A mutation in an \textit{exbD} gene reduces tagetitoxin production by \textit{Pseudomonas syringae} pv. \textit{tagetis}

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\textbf{Abstract:} A mutant of \textit{Pseudomonas syringae} pv. \textit{tagetis} EB037 with limited ability to produce tagetitoxin was isolated after transposon mutagenesis and the mutation was characterized. The mutation occurred in a gene with a high degree of sequence identity to \textit{exbD}. \textit{exbD} is contiguous with \textit{tonB} and \textit{exbB} upstream and with a gene for a TonB-dependent receptor downstream. Using reverse transcription – polymerase chain reaction with RNA from the wild-type and \textit{exbD} mutant strains, we demonstrated that the mutation in \textit{exbD} did not have a polar affect on the expression of downstream genes. The \textit{exbD} mutant was able to grow well in conditions where iron is not freely available. Siderophore production by the \textit{exbD} mutant was similar to that of the wild-type strain. We conclude that the mutation in \textit{exbD} disrupts tagetitoxin production without compromising iron metabolism. The results indicate that tagetitoxin export by \textit{P. syringae} pv. \textit{tagetis} involves an efflux pump that requires a functional TonB system that is not essential for normal iron metabolism.


\textbf{Résumé :} Un mutant de \textit{Pseudomonas syringae} pv. \textit{tagetis} EB037 ayant une capacité limitée à produire de la tagetitoxine fut isolé suite à une mutagénèse par transposon et la mutation fut caractérisée. La mutation était présente dans un gène ayant un haut degré d’identité de séquence à \textit{exbD}. \textit{exbD} est contigu avec \textit{tonB} et \textit{exbB} en amont et avec un gène codant un récepteur dépendant de TonB en aval. Nous avons démontré par réaction en chaîne de la polymérase à transcription inverse avec de l’ARN des souches sauvages et mutantes pour \textit{exbD} que la mutation dans \textit{exbD} n’avait pas d’effet polaire sur l’expression de gènes en aval. Le mutant \textit{exbD} fut capable de bien croître dans des conditions où le fer n’était pas librement disponible. La production de sidérophores par le mutant \textit{exbD} était semblable à celle de la souche sauvage. Nous concluons que la mutation dans \textit{exbD} empêche la production de tagetitoxine sans compromettre le métabolisme du fer. Ces résultats indiquent que l’exportation de la tagetitoxine par \textit{P. syringae} pv. \textit{tagetis} met en jeu une pompe d’écoulement qui nécessite un système fonctionnel TonB qui n’est pas essentiel au métabolisme normal du fer.


\[\text{Traduit par la Rédaction}\]

\textbf{Introduction}

Although iron is an essential nutrient, it is only sparingly soluble and can be toxic to cells in the presence of oxygen. Therefore, iron availability is tightly controlled in most organisms by chelating the iron to specialized proteins. Bacteria have developed several systems to acquire iron in various forms and from different sources (Braun and Braun 2002; Faraldo-Gomez and Sansom 2003; Poole and McKay 2003; Postel and Kadner 2003; Hoegyt et al. 2005). For example, Gram-negative bacteria secrete iron-complexing chemicals, known as siderophores, that bind iron from various sources and are taken up as iron complexes by cells via specific active transport systems. This is accomplished by the TonB system, a complex of three proteins, TonB, ExbB, and ExbD, which are necessary to drive energy-dependent uptake of iron through the bacterial outer membrane (Braun and Braun 2002; Postle and Kadner 2003).

While much of what is known about the mechanism of TonB-dependent transport has come from studies in \textit{Escherichia coli}, similar transport systems have been described in animals, insects, and plant pathogens. TonB-system mutants of the animal pathogens \textit{Bordetella bronchiseptica}, \textit{Bordetella pertussis}, \textit{Brucella melitensis}, and \textit{Pasteurella multocida} either fail to grow or have impaired growth in iron-limiting media (Nicholson and Beall 1999; Pradel et al. 2000; Bosch...
et al. 2002; Danese et al. 2004). An exbD mutant of *Photobacterium hydroburni temperata*, an insect pathogen vectored by *Heterorhabditis* species of nematodes, is unable to grow well in conditions where iron is not freely available (Watson et al. 2005). *Pseudomonas putida* tonB, exbB, or exbD mutants were unable to grow on iron-limiting media (Bitter et al. 1993; Godoy et al. 2001, 2004). Mutational analysis of the tonB–exbB–exbD1–exbD2 cluster of the plant pathogen *Xanthomonas campestris* pv. *campestris* demonstrated that functional tonB, exbB, and exbD1 genes were required for iron transport, however, exbD2 was not (Wiggerich et al. 1997). Deletion of two *Caulobacter crescentus* genes homologous to exbB and exbD not only resulted in impaired growth on Fe$^{3+}$–rhodotorulate as the sole iron source, it also abolished maltodextrin binding and transport, as well as growth on maltodextrins larger than maltotetraose (Neugebauer et al. 2005).

Mutations in TonB-system genes can result in partial or no effect on iron transport in bacteria that possess multiple TonB systems. For example, studies with *Pseudomonas aeruginosa* demonstrated that tonB1, which is not physically linked to exbB or exbD homologues on the chromosome, is required for growth on iron-limiting media, while inactivation of the contiguous genes tonB2, exbB, or exbD in the same organism had no adverse effect on iron or heme acquisition (Zhao and Poole 2000). The ability to utilize haemin or the siderophores vibriobactin or ferrichrome as a sole iron source by *exbB1* or *exbB2* mutants of *Vibrio cholerae* was not altered as compared with the wild-type strain. However, mutations in both of these genes resulted in a *V. cholerae* mutant that could not utilize these iron sources (Occhino et al. 1998). The two TonB systems in *V. cholerae* were found to have unique as well as common functions, with TonB1 specifically mediating the utilization of the siderophore schizokinen, while TonB2 was required for utilization of enterobactin (Seliger et al. 2001).

The species *Pseudomonas syringae* is divided into 57 pathovars that are pathogenic for numerous monocot and dicot crops (Gardin et al. 1997). Several of the *P. syringae* pathovars produce phytotoxins that produce chlorosis (e.g., coronatine, phaseolotoxins, tabtoxin, tagetitoxin) or necrosis (e.g., syringomycin, syringopeptin) (Bender et al. 1999). Although TonB systems in *P. syringae* have not been characterized, examination of the three *P. syringae* pathovar genomes currently sequenced (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005) indicates that these pathovars each have at least four putative TonB systems. We describe here a TonB system in *P. syringae* pv. *tagetis* that when disrupted by a mutation in *exbD* fails to alter iron metabolism but disrupts the production of tagetitoxin, a chlorosis-inducing phytotoxin that inhibits chloroplast RNA polymerase (Matthews and Durban 1990).

**Materials and methods**

**Bacterial culture**

*Pseudomonas syringae* pv. *tagetis* EB037 (Encore Technologies, Plymouth, Md., USA) strains and *Pseudomonas putida* DOT-T1E exbB:aphA3 (Godoy et al. 2004) were routinely grown at 28 °C in King’s B medium (KB) (King et al. 1954) broth or agar. *Escherichia coli* EPI300 (Epiconcentre, Madison, Wis., USA) was routinely grown at 37 °C in Luria–Bertani (LB) (Sambrook et al. 1989) broth or agar. All broth cultures were grown with shaking at 250 r/min. The following antibiotics were added to growth media as appropriate (μg mL$^{-1}$): chloramphenicol, 10; kanamycin, 25; and rifampicin, 25.

**Toxin isolation and identification**

*Pseudomonas syringae* pv. *tagetis* EB037 was grown in tagetitoxin-production medium and tagetitoxin was extracted as previously described (Mitchell and Hart 1983), with the following modifications. The spent medium from three 1 L cultures was first subjected to anion-exchange chromatography on DEAE Sephadex™ (Sigma Chemical Co., St. Louis, Mo., USA); the biologically active fractions were identified using a tagetitoxin bioassay (described below) and were combined and subjected to partition chromatography on Sephadex™ LH-20. The biologically active fractions were combined and subjected to another round of anion-exchange and partition chromatography. The resulting biologically active fractions were examined by thin layer chromatography (TLC) on cellulose plates with ninhydrin and molybdate detection (Mitchell and Hart 1983), and the appropriate fractions were combined. The final product was analyzed by $^1$H NMR spectrometry in D$_2$O on a Bruker 400 instrument (Bruker, Rheinstetten, Germany), as previously described (Mitchell et al. 1989), and was compared with parallel data recorded on a 2 mg sample of pure tagetitoxin previously isolated from *P. syringae* pv. *tagetis* DAR 26807 (Mitchell and Hart 1983).

**Tagetitoxin bioassay**

A sunflower (*Helianthus annuus* L. ‘Autumn Beauty’) bioassay for the detection of tagetitoxin (Gronwald et al. 2005) was used with the following modifications. Plants were grown in 50-cell flats containing Jiffy Mix Plus (Jiffy Products of America, Batavia, Ill., USA) at 24 °C under a photosynthetic photon flux density of 600 μmol m$^{-2}$ s$^{-1}$ for a 16 h photoperiod. After 7 days, either bacterial cells were inoculated into the plant just below the cotyledons by stabbing the stem with a 25 gauge hypodermic needle carrying cells lifted from agar cultures or 50–100 μL of cell-free culture filtrates were injected into each of the cotyledons. Plants were evaluated for apical chlorosis at 5–7 days after treatment. Bioassays were conducted in triplicate and repeated at least three times when evaluating bacterial strains that proved nontoxicogenic.

**Molecular techniques**

Routine DNA manipulations were performed as previously described (Sambrook et al. 1989). Preparations of genomic and plasmid DNA were performed using the Blood & Cell Culture DNA and QIAprep Spin Miniprep kits, respectively, according to manufacturer’s (Qiagen, Valencia, Calif., USA) instructions. Oligonucleotide primers were designed using Primer3 software (Rozen and Skaletsky 2000) at the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ www.cgi) and synthesized by Invitrogen (Grand Island, N.Y.).

Transposon mutagenesis was conducted using the EZ::TN™<KAN-2> Transposon kit following the manufacturer’s (Epiconcentre) instructions. Briefly, the EZ::TN <KAN-2> transposon – EZ::TN transposase complex was introduced
by electroporation into *P. syringae* pv. *tagetis* EB037, a naturally rifampicin-resistant, tagetitoxin-producing strain. Colonies of *P. syringae* pv. *tagetis* EB037 resistant to rifampicin and kanamycin were screened for alternations in tagetitoxin production by stabbing cells into sunflower plants, as described above. Nontoxicogenic mutants were then tested for toxin production in culture. To do so, 100 µL of an overnight 5 mL KB broth culture of the test strain was used to inoculate 5 mL of Woolley’s medium containing kanamycin. After 48 h of growth, cell-free culture filtrates were prepared by pelleting the cells by centrifugation at 2500g for 15 min and filtering the supernatant through a 0.2 µm membrane syringe filter. The cell-free culture filtrates were then injected into sunflower plants, as described above.

The genomic region of mutant *P. syringae* pv. *tagetis* EB03709, a nontoxicogenic, kanamycin-resistant strain derived from *P. syringae* pv. *tagetis* EB037 and believed to be harboring the EZ::TN <KAN-2> transposon, was rescued using the CopyControl™ Fosmid Library Production kit according to the manufacturer’s (Epicentre) instructions. The DNA sequences of the regions flanking the transposon element were confirmed by sequencing from six pCCEB03709 foscids (pCCEB03709-1 – pCCEB03709-6) isolated from different kanamycin- and chloramphenicol-resistant *E. coli* EPI300 isolates produced in the DNA recovery process. Sequencing was conducted at the Centre for Biosystems Research DNA Sequencing Facility, University of Maryland, College Park, Md., USA, on an ABI DNA Sequencer (Applied Biosystems, Foster City, Calif., USA) with the primers complementary to the insertion element that were supplied with the mutagenesis kit. Further extension of nucleotide sequences was done by using pCCEB03709 as template DNA with primers ExbD2-F1 (5′-GACGATGAGAGCTTTGTTGTAGGT) and KAN-2-RP-2 (5′-CTCAAATCTCTGTAGTTACATTGC), which specifically probed the kanamycin resistance gene introduced into the genome of EB03709 by transposon mutagenesis with EZ::TN <KAN-2>. The probe was labeled by the NEBlot® Phototope® kit (New England BioLabs, Beverly, Mass., USA) in accordance with the manufacturer’s instructions. The rest of the hybridization procedure was done as previously described (Sambrook et al. 1989). The Phototope-Star detection kit (New England BioLabs) was used for developing, and X-OMAT films (Eastman Kodak Co., Rochester, N.Y., USA) were used for autoradiography.

**RNA isolation and RT–PCR**

Total RNA was isolated from overnight KB broth cultures of *P. syringae* strains EB037 and EB03709 using the RNAeasy® mini kit (Qiagen). Reverse transcription (RT) PCR assays were conducted using the OneStep RT–PCR kit, following manufacturer’s (Qiagen) instructions. Primer pair TonBRec-F1 (5′-GTAACCTCAACGGCTGTACGC) and TonBRec-R1 (5′-CTGGGATGATGCTTCTCGACA) was used for the amplification of TonB-dependent receptor cDNA. The final volume of reactions was 50 µL and contained 100 ng RNA. RT was conducted at 50 °C for 30 min. Amplification of cDNA was conducted with 30 cycles (each) of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. Reactions were terminated after a final 10 min elongation at 72 °C. Amplification products were sized by agarose-gel electrophoresis.

**Iron limitation effects on colony development and cell growth rate**

Assays were conducted to determine if *P. syringae* pv. *tagetis* EB03709 was restricted in growth under iron-limitation conditions. In the first set of experiments, overnight cultures of *P. syringae* pv. *tagetis* strains EB037 and EB03709 and *P. putida* DOT-T1E exbB:aphA3 (an exbB mutant previously demonstrated to have restricted growth under iron-limiting conditions (Godoy et al. 2004)) were grown overnight in basal medium 2 broth (Gilleland et al. 1974) modified with 0.11 mol L−1 glucose and 0.008 mol L−1 MgSO4 (BM 2-1), and the cells were pelleted and resuspended in sterile, deionized water (SDW) to an optical density at 600 nm (OD600) of 1.0. The cell suspensions were further diluted 1 × 10−5, and 50 µL of the final dilution was homogeneously spread across the surface of LB agar with or without 0.37 mmol L−1 FeCl3. The plates were incubated at 28 °C and observed for colony development after 3 days of growth.

To compare the effects of iron limitation on the growth of *P. syringae* pv. *tagetis* in liquid culture, strains were grown overnight in 5 mL KB broth cultures and the cells pelleted, washed in SDW, and resuspended in SDW to an OD600 of 2.0. Two mililitres of the cell suspension was used to inoculate 50 mL BM 2-1 broth without iron, without iron but with EDDHA (ethylenediamine-di(o-hydroxyphenylacetie acid); ICN Pharmaceuticals, Inc., Plainview, N.Y., USA) at a concentration of 8.3 µmol L−1, or with iron at a concentration of 37 µmol L−1 FeCl3. The cultures were incubated at 28 °C, and the OD600 was measured for subsamples taken at 0, 12, 24, and 36 h of growth.

**Siderophore production**

Siderophore production was assayed with the chrome-azurul S (CAS) agar plate and liquid broth assays as previously described (Payne 1994), with the following modifications: Woolley’s medium with glucose was substituted for M9 minimal medium, and asparagine was added to the liquid cultures at a final concentration of 15 mmol L−1 (Bultreys and Gheysen 2000). For agar plate assays, cells from overnight cultures of *P. syringae* pv. *tagetis* strains EB037 and EB03709 grown in 5 mL KB broth were pelleted, washed with SDW, resuspended in SDW to an OD600 of 1.0, and 10 µL of the cell suspensions was spotted on the modified CAS agar. Cultures were incubated at 28 °C for 2 days, and then the size of the yellow–orange halos produced was recorded. For liquid assays, cells from overnight cultures grown in modified CAS broth were pelleted by centrifugation at 2500g for 15 min, the supernatant filtered through a 0.2 µm membrane syringe filter, and 0.5 mL of the filtrate com-

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Institute; http://www.ebi.ac.uk/clustalw/index.html). The alignment of the N terminus to the C terminus in ClustalW (European TonB, ExbB, and ExbD, were assembled and aligned from NC004578), for analyses. An additional 42 residues were excluded from the ExbB and 153 residues, 13 of which were trimmed from the termini. The ExbB data set contained 625 residues, 388 of which were trimmed from the termini; and the ExbD data set contained 2401 bp nucleotide sequence containing the full cds of Vibrio cholerae pv. Pf-5 (CP000076), and P. aeruginosa 1148a (NC005773), respectively, using Blastx and Blastp via the NCBI database (http://www.ncbi.nlm.nih.gov/). The 2401 bp putative TonB system proteins in pseudomonads: P. putida DOT-T1E (AF315582), P. fluorescens PfO-1 (CP000094), and P. aeruginosa PAO1 (AE004091). Vibrio cholerae N16961 (AE003853) was chosen as an outgroup for rooting purposes.

Phylogenetic analysis

Additional amino acid sequences were obtained from the GenBank DNA and nonredundant protein sequence databases, respectively, using Blastx and Blastp via the National Center for Biotechnology Information (NCBI) internet homepage (http://www.ncbi.nlm.nih.gov/). The 2401 bp joining in PAUP* and evaluated by 10 000 bootstrap replicates each. Distance searches were conducted by neighbor joining in PAUP* and evaluated by 10 000 bootstrap replicates.

Analysis of DNA and protein sequences

The DNA and deduced protein sequences were compared with GenBank DNA and nonredundant protein sequence databases, respectively, using Blastx and Blastp via the National Center for Biotechnology Information (NCBI) internet homepage (http://www.ncbi.nlm.nih.gov/). The 2401 bp nucleotide sequence containing the full cds of exbB and exbD and the partial cds of tonB and a TonB-dependent receptor has been deposited in GenBank under accession No. AY228178.

Maximum parsimony (MP) analyses were conducted in PAUP* v.4.0b10 (Swofford 2002) for analyses. Characters were unordered and given equal weight. Support for the branching topology was evaluated by bootstrap analysis derived from 10 000 replicates with 10 random addition replicates each. Distance searches were conducted by neighbor-joining in PAUP* and evaluated by 10 000 bootstrap replicates.

Results and discussion

Toxin isolation and identification

Based on the sunflower bioassay, the bioactive product isolated from P. syringae pv. tagetis EB037 gave an apical chlorosis response identical to that of tagetitoxin. On TLC analysis, the purified product migrated and stained with ninhydrin and molybdate, as previously reported for tagetitoxin (Mitchell and Hart 1983) (data not shown). All of the proton peak multiplicities and couplings were very clearly displayed in the nuclear magnetic resonance (NMR) spectrum of the P. syringae pv. tagetis EB037 product (data not shown). There were no significant NMR signals other than those reported for the authentic compound (Table 1). Our TLC and NMR results for the chlorosis-inducing compound from P. syringae pv. tagetis EB037 were essentially identical to those reported for tagetitoxin from strain P. syringae pv. tagetis DAR 26807 (Mitchell and Durbin 1981; Mitchell and Hart 1983; Mitchell et al. 1989). However, we did observe that the yields of tagetitoxin from P. syringae pv. tagetis EB037 liquid cultures were substantially lower than those obtained earlier from P. syringae pv. tagetis DAR 26807 (direct comparative data not available), which resulted in greater difficulties in purification. Recently, tagetitoxin was isolated from strain P. syringae pv. tagetis EB037 (Gronwald et al. 2005); however, the authors proposed that the previously published structure (Mitchell et al. 1989) for the toxin is incorrect. Although the actual chemical structure has been questioned, this study and recently published results (Gronwald et al. 2005) demonstrate that P. syringae pv. tagetis EB037 produces only one chlorosis-inducing metabolite, tagetitoxin.

Transposon mutagenesis

After random insertion of the EZ::TN <KAN-2> transposon into P. syringae pv. tagetis EB037, 17 kanamycin-resistant derivatives were selected on the basis of the loss of the ability

Table 1. Proton nuclear magnetic resonance (1H NMR) data for tagetitoxin purified from the cell-free culture filtrate of Pseudomonas syringae pv. tagetis EB037.

<table>
<thead>
<tr>
<th>Carbon position*</th>
<th>Chemical shift δ ppm</th>
<th>Tagetitoxin standard†</th>
<th>EB037 product</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.22 (d, J = 13.7 Hz)</td>
<td>3.22 (d, J = 13.7 Hz)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.98 (d, J = 13.7 Hz)</td>
<td>2.94 (d, J = 13.7 Hz)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.47 (d, J = 4.1 Hz)</td>
<td>4.36 (d, J = 4.1 Hz)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.04 (dd, J = 4.1, 12.3 Hz)</td>
<td>5.14 (dd, J = 3.95, 12.0 Hz)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.44 (dd, J = 7.3, 12.3 Hz)</td>
<td>3.46 (dd, J = 8.0, 12.1 Hz)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.71 (dd, J = 7.4, 11.6 Hz)</td>
<td>4.64 (dd, J = 8.1, 11.3 Hz)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.98 (s)</td>
<td>1.98 (s)</td>
<td></td>
</tr>
</tbody>
</table>

Note: s, singlet; d, doublet; dd, double doublet; and J, coupling constant.
*Structure numbering as previously reported by Mitchell et al. (1989).
†Each referenced to HOD (partially deuterated water) at δ 4.75 ppm.
‡From a 2 mg crystalline sample.
to induce chlorosis in sunflower plants 6 days after the plants were stem inoculated with cells of the mutants. The frequency of detection of nontoxigenic mutants was one in every 400 kanamycin-resistant mutants screened in the sunflower bioassay. The cell-free 48 h spent culture of one mutant, \textit{P. syringae pv. tagetis} EB03709, induced faint but transient chlorosis in sunflower seedlings that was not visible 6 days after treatment (Fig. 1), indicating that much lower levels of tagetitoxin were produced by the EB03709 mutant than by the wild-type strain. Trace amounts of phytotoxins produced by a \textit{P. syringae pv. tagetis} strain harboring a mutation in \textit{syrD}, a gene that encodes for a protein that is predicted to function as an ATP-driven efflux pump for syringomycin and syringopeptin, were attributed to cell lysis (Grgurina et al. 1996). Similarly, small amounts of toxin in the cell-free spent media of 48 h cultures of \textit{P. syringae pv. tagetis} EB03709 may represent toxin released from cell lysis. Furthermore, internal levels of tagetitoxin must be tightly regulated, as the RNA polymerase of the producing organism is sensitive to the toxin (Mathews and Durbin 1990). Consequently, the quantity of tagetitoxin in the 48 h cultures of a mutant deficient in toxin efflux would be expected to be small, as was observed.

**Sequence analysis**

Southern blot analysis of the \textit{EcoRV}-digested DNA isolated from \textit{P. syringae pv. tagetis} EB03709 and probed with an insert-specific probe demonstrated that EB03709 contained a single copy of \textit{EZ::TN <Kan-2>} (data not shown). After
Fig. 4. The bootstrapping phylogram recovered from maximum parsimony (MP) analyses of concatenated data sets for three characterized or putative TonB system proteins in pseudomonads. Numbers above a branch represent support >50% for those nodes: the first number represents the bootstrapping values for MP, followed by bootstrapping values for neighbor joining. Pseudomonas syringae pv. tagetis EB037 is indicated in bold type. For strains with multiple TonB systems, the last letter in the strain name was assigned in alphabetically ascending order as the TonB system appeared in the GenBank accession number. Numbers to the right of the brackets are clade designations. GenBank accession Nos. are as follows: AY228178, P. syringae pv. tagetis EB037; NC004578, P. syringae pv. tomato DC3000; CP000075, P. syringae pv. syringae B728a; NC005773, P. syringae pv. phaseolicola 1148a; AF315582, P. putida DOT-T1E; CP000094, P. fluorescens PfO-1; CP000076, P. fluorescens Pf-5; and AE004091, P. aeruginosa PAO1. Vibrio cholerae N16961, GenBank accession No. AE003853, was chosen as an outgroup for rooting purposes.

![Diagram of phylogram](image)

digestion of chromosomal DNA of *P. syringae* pv. tagetis EB03709, inverse PCR was employed to amplify sequences adjacent to the EZ::TN <KAN-2> transposon. Blast analyses of the sequences obtained showed that the EZ::TN <KAN-2> transposon in *P. syringae* pv. *tagetis* EB03709 was located in a gene with a high degree of sequence similarity to *exbD* and shared the greatest identity with putative *exbD* genes identified in *P. syringae* pv. *syringae* B728a (90%) and *P. syringae* pv. *tomato* DC3000 (87%). Sequence analysis of the DNA upstream from *exbD* identified partial and complete operons with similar levels of homology to putative *tonB* and *exbB* genes (Fig. 2), respectively, in *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000. Furthermore, downstream from *exbD* appears to be a partial operon with high levels of identity to putative TonB-dependent receptor genes in *P. syringae* pv. *phaseolicola* 1448a (90%), *P. syringae* pv. *syringae* B728a (88%), and *P. syringae* pv. *tomato* DC3000 (87%). Reverse transcription analysis was conducted to determine if the EZ::TN <KAN-2> insert at *exbD* had a polar effect on the downstream operon. An RT–PCR amplification product of the predicted size was obtained in reactions with RNA from *P. syringae* pv. *tagetis* strains EB037 and EB03709, with reverse transcriptase, and with primers designed to amplify a 425 bp region of the TonB-dependent receptor gene (Fig. 3). No amplification product was produced when RNA or reverse transcriptase
were not included in the reaction mix. These results indicate that the EZ::TN <KAN-2> transposon does not exert a transcriptional polar effect on downstream genes.

Phylogenetic analysis

A total of 631 characters were included in the combined TonB, ExbB, and ExbD analyses, of which 557 were parsimony informative, 33 were variable but parsimony uninformative, and 41 were constant. Four of the most parsimonious trees of length 2632 were found by MP; confidence interval = 0.83; retention index = 0.89. The TonB systems identified in pseudomonads fell into five distinct clades, with each of the TonB systems identified in *P. syringae* pv. tomato DC3000, *P. syringae* pv. syringae B728a, and *P. syringae* pv. phaseolicola 1448a allied in different clades (Fig. 4). The TonB system in *P. syringae* pv. tagetis EB037 belongs to a distinct lineage of proteins (clade III) that, thus far, has only been identified in some phytotoxin-producing pathovars of *P. syringae*: *P. syringae* pv. tomato DC3000 produces coronatine (Moore et al. 1989), *P. syringae* pv. syringae B728a produces syringomycin and syringopeptin (Scholz-Schroeder et al. 2001), and *P. syringae* pv. phaseolicola 1448a produces phaseolotoxin (Joardar et al. 2005). As this is the first report of an exbD mutant of *P. syringae*, it is not known if mutations in the same TonB system of strains DC3000, B728a, or 1448a affect toxin production.

Phenotypic characterization of *P. syringae* pv. tagetis EB03709

Colony development of the exbB mutant *P. putida* DOT-T1E exbB:aphA3 after 3 days of growth in LB solid medium not supplemented with iron was inhibited compared to its growth in medium supplemented with iron at a concentration of 0.37 mmol L⁻¹ FeCl₃ (data not shown). However, the presence or absence of FeCl₃ in the medium had no effect on colony development by *P. syringae* pv. tagetis strains EB037 or EB03709. While bacterial growth of the *P. syringae* pv. tagetis strains in BM 2-1 minimal medium decreased with increasing stringencies of iron limitation, the growth of strain EB037 and EB03709 within treatments was similar (Fig. 5). Numerous mutational studies have demonstrated the importance of the TonB system in iron transport. However, growth of *P. syringae* pv. tagetis EB03709 under the iron-limiting conditions in this bioassay was not compromised.

Siderophore production in Gram-negative bacteria increases in response to iron limitation (Vasil and Ochsner 1999; Hantke 2001). Consequently, mutations in TonB system genes that alter iron uptake can also result in increased production of siderophores (Wiggerich et al. 1997). In fact, screening for the hyperproduction of siderophores has been used to identify TonB system mutants (Watson et al. 2005). Siderophore production by *P. syringae* pv. tagetis strains EB037 and EB03709 was not significantly different whether determined by the size of the yellow–orange halos produced after 3 days of growth on modified CAS agar (2.1 ± 0.2 cm and 2.2 ± 0.3 cm, respectively) or if determined by the absorption at 630 nm of liquid CAS assays (1.04 ± 0.15 and 1.00 ± 0.06, respectively). Thus, the fact that siderophore production by *P. syringae* pv. tagetis EB03709 was similar to that by *P. syringae* pv. tagetis EB037 is additional evidence that iron metabolism was not altered in this exbD mutant.

Mutations in one TonB system are insufficient to cause alterations in growth under iron-limiting conditions in Gram-negative bacteria that possess more than one TonB system (Ochchino et al. 1998; Seiger et al. 2001). As the *P. syringae* pathovars contain at least four homologous TonB systems, the absence of alterations in iron metabolism of *P. syringae* pv. tagetis EB03709 may result from the mutation in exbD being complemented by other TonB systems present in this bacterium. However, such complementation can not explain the nontoxigenic phenotype expressed by this mutant.

While TonB systems are generally involved in uptake across the outer membrane, there is evidence that TonB proteins and TonB systems are involved in cellular export. For example, the export of the exotoxin aerolysin by *Aeromonas hydrophila* involves a TonB-like protein (Howard et al. 1996). A tonB mutant of *P. aeruginosa* deficient in iron siderophore uptake was hypersensitive to a wide variety of antibiotics; however, compensation for the iron deficit failed to alleviate the drug hypersensitivity (Zhao et al. 1998). Based on drug accumulation assays, it was determined that the tonB mutant had decreased levels of drug efflux (Zhao et al. 1998). In *P. putida* DOT-T1E, high levels of resistance to toluene, *p*-hydroxybenzonic acid, and several antibiotics have been attributed to multidrug efflux pumps (Godoy et al. 2001). In addition to not being able to grow in low-iron medium when EDDHA is added, TonB system mutants of *P. putida* DOT-T1E are sensitive to toluene, *p*-hydroxybenzonic acid, and the antibiotics ciprofloxacin, cefotaxime, and imipenem (Godoy et al. 2001, 2004). While an iron-rich medium

![Fig. 5. The effect of iron availability on the growth rates of *Pseudomonas syringae* pv. *tagetis* strains. Overnight cultures of strains EB037 (●) and EB03709 (○) were grown in BM 2-1 medium and subcultured into the same medium to a starting OD₆₀₀ of 0.04. Cultures were grown at 28 °C with shaking, and the OD₆₀₀ was measured and recorded at the times indicated. +Fe = BM 2-1 medium with iron (37 µmol L⁻¹ FeCl₃); −Fe = BM 2-1 medium without iron; and −Fe, +EDDHA = BM 2-1 medium without iron and with 8.3 µmol L⁻¹ EDDHA (ethylenediamine-di(ß-hydroxyphenylacetic acid)). Values represent the average of three replicates ± 1 standard deviation. Similar results were obtained for two additional experiments.](https://example.com/fig5.png)
allows normal cell growth of the TonB system mutants, the level of sensitivity to toluene, p-hydroxybenzonic acid, and the above antibiotics remains unchanged.

Based on these previous studies, which show an involvement of TonB systems with efflux systems, and our results which demonstrated that a mutation in an exbD gene disrupts tagetitoxin production without compromising iron metabolism, we believe that tagetitoxin export by P. syringae pv. tagetis involves an efflux pump that requires a functional TonB system that is not essential for normal iron metabolism.

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References


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