated that PAISt mobilizes from *S. turgidiscabies* and integrates into the chromosome of nonpathogenic *Streptomyces* spp. including *S. coelicolor*, *S. diastochromogenes* and *S. lividans.* In the case of *S. diastatochromogenes,* acquisition of PAISt was sufficient to confer a pathogenic phenotype on the organism. Integration of PAISt occurs at the palindromic sequence TTCATGAA located within the 3′ end of the bacitracin resistance gene (*bacA*) in *S. turgidiscabies* Car8 and all recipient strains (Kers et al., 2005).

Streptomycete PAISt transconjugants typically contain the approximately 660 kb element, or a truncated version of approximately 100 kb (Kers et al., 2005). These data suggest that PAISt is a very large GEI that has the ability to mobilize and site specifically insert during conjugation, meeting the criterion of an ICE. However, two properties of PAISt mobilization are unusual relative to described ICEs: these elements typically insert into the 3′ end of tRNA encoding genes and do not mobilize in a modular fashion (Burrus et al., 2002). Here we provide the complete sequence of PAISt and an analysis of gene content relative to the pathogenic phenotype and mobility.

2. Materials and methods

2.1. Annotation of the PAISt

A pseudomolecule representing the chromosome of *S. turgidiscabies* Car8 was built from the scaffolds of the *S. turgidiscabies* Car8 genome-sequencing project (GenBank Accession No.: AEJB00000000). MUMmer 3.0 software (Kurtz et al., 2004) was used to order the scaffolds using the finished sequence of the *S. scabies* 87–22 chromosome as a reference (GenBank Accession No.: FN554889). Previously published sequence data from the PAISt (Kers et al., 2005) was used to delimit the boundaries of the PAISt in the pseudomolecule. The landmark sequences included in the analysis were: *nec1*, the *txt* genes, and the 3′ end of *bacA* (Genbank Accession Nos.: AY707080, AY707081 and AY707082, respectively). The 674,223 bp PAI sequence was initially fragmented into 18 contigs with numerous low coverage regions. The relevant sequence was closed to a single, high quality finished contig using 149 Sanger clone primer walks and resulted in a 717,473 bp contig, which included the closed PAI sequence.

Coding sequences in the PAISt were predicted using GLIMMER 3.2 (Delcher et al., 1999) trained with *S. scabies* 87–22 genes. Predicted genes were automatically annotated using Blast2GO (Conesa and Gotz, 2008). Manual annotation was performed using the Artemis genome viewer (Rutherford et al., 2000), and the Gene Ontology (2006) and Pfam (Bateman et al., 2004) databases.

2.2. Comparative sequence analysis

Predicted coding sequences were compared at the amino acid level with the genomes of selected actinobacteria and plant pathogenic bacteria (Supplementary Table S1). Homologs were identified with BLAST (Altschul et al., 1990), using the *e*-value of 0.0001 as a threshold for significance, and identity scores were used to build a matrix of best hits. This matrix was used to generate a color-coded BLAST-fingerprinting plot with the *R* statistical package (http://www.r-project.org/). Additionally, sequence comparisons and analysis of gene synteny were carried out using the Artemis Comparison Tool (Carver et al., 2005).

3. Results and discussion

3.1. Modular nature of the PAI

The complete DNA sequence of PAISt was extracted from a draft genome sequence of *S. turgidiscabies* Car8 (GenBank Accession No.: AEJB00000000). The sequence data revealed that PAISt is an integrated genetic element of 674,223 bp containing two non-overlapping modules of 105,364 and 568,859 bp (Fig. 1, Supplementary Data S2). These data agree with the experimentally estimated sizes (660 and 100 kb) of the versions of PAISt transferred from *S. turgidiscabies* Car8 to *S. coelicolor* (Kers et al., 2005). Three copies of the 8 bp palindrome sequence TTCATGAA delimited the two modules of PAISt (Fig. 1); this sequence is also the experimentally demonstrated genomic integration site (*att*-site) of the element (Kers et al., 2005). *att*-L was located within the 3′ region of *bacA* (1433,070 bp on the *S. turgidiscabies* Car8 pseudomolecule), while *att*-I (1538,434 bp) and *att*-R (2107,293 bp) were each embedded in a copy of the 3′ end of *bacA,* suggesting a conserved recombination event. The average G + C content of PAISt was 68%, which is somewhat lower than that typical of streptomycete genomes (72%). PAISt is predicted to contain 647 coding sequences (CDS) (Supplementary Data S2) with an average length of 883 bp.

3.2. Tyrosine integrase and excisionase candidates

Previous studies demonstrated that PAISt transfers from *S. turgidiscabies* Car8 by conjugation to other *Streptomyces* spp. and integrates through a site-specific recombination mechanism (Kers et al., 2005), consistent with the hypothesis that PAISt is an ICE. Typically, an ICE encodes a tyrosine-recombinase adjacent to an *att* site (Burrus and Waldor, 2004; Ramsay et al., 2006; Scott and Churchward, 1995). The CDS stPAI0647 lies just upstream of *att*-R (Fig. 1, Supplementary Data S2). PSI-BLAST (three iterations) of the 466 amino acid sequence against the non-redundant database in Genbank indicated that stPAI0647 is 18% identical to a tyrosine recombinase found in the firmicute *Allicyclobacillus acidocaldarius* (GenBank Accession No.: ZP_03494792). There are five additional putative integrases on PAISt (stPAI0447c, stPAI0567, stPAI0570, stPAI0575, and stPAI0632) but none are located near *att* sites (Supplementary Data S2). Interestingly, a copy of the *att*
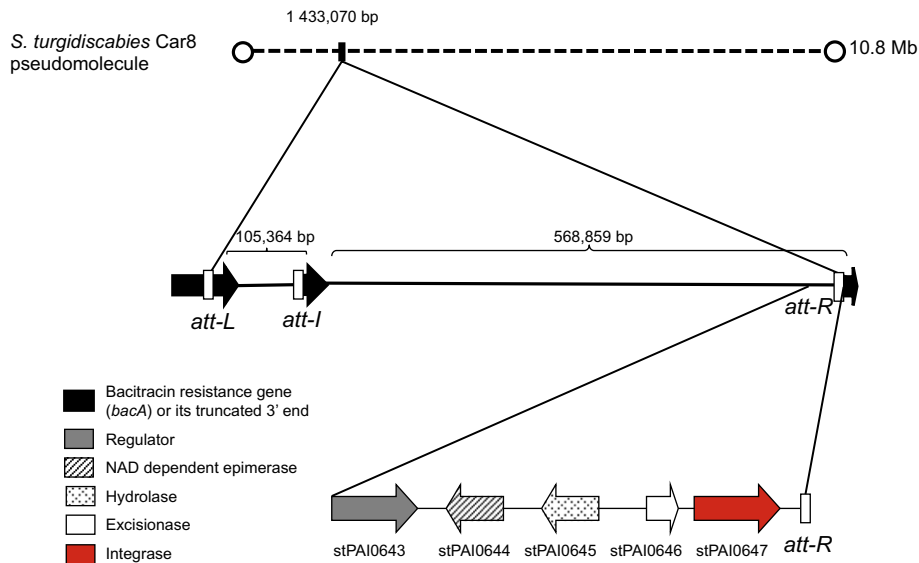
Fig. 1. The characteristics of PAISt and its location in the pseudomolecule of *S. turgidiscabies* Car8. PAISt is organized as a complex ICE within the chromosome of *S. turgidiscabies* Car8 (top portion of the figure). The 8 bp integration (*att*) sites are imbedded within the duplication of the 3′ end of *bacA* (black arrows in the middle portion). A putative integrase (stPAI0674; IntSt) and a putative excisionase (stPAI0646; XisSt) are encoded adjacent to *att*-R (bottom portion). Upstream from IntSt and XisSt is a gene encoding a putative transcriptional regulator (stPAI0643) that could be involved in regulating excision and integration.

site is imbedded in the C-terminal region of stPAI0570; however, there is no evidence of recombination (duplication of the 3′ end of the bacitracin resistance gene). These data suggest, but do not prove, that stPAI0647 (IntSt) serves to site specifically integrate PAISt into the *att* site in the *bacA* gene. It is worth noting that tyrosine recombinases are responsible for integration and excision of a wide array of integrative elements and usually, but not exclusively, catalyze integration into the 3′ end of tRNA genes (9).

ICE integrases often function in conjunction with excisionase proteins (Xis) which share little homology at the amino acid level but are typically small proteins with basic isoelectric points (Lee et al., 2007). Adjacent to IntSt there is an ORF (stPAI0646) that encodes a putative 118 amino acid protein with a basic isoelectric point (13.8 kDa; p*I* 11.76). Based on the location, size and p*I* of the protein encoded by stPAI0646, it is reasonable to suggest that this gene encodes a protein (XisSt) that functions in the excision of PAISt (Supplementary Data S2).

Bioinformatic data presented here, in combination with experimental data presented previously (Kers et al., 2005), suggest that IntSt and XisSt are responsible for the excision and integration of the entire PAISt (674,223 bp) and the smaller module (105,364 bp). However, it may be that the whole element is transferred and only the smaller module is stably integrated, in some cases. Based on the organization of PAISt, it is expected that the 568,859 bp module (Fig. 1) also should be integrated into recipient genomes. However, in experiments demonstrating integration, the selectable marker was located within the 105,364 bp module (at *nec1*), therefore transfer of the 568,859 bp module would not have been detected (Kers et al., 2005). Regardless, the possibility that IntSt and XisSt might be able to excise and integrate multiple modules of PAISt is noteworthy and worthy of experimental follow up.

3.3. Characteristics of the 105,364 bp PAI module

Interestingly, the entire 105,364 bp PAISt module is identical to a nonmobile GEI (PAISs1) located in the chromosome of the plant pathogen *S. scabies* 87–22 (Figs. 2 and 3). As in *S. turgidiscabies* Car8, PAISs1 is integrated into the 3′ end of the *bacA* gene (SCAB77601) in *S. scabies* 87–22. Importantly, the *att* site at the 3′ end of the *S. scabies* 87–22 PAISs1 is mutated from TTCATGAA to TGTATGAA and contains a degenerated version of IntSt (SCAB76721; Genbank accession YP_003493179). It is possible that the mutation in the *att* site of PAISs1 and the gene erosion of the integrase cause the fixation of this GEI in *S. scabies* 87–22.

The CDSs stPAI001 – stPAI0081 constitute the 105,364 bp module encoded in *S. turgidiscabies* Car8 (Supplementary Data S2). Most of the highest scoring BLAST hits are from the genus *Streptomyces,* but a number of other actinomycetes are represented. The large number of putative secreted hydrolytic proteins, particularly glycoside hydrolases, in this module is noteworthy and consistent with the degradation of plant tissue caused by plant pathogenic streptomycetes (Loria et al., 2006). Predicted hydrolase genes were interspersed with putative ABC transporter genes, some of which are predicted to encode sugar transporters. Many regulators, including LacI, IclR, TetR, MarR, XRE, PadR, and two-component families, are also encoded in this region. There are a total of five LacI and IclR family regulators in the 105,364 module (Supplementary Data S2), consistent with their role in regulating carbohydrate metabolism (Nguyen and Saier, 1995).

Encoded in this region is TomA, a putative secreted virulence protein that has been characterized as a tomatinase in *S. scabies* (Seipke and Loria, 2008) and is known to be
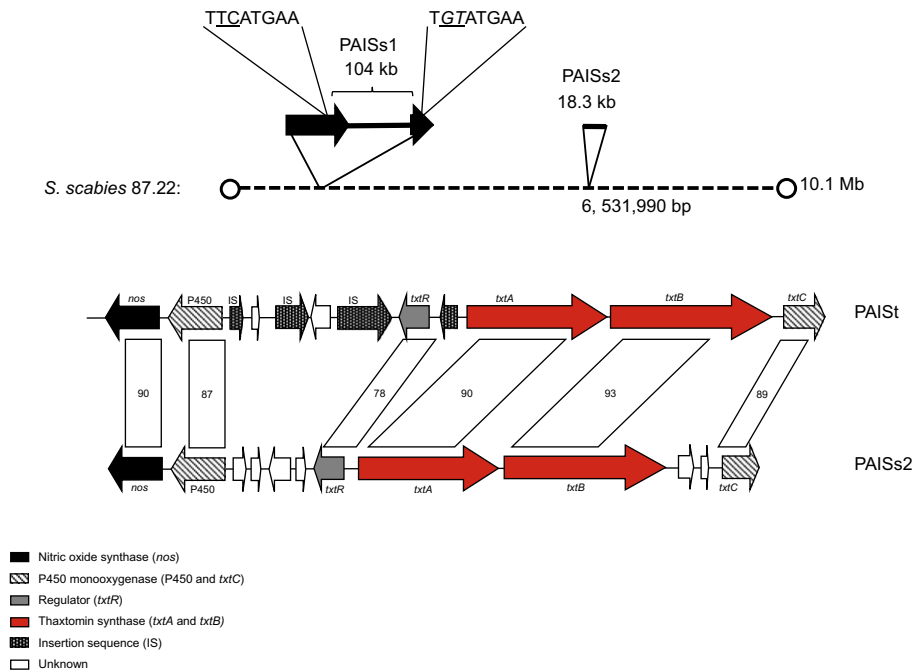
Fig. 2. The GEIs in *S. scabies* 87–22 and syntenic regions in the *S. turgidiscabies* PAISt and *S. scabies* PAISs2. The *S. scabies* 87–22 chromosome (top portion of the figure) contains the GEIs PAISs1 (104 kb) and PAISs2 (18.3 kb). The 3′ end of PAISs1 has a degenerate integration site (underlined). The thaxtomin biosynthesis genes are encoded in syntenic regions in the *S. turgidiscabies* Car8 PAISt and *S. scabies* 87–22 PAISs2 (bottom portion). White bars indicate homology and percentage identity at the amino acid level. The predicted functions of genes in the depicted regions are provided in the legend.

conserved in the pathogen *S. acidiscabies* (Loria, 2006). This protein also has a homolog in the actinobacterial plant pathogen *Clavibacter michiganensis* subsp. *michiganensis* (CMM) (Gartemann et al., 2008), but is better known as a virulence factor in plant pathogenic fungi (Ito et al., 2004; Martin-Hernandez et al., 2000). Interestingly, TomA is embedded in a region (stPAI0021–stPAI0035) in which nine out of 15 CDSs encode proteins with best hits to CMM (Supplementary Data S2). The CDSs stPAI0028, and stPAI0031–stPAI0035 are syntenous in the two genomes and are predicted to encode two beta-glycosidases, an ABC sugar transporter, and a TetR family regulator. These data suggest the hypothesis that CMM and plant pathogenic streptomycetes share a virulence mechanism involving carbohydrate metabolism.

The secreted protein Nec1 is another characterized virulence protein that is encoded on the 105,364 bp module (Supplementary Data S2) (Bukhalid and Loria, 1997; Kers et al., 2005). Nec1 lacks homologs in the public databases outside plant pathogenic streptomycetes and has no characterized motifs, yet has a strong virulence phenotype on inoculated plant roots (Joshi et al., 2007a). The DNA sequence of *nec1* is almost identical in diverse plant pathogenic streptomycetes, but has a particularly low G + C content, consistent with very recent horizontal transfer among pathogens (Bukhalid et al., 2002). A previous study used Southern analysis to demonstrate conservation of the *nec*1 gene and a 26 kb flanking region across plant pathogenic streptomycetes in the diastatochromogenes cluster (Bukhalid et al., 2002); these data are consistent with the sequence analysis provided in this study.

3.4. Characteristics of the 568,859 PAI module

The CDSs stPAI0082–stPAI0647 constitute the 568,859 bp module in *S. turgidiscabies* Car8 (Fig. 1, Supplementary Data S2). Overall, this module is characterized by a preponderance of hypothetical proteins, which is not unusual for ICEs. However, a region of synteny to the *S. scabies* 87–22 genome also exists in this module (Fig. 2, Fig. 3). This syntenic region is a GEI in *S. scabies* 87–22 (PAISs2) that encodes six characterized thaxtomin A biosynthetic and regulatory genes (Healy et al., 2002; Healy et al., 2000; Johnson et al., 2009; Joshi et al., 2007b). A region of synteny to the *Rhodococcus fascians* D188 genome lies adjacent to the thaxtomin biosynthetic pathway in *S. turgidiscabies* and is composed of the previously described *fas* operon (Supplementary Data S2, *fas1–5*); these virulence genes are not present in *S. scabies* 87–22 (Joshi and Loria, 2007).

The thaxtomin and *fas* biosynthetic pathways are the only characterized virulence genes on the larger PAI module. However, the 568,859 bp PAISt module contains a number of gene clusters with synteny to other actinomycetes (Fig. 3). An interesting cluster of five genes (stPAI0309–stPAI0313) encodes proteins syntenic to the plant symbiotic actinobacterium *Frankia* sp. CcI13 that may constitute a metabolic pathway (Supplementary Data S2). Another interesting region in the PAISt is a putative lantibiotic biosynthetic pathway (stPAI0324, stPAI0325, stPAI0326 and stPAI0327). This region is syntenic with a predicted biosynthetic operon in the saprophytic streptomycete *S. griseus* (Supplementary Data S2). Since
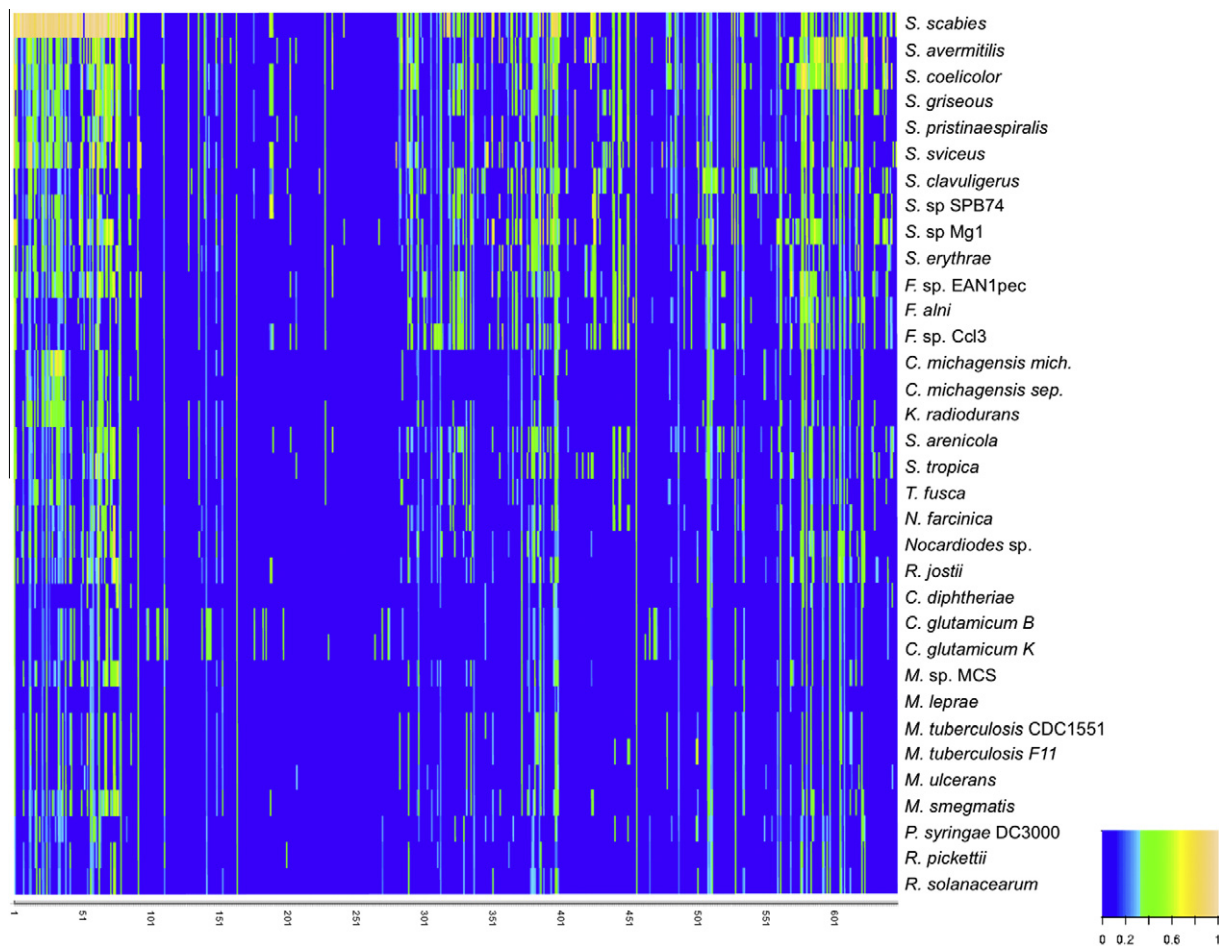
Fig. 3. BlastP-fingerprinting plot of the PAISt. Coding sequences of PAISt are denoted by the horizontal line at the bottom of the plot (0–675 kb). The predicted proteome of PAISt was compared to predicted proteomes of selected bacteria (primarily actinobacteria) listed on the right side of the plot. See Table S1 for complete names and genome accession numbers. Percentage identity is indicated by colors shown in the legend at the bottom of the figure.

lantibiotics are active against other Gram-positive bacteria (McAuliffe et al., 2001; Willey and van der Donk, 2007), this pathway might provide an advantage to *S. turgidiscabies* Car8 in rhizosphere colonization or in competing for nutrients in necrotized plant tissue. Discrete regions at the 3′ end of PAISt displayed high identity with the saprophytes *S. avermitilis* and *S. coelicolor* (Fig. 3). Alignment of PAISt with the *S. avermitilis* and *S. coelicolor* chromosomes revealed syntenic clusters of genes coding for regulators and transporter systems (Fig. 4, Supplementary Data S2). Interestingly, these regions are located on the arms of both the *S. avermitilis* and *S. coelicolor* chromosomes, suggesting that their functions are niche specific (Bentley et al., 2002).

3.5. Comparison with actinomycete ICEs

Previously described actinomycete ICEs share homologs that function in excision and integration, replication, conjugative transfer, and regulation, as well as a structural organization (te Poele et al., 2008a,b). Actinomycete ICEs have the ability to excise from the chromosome in donor cells, replicate autonomously like a plasmid, transfer to recipient cells as double-stranded DNA and, finally, integrate into the recipient's chromosome (te Poele et al., 2008a,b). In PAISt, IntSt, XisSt and a putative transcriptional regulatory gene (stPAI0643) cluster at an *att* site (Fig. 1), which is consistent with the organization of other actinomycete ICEs (te Poele et al., 2008a,b). However, conjugation, replication and regulatory proteins typically associated with actinomycete ICEs and encoded near the *int* and *xis* genes, are lacking in PAISt (Supplementary Data S2). There are 11 genes clustered within the region stPAI106–stPAI185 that are syntenic to genes on an integrated plasmid in the actinobacterium *Corynebacterium glutamicum* ATCC 13032 (Supplementary Fig. S3), which is a species used to produce amino acids in large-scale fermentation (Hermann, 2003). This region of synteny displays a peculiar distribution of several genes that encode putative DNA-processing proteins that could participate in mobilization or replication of PAISt (Supplementary Data S2). However, none of these proteins are homologous with the main transfer protein (TraB) of actinomycete ICE elements and *Streptomyces* plasmids. TraB proteins are similar to the FtsK-SpoIIIE family of septal DNA
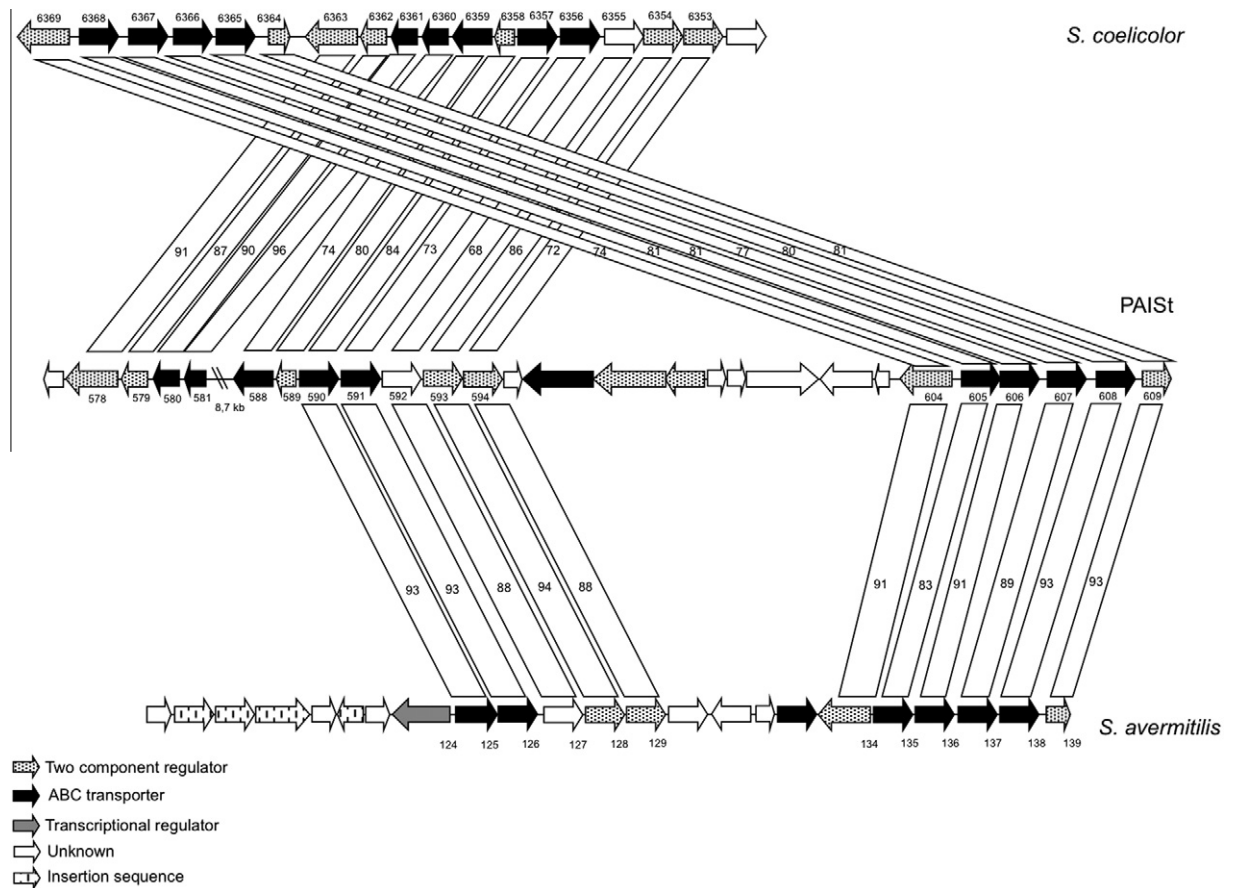
Fig. 4. Synteny of PAISt with multiple two-component response regulators and ABC transporters genes in *S. coelicolor* and *S. avermitilis*. White bars indicate homology and percentage identity at the amino acid level. The predicted functions of genes are provided in the legend.

translocases and are responsible for transferring actinobacterial plasmids intercellularly in the novel double-stranded form mentioned earlier (te Poele et al., 2008a). With the caveat that conjugation functions may yet be identified on PAISt, the results here raise the possibility that PAISt transmission may instead occur via mobilization by an additional undetermined conjugal element present in *S. turgidiscabies*. If PAISt is in fact found to be self-transmissible, the lack of TraB homologs would suggest that the mechanism of PAISt conjugal transfer will differ significantly from that of other actinomycete ICEs.

3.6. Possible recombination events

DNA sequence comparisons suggest at least two possible scenarios for recombination events that led to the formation of the PAIs in *S. turgidiscabies* and *S. scabies* (Fig. 1). It may be that the PAISt was originally an ICE in *S. scabies* that progressively acquired novel DNA. The entire island could have been transferred directly from *S. scabies*, or via other donors, to *S. turgidiscabies*. In *S. scabies,* gene erosion and recombination might have resulted in the loss of the recombinase, conjugation genes, and the *fas* operon, as well as the degeneration of the integration sites. Subsequently, the PAI might have undergone recombination

events that separated the island into two modules, PAISs1 (containing *nec1* and *tomA*) and the PAISs2 (containing the thaxtomin biosynthetic cluster), fixing this element in the bacterium. Furthermore, the functional integrase on PAISt may have served in the acquisition of DNA into the element (Biskri et al., 2005; Fonseca et al., 2008).

Another possible scenario is that *S. turgidiscabies* acquired a small version of the island from *S. scabies* (105,364 bp PAI module). The thaxtomin biosynthetic cluster and *fas* operon could have been acquired subsequently during independent recombination events, forming genomic "islets" within the PAISt. Transposons and ISs in the vicinity of the thaxtomin genes and *fas* operon suggest that these regions might have been acquired through transposition. Of course, neither of these scenarios addresses the intra species genomic variability in these species, a subject of future investigations.

4. Conclusions

To the best of our knowledge, PAISt is the largest mobile GEI described to date. It is very likely that PAISt has played a pivotal role in the evolution of *S. turgidiscabies*. In fact, it is tempting to speculate that acquisition of PAISt provided the genetic potential for *S. turgidiscabies* to develop a

pathogenic relationship with plants. This PAI contains most of the characterized virulence genes in plant pathogenic streptomycetes (Loria et al., 2006). Thaxtomin A, a potent cellulose biosynthesis inhibitor, is required for virulence in scab-causing streptomycetes and likely functions to facilitate inter and intracellular penetration of the plant host by this filamentous pathogen. Interestingly, thaxtomins are only produced by plant pathogenic streptomycetes. The *fas* genes on PAISt are homologous to the *fas* operon in the actinobacterial plant pathogen *R. fascians* and encode biosynthesis of cytokinins that have a virulence phenotype in both pathogens (Joshi and Loria, 2007). The secreted protein Nec1 has a dramatic virulence phenotype in root infection and is highly conserved among plant pathogenic streptomycetes. The secreted protein TomA, also highly conserved among plant pathogenic streptomycetes, has homologs in the actinobacterial plant pathogen CMM (Seipke and Loria, 2008). The PAISt lacks the biosynthetic pathway for a coronafacic acid-like biosynthetic cluster that contributes to virulence in *S. scabies* 87–22 (Bignell et al., 2010). Furthermore, there are many excellent virulence gene candidates on PAISt.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plasmid.2010.11.002.

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