Application of signature-tagged mutagenesis to the study of virulence of *Erwinia amylovora*

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**Abstract**

To identify genes that contribute to the virulence of *Erwinia amylovora* in plants, 1892 mutants were created and screened in pools of ≤ 96 mutants using signature-tagged mutagenesis. Nineteen mutants were not recovered from apple shoots following inoculation, which suggested that the insertions in these mutants affected genes important for bacterial survival *in planta*. DNA flanking the Tn5 insertions in the 19 mutants was sequenced and analyzed by BLAST. One mutant had a Tn5 insertion in *amsE*, a gene involved in the biosynthesis of exopolysaccharide (EPS). Fourteen mutants had insertions in loci that were implicated in biosynthesis or transport of particular amino acids or nucleotides, a site-specific recombinase active during cell division and several putative proteins of unknown function; the flanking DNA of the remaining four mutants lacked significant homology with any DNA in the database. When inoculated individually to hosts, 10 of the 19 mutants caused significantly less disease and multiplied less, as compared with the wild-type strain.

**Introduction**

*Erwinia amylovora*, a member of the *Enterobacteriaceae*, causes fire blight on pear, apple and other plants of the *Rosaceae* (Eastgate, 2000). The bacterium often infects open blossoms, young shoots or leaves through natural openings or wounds. Once inside host tissue, bacteria migrate to other tissues via xylem vessels, initially causing wilting and water-soaked lesions, followed by rapid necrosis (Bogs et al., 1998). How *Erwinia amylovora* survives and causes disease in hosts is still poorly understood. Since the 1980s, the use of molecular tools has facilitated identification of genes needed for or involved in disease development (Oh & Beer, 2005). These include genes involved in the biosynthesis of exopolysaccharide (EPS) (Bellemann & Geider, 1992; Bernhard et al., 1993; Geier & Geider, 1993; Bugert & Geider, 1995, 1997), genes encoding the type III secretion apparatus and proteins secreted via this pathway (Wei et al., 1992, 2000; Wei & Beer, 1993, 1995; Bogdanove et al., 1996; 1998; Gaudriault et al., 1997; Kim & Beer, 1998; Oh et al., 2005), metabolic genes contributing to bacterial virulence including those that protect bacteria from host defenses or antagonistic microorganisms (Zhang et al., 1999; Venisse et al., 2002; Burse et al., 2004).

Here, we report the use of a high-throughput technique, signature-tagged mutagenesis (STM), to identify genes that are likely involved in the infection process by *Erwinia amylovora*. STM has been applied successfully to bacterial pathogens of animals, including *Salmonella typhimurium*, *Yersinia enterocolitica* and *Escherichia coli* (Mecsas, 2002; Saenz & Dehio, 2005). Mutants generated by uniquely tagged transposons are pooled and used as an inoculum for a susceptible host. If a mutant does not survive in the host or compete with fully virulent mutants during pathogenesis, it will not be recovered following inoculation and incubation. Therefore, in theory, any mutant that does not grow as vigorously in the host as the wild-type strain will be identified as a candidate virulence mutant. Mutants of *Erwinia amylovora* were created with a pool of uniquely tagged transposons, and then inoculated to shoots of apple trees and incubated; recovery of the mutants was attempted from the symptomatic tissues. Mutants not recovered were considered mutated in putative virulence genes, and their insertion sites were characterized. Although widely used to identify virulence genes in bacteria pathogenic to animals (Mecsas, 2002; Saenz & Dehio, 2005), to our knowledge, STM has not been applied in studies of plant pathogenic bacteria.
Materials and methods

Bacterial strains, plasmids, media and growth conditions

A pool of DNA sequence-tagged pUTmini-Tn5Km2 plasmids, *Escherichia coli* strains CC118 λpir and S17-1 λpir and protocols for STM were kindly provided by D.W. Holden. *Escherichia coli* and *Erwinia amylovora* strains were cultured in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) with appropriate antibiotics at 37 °C and 28 °C, respectively. MM2Cu was used as a minimal and selective medium for *Erwinia amylovora* (Berereswill *et al*., 1998). The concentrations of antibiotics used in media were as follows: ampicillin (Ap), 100 μg mL⁻¹; carbenicillin (Cb), 400 μg mL⁻¹; kanamycin (Km), 50 μg mL⁻¹; and rifampicin (Rp), 50 μg mL⁻¹.

Generation of unique-tag marked library of mutants of *E. amylovora*

Each individual pUTmini-Tn5Km2 plasmid carries an 80-bp DNA sequence tag comprised of a 40-bp variable central region, which is used to differentiate each plasmid from the others, and two flanking 20-bp invariant ‘arms’ that serve as primer-binding sites for generating probes by PCR (Fig. 1a). The pool of tagged pUTmini-Tn5Km2 vectors, which confer resistance to both ampicillin (Ap) and kanamycin (Km), in *Escherichia coli* CC118 λpir was conjugated with *Erwinia amylovora* Ea273, which is resistant to rifampicin (Rp) (Bauer, 1990). Individual transconjugant colonies were checked on MM2Cu and LB plates with Cb (Berereswill *et al*., 1998). Because *Erwinia amylovora* mutants to Ap resistance spontaneously, Cb was used to select cells of *Erwinia amylovora* that harboured the pUT vector. One thousand eight hundred ninety two individual Cb-sensitive, but Km- and Rp-resistant colonies were selected and stored at −80 °C in 15% glycerol in 96-well microtitre plates.

Colony blots

The overnight cultures of mutants in each plate were transferred onto two nylon membranes (Nalge Nunc International, Naperville, IL) using the CloneMaster™ 96-pin replicator (Immusine Laboratories Inc., obtained from Fisher Scientific Co., Springfield, NJ). The plates holding the membranes were incubated at 28 °C overnight. The membranes were then treated as recommended by the supplier and stored at room temperature (24 ± 4 °C).

Screening the mutant library

Three rounds of PCR were performed to generate a probe representative of each ‘Input Pool’, defined as the culture mixture of all the mutants stored in one microtitre plate (Fig. 1b). The first PCR provided specific templates for generating probe DNA. The second PCR generated enough templates for amplifying probes. The third PCR amplified enough DNA for probe labelling. The primers used for all three PCRs were P2 (5'-TACCTACAAACCTCAAGCT-3') and P4 (5'-TACCCATTCAACAAGC-3') (Hensel *et al*., 1995). During the first PCR, genomic DNA of the Input Pool was used as a template. The 80-bp band from the first PCR was cut out from a 0.8% (w/v) low-melting agarose gel and used directly as a template for the second PCR. The second PCR product was used directly as a template for the third PCR. After digestion of the third PCR reaction mixtures with HindIII, the 40-bp probe DNA was purified from a 1.6% (w/v) low-melting agarose gel using the QIAEXII Gel Extraction Kit.
mutants were determined by inverse PCR (Fuller et al., 1996). Sequences were edited using DNASTAR software (DNASTAR Inc., Madison, WI), and BLAST programs, TBLASTX and BLASTN, were used for homologous sequence searches.

Results and discussion

Sequence analysis of attenuated mutants
The DNA sequences flanking the Tn5 insertions in the mutants were determined by inverse PCR (Fuller et al., 2000). The sequencing was performed at the Cornell University Biotechnology Sequencing Center. The DNA sequences were edited using DNASTAR software (DNASTAR Inc., Madison, WI), and BLAST programs, TBLASTX and BLASTN, were used for homologous sequence searches.

Virulence and growth assay in immature pear fruits and apple shoots
The virulence assay in immature Bartlett pear fruits was as used by Steinberger & Beer (1988). The inoculation procedure for virulence assay in apple trees was as described for screening the pools. The growth assay was similar, except that inoculation was performed with a florist’s frog (Bonasera et al., 2006). The latter inoculation procedure allowed for punching out discs from the same leaf for assays at different times. This tended to minimize the variation in the bacterial populations recovered from different apple leaves. The necrosis of the leaves and the length of lesions in the stems were rated to derive a disease index for each shoot.

Nineteen candidate mutants were identified after two-round screenings
Hybridizations were performed for each of 20 pools, which together contained 1892 Tn5 insertion mutants, with probes generated from the Input Pool and the Output Pool, respectively. Mutants that showed reduced signals in the Output Pool were picked. To reduce the number of false-positive candidates, a second round of screening was performed (Darwin & Miller, 1999) with newly picked mutants. The number of candidate mutants with attenuated signals in the Output Pool was reduced to 19. Southern hybridization results with the 19 mutants, using a probe within the mini-Tn5Km2 sequence, revealed that all mutants have single transposon insertions, which are located throughout the genome (data not shown).

The hybridization results of both Input Pool and Output Pool were compared for a pool of 96 mutant strains (Fig. 2). The signals of mutants D11 and F2 were reduced in the Output Pool compared with those of the Input Pool. The two mutants were tested individually on immature pear fruits for virulence. D11 did not cause disease, while F2 caused similar symptoms and signs as the parent strain (data not shown). The BLAST result for D11, renamed STM6,
indicated that it has an insertion in \textit{amsE}, which is involved in the biosynthesis of amylovoran, a major virulence factor of \textit{Erwinia amylovora} (Table 1; Bugert & Geider, 1995). Meanwhile, B2, B3 and G4 showed weak signals in both pools (Fig. 2). As the colonies grew normally on medium containing kanamycin, the transposon apparently was stable in these three mutants. This result might be due to the loss of tag DNA from the transposon, or inefficient amplification or labelling (D.W. Holden, pers. commun.). B9 and F12 showed weak signals in the Input Pool but not in the Output Pool (Fig. 2), suggesting inefficient labeling of the Input Pool probe.

**Virulence assay and growth in planta**

To determine the effect on the virulence of each mutated gene, the 19 mutants were tested individually for virulence in immature pear fruits. Ten of the mutants showed either

### Table 1. Predicted function of genes of \textit{Erwinia amylovora} putatively altered by 19 signature-tagged transposons

<table>
<thead>
<tr>
<th>Mutant</th>
<th>GenBank access number</th>
<th>Shotgun sequence numbers from the genome of \textit{E. amylovora} Ea273</th>
<th>Homolog (organism, NCBI access number)</th>
<th>Putative function</th>
<th>e-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM6</td>
<td>DX936507</td>
<td>amy585c07.q1ka</td>
<td>\textit{amsE} (\textit{E. amylovora}, Q46635)</td>
<td>Glycosyl transferase in amylovoran biosynthesis</td>
<td>$1 \times 10^{-9}$</td>
</tr>
<tr>
<td>STM7</td>
<td>DX936508</td>
<td>amy250g09.p1k amy61g11.q1ka amy172e05.p1k</td>
<td>\textit{cysB} (\textit{E. coli}, X60293)</td>
<td>Positive transcriptional regulator for cysteine regulon</td>
<td>$2 \times 10^{-15}$</td>
</tr>
<tr>
<td>STM9</td>
<td>DX936510</td>
<td>amy124c01.p1k</td>
<td>\textit{str1199} (\textit{S. thermophilus}, CP000024)</td>
<td>Conserved hypothetical protein</td>
<td>$3 \times 10^{-34}$</td>
</tr>
<tr>
<td>STM10</td>
<td>DX936511</td>
<td>amy48h07.p1k</td>
<td>\textit{phea} (\textit{Pantoea agglomerans}, M74133)</td>
<td>Chorismate mutase in the biosynthesis of phenylalanine and tyrosine</td>
<td>$4 \times 10^{-60}$</td>
</tr>
<tr>
<td>STM4</td>
<td>DX936505</td>
<td>amy319b07.q1k amy51h11.q1k amy380d05.p1k</td>
<td>\textit{pyrD} (\textit{Salmonella typhimurium}, NP_460032)</td>
<td>Dihydroorotate oxidase in pyrimidine biosynthesis</td>
<td>$9 \times 10^{-109}$</td>
</tr>
<tr>
<td>STM8</td>
<td>DX936509</td>
<td>amy124c01.p1ka</td>
<td>\textit{pyrB} (\textit{P. agglomerans}, AAP1338)</td>
<td>Aspartate transcarbamylase catalytic subunit in pyrimidine biosynthesis</td>
<td>$7 \times 10^{-105}$</td>
</tr>
<tr>
<td>STM25</td>
<td>DX936520</td>
<td>amy402d03.q1k</td>
<td>\textit{carB} (\textit{E. coli}, NP_751995)</td>
<td>Carbamoyl-phosphate synthase large chain in pyrimidine and arginine biosynthesis</td>
<td>$4 \times 10^{-33}$</td>
</tr>
<tr>
<td>STM29</td>
<td>DX936521</td>
<td>amy536b12.p1k</td>
<td>\textit{purN} (\textit{E. coli}, AAC14580)</td>
<td>Phosphoribosylglycinamide formyltransferase 1 in purine biosynthesis</td>
<td>$6 \times 10^{-53}$</td>
</tr>
</tbody>
</table>

(1) Gene involved in known function

(2) Genes involved in amino acid and nucleotide biosynthesis

(3) Genes involved in nutrient transportation

(4) Genes involved in other functions

**e-value is a statistical way to show homology. If the e-value for a homolog is $> 1 \times 10^{-30}$, this match was considered nonsignificant (Jones et al., 2000).**
no symptoms (STM5, 6, 9, 10, 25) or slower development of disease and reduced symptoms (STM4, 7, 8, 22, 29) 2 days after inoculation (data not shown). Three days later, all mutants, except for STM6 and STM25, which never initiated disease, caused similar symptoms in pears as the wild-type strain Ea273 (data not shown).

To further confirm their attenuated virulence, the ten mutants that had shown reduced symptoms in immature pear fruits were inoculated to apple shoots. Symptoms started to appear 5 days after inoculation and lesions stopped extending c. 2 weeks after inoculation. Shoots that had been inoculated with the wild-type strain showed obvious symptoms and signs of disease with a disease index 5.83 ± 0.4 (Table 2). STM29 showed disease symptoms and lesions similar to, but slightly less severe (disease index = 4.17 ± 1.01), than those of the wild-type control (Table 2). In contrast, STM7 caused much less severe disease than the wild-type strain, with a disease index of 1.83 ± 0.7 (Table 2). No obvious disease symptoms or signs resulted from inoculation with the other mutants (Table 2).

STM mutants were selected for loss of capacity to survive in a given host when inoculated together with a group of other mutants. Therefore, genes identified by STM should be required for either independent or competitive survival in hosts (Autret & Charbit, 2005). To test whether the growth of the 10 mutant strains is compromised in apple tissue, bacterial populations in inoculated leaves were analyzed immediately and 3 days after inoculation. Immediately after inoculation, all the strains were present in the tissues in similar numbers (Fig. 3). Three days after inoculation, the wild-type strain, Ea273, had multiplied at least four orders of magnitude; no living cells were detected in shoots inoculated with STM6 and 25, which is consistent with their failure to cause disease in immature pear fruits and apple shoots. The growth of STM4 was only slightly reduced in apple tissues. The growth of STM5, 22 and 29 was also close to the wild-type level, indicating that the genes disrupted by these mutations contribute less to the survival of Erwinia amylovora in hosts. The growth rate of the other mutants was significantly lower than that of Ea273 (Fig. 3).

Sequence analysis of candidate mutants

The DNA flanking the Tn5 insertions in the 19 mutants were classified into four groups based on their possible functions as ascertained by reference to Genbank (Table 1).

(1) Known virulence gene.

The insertion in STM6 affects amnE, which had been characterized previously as encoding glycosyl transferase, which functions in the biosynthesis of amylovoran (Bugert & Geider, 1995).

(2) Genes involved in amino acid and nucleotide biosynthesis.

The insertions in STM7, 10 and 25 are in loci that likely affect the biosynthesis of amino acids. The interrupted locus of STM7 encodes a protein that is homologous to a positive transcriptional regulator of the cysteine regulon. The homologue of the interrupted locus of STM10 encodes a chorismate mutase, which functions in the biosynthesis of phenylalanine and tyrosine. In STM25, the Tn5 was inserted into a locus homologous to the large chain of carbamoyl-
phosphate synthase that functions in the biosynthesis of arginine and pyrimidine.

STM4 and STM8 have transposon insertions in loci that encode proteins homologous to dihydroorotate dehydrogenase and the catalytic subunit of aspartate transcarbamylase, respectively. Both enzymes function in the de novo biosynthesis of pyrimidine. The factor affected by mutant STM29 is homologous to phosphoribosylglycinamidoformyltransferase, an enzyme functioning in the de novo biosynthesis of purine.

As all of the above mutations affected virulence in host plant tissues, we conclude that certain amino acids and nucleotides are critical to the infection process of Erwinia amylovora, and these amino acids are not present or available to Erwinia amylovora from hosts.

(3) Genes involved in nutrient transport.

The mutations in STM17 and STM20 may interrupt the transport of nutrients into cells of Erwinia amylovora. The interrupted gene in STM17 is similar to uraA of S. typhimurium, which encodes a uracil transport enzyme that functions in the uptake of uracil. UraA may help Erwinia amylovora utilize uracil from hosts to synthesize pyrimidines through a salvage pathway. The Tn5 insertion in STM20 may affect a factor homologous to yieG of Yersinia pestis, which may be associated with transport. These results suggested that membrane transporters also play a role in the infection process by Erwinia amylovora.

(4) Genes involved in other functions.

BLAST results showed that the sequences of STM1, 5, 14, 15 and 22 correspond to genes in other organisms, whereas the remaining four mutants lack significant homology for identification. The Tn5 insertion in STM1 is in a gene similar to xerC of Pseudomonas fluorescens. Mutation of xerC reportedly results in reduced root colonization when the mutant was inoculated together with its parent strain, whereas it grew comparably to the parent strain when it was tested individually (Dekkers et al., 1998). This implies that perhaps STM1 does not competitively colonize inoculated apple shoots as effectively as Ea273.

The available Tn5 flanking sequence of STM22 has substantial similarity to AJ289879, a known DNA sequence of Erwinia amylovora in Genbank, which contains genes ycfA and ycfB that are responsible for the yellow colony color of Erwinia amylovora on MM2Cu medium (Zhang et al., 2000). The color of STM22 colonies was white, rather than yellow, when tested on MM2Cu (data not shown). However, it is unlikely that STM22 controls the formation of the yellow pigment through ycfAB as Tn5 appears to be inserted 424-bp downstream of ycfAB. It may affect another gene that encodes a protein functioning in the same pathway for synthesis of YcfAB. Zhang et al. (2000) showed that the yellow pigment produced by Erwinia amylovora on MM2Cu may be related to a low-molecular-weight compound, termed CP340, which has an absorption maximum at 340 nm; however, they did not provide evidence that the yellow pigment contributes to virulence. In the present study, we showed that the STM22 mutation affects both virulence and yellow color formation on MM2Cu. Further investigation is needed to determine just how the loci affected by the STM22 insertion affects colony colorization.

The flanking sequences of the mutations in STM5, 14 and 15 are homologous to putative ORFs in S. typhimurium, Escherichia coli and Xanthomonas campestris, which encode a putative inner membrane protein, a putative aminotransferase and a DNA helicase-related protein, respectively.

Interestingly and rather surprisingly, we identified no known genes of the type III secretion system by STM. We consider that the product from one mutant in the same inoculation pool may have complemented the function of a second mutant and helped it survive in the host, effectively masking the phenotype of the mutant. HrpL is the transcriptional activator of genes encoding the type III secretion system, and a strain with impaired HrpL could not survive in the host (Wei & Beer, 1995). However, Jock and her colleagues recovered equal numbers of both strains in the recovered population after cooinoculating immature pear fruits with an hrpL-deficient strain and a wild-type strain of Erwinia amylovora at different ratios (Jock et al., 2003). Thus, a strain mutated in genes encoding a component of the type III secretion system may be difficult or impossible to recover from STM screenings. However, as genes encoding the type III secretion apparatus were identified when STM was applied to S. typhimurium (Hensel et al., 1995; Shea et al., 1996), perhaps, we screened too few mutants.

Recently, Zhao et al. (2005) identified 394 putative virulence genes of Erwinia amylovora based on screening 19,200 clones on immature pear fruit disks using in vivo expression technology (IVET). The IVET results identified several types of genes previously known to be involved in the pathogenicity of Erwinia amylovora and others, previously unknown, which could be considered candidate virulence genes. The genes identified by IVET actually are genes, whose expression is enhanced by inoculation of host plant tissue (immature pear fruit). Genes with enhanced expression may function in virulence, or perhaps their expression may be enhanced coincidentally. IVET was efficient for identifying virulence genes of Erwinia amylovora, as 2% of the clones screened were identified. Here, we report the use of STM, another high-throughput technique, for identifying genes involved in the infection process by Erwinia amylovora; 1% of the screened mutants were identified as having disrupted genes that function in amylovoran biosynthesis, metabolism, metabolite transport and colonization, including the genes ansE, pheA, uraA and xerC. In contrast to the IVET work, the STM approach involved inoculation of apple shoots on trees growing in a greenhouse as the host.
screening system. Shoot infection often accounts for the devastating damage caused by fire blight in orchards under natural conditions (van der Zweit & Beer, 1999). Thus, the genes identified by STM perhaps are more likely to be involved in the natural infection process of Erwinia amylovora than at least some of those identified by IVET.

In conclusion, STM was effective in Erwinia amylovora in identifying one well-known virulence gene, amnE, and nine new loci that resulted in reduced virulence, relative to the wild-type parent, when the mutated strains were inoculated to hosts. However, the STM approach was somewhat disappointing, as it did not identify more of the genes known previously as involved in the virulence, and it required considerable technical expertise, the use of radioactive nuclides and host material that was rather difficult to maintain. A recent report (Shah et al., 2005) of the use of PCR-based STM with Salmonella gallinarum suggests that this modification makes STM easier to pursue and quite effective in identifying genes involved in virulence. To our knowledge, the present study is the first instance in which STM has been used to identify virulence genes of a plant pathogen. Further study of the genes identified by STM may promote a better understanding of the pathogenic process initiated by Erwinia amylovora in hosts.

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