The gene \textit{stlA} encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in \textit{Photorhabdus luminescens} TT01

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\textit{Photorhabdus} is a genus of Gram-negative bacteria from the family \textit{Enterobacteriaceae}. Members of \textit{Photorhabdus} have a complex life cycle during which the bacterium has a pathogenic interaction with insect larvae whilst also maintaining a mutualistic relationship with nematodes from the family Heterorhabditidae. During growth in the insect, \textit{Photorhabdus} bacteria produce a broad-spectrum antibiotic identified as 3,5-dihydroxy-4-isopropylstilbene (ST). The biochemical pathway responsible for the production of this antibiotic has not been characterized. In this report, a mutant strain of \textit{Photorhabdus luminescens} subsp. laumondii TT01, BMM901, has been isolated, by transposon mutagenesis, that is unable to produce the ST antibiotic. Using \textit{in silico} studies, feeding experiments and biochemical analyses, it is shown that the gene mutated in this strain, \textit{stlA}, encodes phenylalanine ammonia-lyase (PAL). PAL catalyses the non-oxidative deamination of L-phenylalanine to \textit{trans}-cinnamic acid and the enzyme is ubiquitous in plants, where it is involved in the production of phenylpropanoids such as lignin and phytoalexins. However, this is the first report of PAL activity in a member of the \textit{Proteobacteria}.

\textbf{INTRODUCTION}

\textit{Photorhabdus} is a genus of Gram-negative entomopathogenic bacteria. \textit{Photorhabdus} is a member of the family \textit{Enterobacteriaceae} and is therefore closely related to many well-known pathogens, e.g. \textit{Escherichia coli}, \textit{Salmonella enterica}, \textit{Erwinia} species and \textit{Yersinia pestis}. In addition to being highly virulent to insect larvae, \textit{Photorhabdus} bacteria also maintain a mutualistic association with nematodes from the family Heterorhabditidae. \textit{Photorhabdus} species are normally found colonizing the gut of the free-living, soil-dwelling infective juvenile (IJ) stage of the nematode. The IJ actively seeks out and penetrates a potential host before migrating to the insect blood system (the haemolymph). The \textit{Photorhabdus} bacteria are regurgitated into the haemolymph, where they rapidly grow and divide, killing the insect within 48 h. The nematodes grow and develop using the bacterial biomass within the cadaver as their food source and, after two to three generations, a new population of bacterial biomass within the cadaver is their food source. The nematodes grow and develop using the lymph, where they rapidly grow and divide, killing the insect. The \textit{Photorhabdus} migrates to the insect blood system (the haemolymph).

\textit{Photorhabdus luminescens} subsp. \textit{laumondii} TT01 has been shown to produce a \textit{\beta}-lactam antibiotic, carbapenem, during the exponential phase of growth (Derzelle \textit{et al.}, 2002). Members of \textit{Photorhabdus} have also been reported to produce a variety of colicin-like antimicrobial proteins and both the carbapenem and the colicins have been shown to have activity against Gram-negative bacteria (Derzelle \textit{et al.}, 2002; Sharma \textit{et al.}, 2002). In addition, all strains of \textit{Photorhabdus} tested have been shown to produce an antibiotic activity identified as 3,5-dihydroxy-4-isopropylstilbene (ST) (see Fig. 1) (Hu \textit{et al.}, 1997; Li \textit{et al.}, 1995; Richardson \textit{et al.}, 1988). The ST antibiotic is produced as the bacteria enter the stationary phase of growth [both in the insect (\textit{in vivo}) and in nutrient broth (\textit{in vitro})] and it has been suggested that the role of the ST antibiotic is to protect the insect cadaver from predation and saprophytic attack (Hu \& Webster, 2000). In support of this, it has been demonstrated that ST has anti-bacterial, anti-fungal and anti-nematode activity (Akhurst, 1982; Han \& Ehlers, 1999; Hu \textit{et al.}, 1999). However, the biochemical pathway responsible for the production of this antibiotic has not yet been characterized.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is an enzyme that catalyses the non-oxidative deamination of the amino acid L-phenylalanine to \textit{trans}-cinnamic acid. Cinnamic acid is a precursor for the production of several classes of plant phenylpropanoids, including lignins,
flavonoids and stilbenes such as resveratrol (Jeandet et al., 2002; Weisshaar & Jenkins, 1998). Although ubiquitous in higher plants, PAL activity is extremely rare in prokaryotes (Moore et al., 2002). The only example of prokaryotic PAL activity is in the actinomycete 'Streptomyces maritimus', where the encP gene has been shown to encode a protein with PAL activity (Xiang & Moore, 2002). EncP is required to supply cinnamic acid for the production of benzoyl-CoA, the starter molecule for the biosynthesis of the bacteriostatic agent enterocin (Piel et al., 2000; Xiang & Moore, 2002, 2003).

In this report, we describe the identification and characterization of a PAL from the bacterium P. luminescens TT01. This is the first report of PAL activity in a member of the Proteobacteria. We also demonstrate that cinnamic acid is required for the production of the ST antibiotic in P. luminescens and propose a model for the biochemical pathway leading to ST production.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. We have used a spontaneous rifampicin-resistant mutant of P. luminescens TT01 as the wild-type in all experiments. Bacteria were routinely cultured in Luria–Bertani (LB) broth or on LB agar (LB broth plus 1.5% w/v agar) at 28 °C for P. luminescens or 37 °C for E. coli, unless otherwise stated. When required, antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg ml⁻¹; kanamycin (Kn), 30 μg ml⁻¹; and rifampicin (Rif), 50 μg ml⁻¹.

Transposon mutagenesis and the overlay method. A spontaneous Rif-resistant mutant of P. luminescens TT01 was mated with E. coli S17-1 carrying the plasmid pUT-Kn2 (a gift from D. Holden, Imperial College, London, UK), a derivative of pUT-Kn (Herrero et al., 1990). Both strains were grown in LB broth to an OD₆₀₀ of 0.6 and the donor and recipient bacteria were mixed at a ratio of 1:4. The cells were left overnight at room temperature and Rif⁸ Kn⁸ exconjugants were selected by plating the conjugation mixture on selective agar. Individual mutants were tested for antibiotic production by picking colonies onto fresh LB agar and incubating the plates at 28 °C for 4 days before the plates were overlaid with 0.1 ml of an overnight culture of Micrococcus luteus ATCC 4698 in 10 ml soft agar (LB broth plus 0.7% w/v agar). Plates were then incubated overnight at 28 °C and antibiotic production was scored by the presence of a zone of inhibition of M. luteus growth around the P. luminescens mutant colony.

DNA manipulations. Chromosomal DNA was isolated from P. luminescens and E. coli strains using standard procedures (Sambrook et al., 1989). The chromosomal DNA flanking the transposon was identified, as previously described, by cloning into pBR322 and sequencing (Joyce & Clarke, 2003).

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>P. luminescens TT01</td>
<td>Spontaneous Rif⁸ mutant</td>
<td>Lab stock</td>
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<tr>
<td>P. luminescens BMM901</td>
<td>P. luminescens TT01 stIA::Kn</td>
<td>This study</td>
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<tr>
<td>E. coli S17-1 (zipir)</td>
<td>E. coli lysogenized with zipir</td>
<td>Lab stock</td>
</tr>
<tr>
<td>E. coli EC100</td>
<td>F⁻ mcrA Δ(mrr–hsdRM–mcrBC) 380ΔlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara-leu)7697 galU galK l–rpsL nupG</td>
<td>Epicentre</td>
</tr>
<tr>
<td>M. luteus ATCC 4698</td>
<td>Wild-type</td>
<td>ATCC</td>
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<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBR322</td>
<td>ori colE1, Ap⁸, Tet⁸</td>
<td>Lab stock</td>
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<tr>
<td>pTRC99a</td>
<td>ori colE1, Ap⁸</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pTRC99a-His</td>
<td>ori colE1, Ap⁸</td>
<td>M. Blight</td>
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<tr>
<td>pBMM901</td>
<td>pTRC99a, pTRc–stlA</td>
<td>This study</td>
</tr>
<tr>
<td>pBMM902</td>
<td>pTRC99a-6His, pTRc–stlA (N-terminal His tag)</td>
<td>This study</td>
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Cloning of stlA into pTRC99a and pTRC99a-His. The stlA gene was amplified from P. luminescens TT01 chromosomal DNA by PCR using oligonucleotides JW124 (5'-CGATCCGAGGCTAAAAGCAGTTGAGCCC-3') and JW125 (5'-CGATCCGATGATTATTCTTCCAGATGATTCTTG-3'), for cloning into the NcoI and XbaI sites of pTRC99a (Amerham Pharmacia Biotech) and a derivative, pTRC99a-His (a gift from Dr Mark Blight, CNRS, Orsay, France), resulting in pBMM901 and pBMM902, respectively. The integrity and accuracy of all plasmid clones were confirmed by DNA sequencing.

TLC analysis of culture supernatants. An overnight culture of the bacterial strain being tested was inoculated (1:100) into 100 ml LB and incubated at 28 °C for 48 h. When required, cinnamic acid (Sigma-Aldrich) was added to the LB to a final concentration of 33 μg ml⁻¹. The cells were removed by centrifugation at 12 000 r.p.m. for 10 min and the culture supernatant was aliquotted into four 25 ml samples. The supernatant was then extracted three times with 20 ml ethylacetate (HPLC grade) and the ethylacetate extractions were collected into a large round-bottomed flask. The sample was then dried down in a rotary evaporator using partial pressure and a temperature of approx. 55–60 °C and resuspended in 500 μl methanol. The extract was transferred to a 1·5 ml Eppendorf tube and stored at −20 °C until required. A small aliquot (30 μl) of each extract was applied to a TLC plate [aluminium-backed silica gel 60 F254 (fluorescence wavelength) (Merck)] using a glass micro-syringe. The TLC was run using a developing solvent of chloroform : methanol (98:5:1:5) and the plates were analysed under UV light (Hu et al., 1997). To overlay with M. luteus, the TLC plate was placed on the surface of an LB agar plate and covered with 10 ml of a soft agar containing 0·1 ml of an overnight culture of M. luteus. The agar plate was then incubated at 30 °C for 2–3 days and antibiotic activity was observed as an inhibition of growth over the stilbene band on the TLC plate.

Purification of His–StlA. E. coli EC100 containing the pBMM902 plasmid was cultured overnight in LB at 37 °C and inoculated (1:100) into 50 ml fresh LB. The cells were grown at 37 °C until an OD₆₀₀ of 0·5 and 100 μM IPTG was added to induce expression of the stlA gene. After 3 h induction, the cells were harvested by centrifugation and the pellet was stored at −20 °C. The cell pellet was resuspended in 2 ml BugBuster solution (Novagen) containing 50 U Benzonase and 100 μg lysozyme. The cell suspension was left at room temperature for 30 min and cell debris was removed by centrifugation at 13 300 r.p.m. for 15 min at 4 °C. The clarified supernatant was mixed with 250 μl Ni-NiTA resin (Novagen) and incubated at 4 °C with constant mixing for 60 min. At this stage, the mixture was loaded into a column and the resin was washed twice with 8 volumes of wash buffer (50 mM sodium phosphate buffer, pH 8·0, 300 mM NaCl, 20 mM imidazole). The His–StlA protein was then eluted in four 0·5 ml aliquots of elution buffer (50 mM sodium phosphate buffer, pH 8·0, 300 mM NaCl, 250 mM imidazole) and the fractions were analysed by SDS-PAGE. Fractions containing StlA were pooled and dialysed, using a Slide-A-Lyser (Pierce), against 50 mM sodium phosphate buffer (pH 8·0), 300 mM NaCl to remove the imidazole. Glycerol was added to a final concentration of 20% (v/v) and the sample was aliquotted and stored at −20 °C. The protein concentration was then determined with the Bradford reagent assay (Sigma) using BSA as the standard. To measure PAL activity, we added 0·1 M sodium borate buffer (pH 8·8) and 20 mM l-phenylalanine to an aliquot of the lysate to give a final reaction volume of 1 ml and the reaction tube was incubated at 30 °C. The production of cinnamic acid was detected by measuring the increase in A₂₉₀ (Kyndt et al., 2002). The specific activity of PAL in total cell extract was calculated using an absorbance coefficient for cinnamic acid of 10 000 M⁻¹ cm⁻¹. For the PAL assay using purified protein, the reaction (1 ml in total) was performed in 50 mM sodium phosphate buffer (pH 8·0), 300 mM NaCl at 30 °C with 10 mM l-phenylalanine as the substrate and 5 μg purified StlA protein. The Km and v₅₀ of the purified His–StlA were calculated using the direct linear plot method described by Eisenthal & Cornish-Bowden (1974) using the EnzPack software package.

RESULTS

Isolation and characterization of mutants that do not produce antibiotic

The first step in our efforts to describe the biochemical pathway responsible for ST production in Photobacterus was to isolate mutants in P. luminescens that were no longer able to produce the ST antibiotic. Therefore, we mutated P. luminescens TT01 with a transposon, delivered from the plasmid pUT-Kn2, and isolated 600 independent Kn-resistant mutant colonies. Photobacterus produces several antibiotics during growth in vitro; carbapenam and colicins that are active against Gram-negative bacteria and the ST antibiotic that has been reported to be active against both Gram-negative and Gram-positive bacteria (Akhurst, 1982; Derzelle et al., 2002; Sharma et al., 2002). Therefore, it is possible to assay specifically for ST activity by overlaying the colonies with a Gram-positive bacterium, e.g. M. luteus. Each of the isolated mutants was then tested for antibiotic production using the overlay method (see Methods) and, in this way, we identified a mutant strain, called BMM901, that was unable to produce the ST antibiotic (Fig. 2a). Southern blot analysis confirmed that BMM901 contained a single transposon insert (data not shown).

The gene interrupted in BMM901 was identified by digesting chromosomal DNA with EcoRV and cloning the Kn-resistance gene carried by the transposon into pBR322. The annotated genome of P. luminescens TT01 is available at http://genolist.pasteur.fr/PhotoList (Duchaud et al., 2003). DNA sequencing of the regions flanking the Kn-resistance gene followed by BLASTX analysis against the TT01 genomic sequence revealed that the precise location of the insertion was in the gene plu2234. The transposon was inserted immediately downstream from codon 349 in the plu2234 gene, which is predicted to encode a protein of 532 amino acids. The protein encoded by plu2234 is predicted to encode a protein with similarity to histidine ammonia-lyase (HAL) (see http://genolist.pasteur.fr/PhotoList).

BMM901 is defective in ST production

It has previously been reported that ST can be visualized after TLC by using UV light (Hu et al., 1997). Therefore, to
confirm that the lack of antibiotic production in BMM901 was due to an absence of the ST antibiotic, we carried out a TLC analysis of organic extracts of cell-free supernatants from overnight cultures of TT01 and BMM901. From this analysis, it is clear that two compounds were missing from the BMM901 supernatant (Fig. 2b; UV). The major compound (Fig. 2b; indicated with an arrow) migrated with an $R_f$ of approximately 0.60 and this is very close to the $R_f$ reported previously for ST ($R_f = 0.59$) (Hu et al., 1997). To confirm that this band was ST, we placed the TLC plate on the surface of an LB agar plate and covered it with 10 ml soft agar containing 0.1 ml of an overnight culture of M. luteus. The agar plate was then incubated at 30°C for 2–3 days and antibiotic activity was determined by observing inhibition of bacterial growth over certain regions of the TLC plate (Fig. 2b; Overlay). It is clear that the major compound present in TT01 supernatants and absent from BMM901 supernatants strongly inhibits the growth of M. luteus, confirming that this compound is ST. Although the nature of the minor compound has not yet been determined, it is possible that it is an intermediate of ST production. Furthermore, we did not detect any other compounds with antimicrobial activity in supernatants of TT01 cultures using the extraction protocol outlined in this study.

To confirm that the defect in ST production in BMM901 is entirely due to the mutation in plu2234, we cloned plu2234 into the vector pTRC99a and the resulting plasmid, pBMM901, was transformed into BMM901 and ST production was determined by TLC analysis (Fig. 2b). It is clear that the defect in ST production in BMM901 is entirely due to the mutation in plu2234. In addition to restoring production of ST, complementation of BMM901 with pBMM901 also restored the production of the minor compound shown to be absent from supernatants of BMM901.

As plu2234 is the first gene shown to be required for the production of the stilbene antibiotic in P. luminescens, we propose to rename plu2234 as stlA (stilbene A).

**DNA sequence analysis of the stlA locus**

To determine whether any of the genes in the vicinity of stlA might be involved in antibiotic production, we carried out an in silico analysis of the proteins that are predicted to be encoded by the genes surrounding stlA, using the BLASTP algorithm available at http://www.ncbi.nlm.nih.gov/blast/ (Fig. 3). The gene plu2233, immediately upstream from stlA, is predicted to encode a protein with oxidoreductase activity [best BLASTP hit, 77% identity with probable oxidoreductase in Yersinia pseudotuberculosis (YP_070712); score = e^{-107}]. Oxidoreductases are often involved in the detoxification of molecules and these genes may encode

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**Fig. 2.** Antibiotic production by P. luminescens TT01 and BMM901. (a) Cells from overnight cultures of TT01 and BMM901 were spotted onto fresh LB agar plates. The plates were incubated at 28°C for 4 days and antibiotic production was determined by overlaying the colonies with soft agar containing M. luteus. (b) Supernatants from cultures of TT01 and BMM901 (with or without plasmid pBMM901, as indicated) grown in LB broth for 4 days at 28°C were analysed for ST production by TLC. After development of the TLC plate, ST was visualized under UV light (ST indicated by arrow) and antibiotic activity was determined by overlaying the TLC plate with M. luteus.

**Fig. 3.** The stlA genetic locus in P. luminescens TT01. The fragment of DNA shown corresponds to nucleotides 2624343–2631262 (plu2231–plu2236) of the P. luminescens TT01 genome available at http://genolist.pasteur.fr/PhotoList.
proteins involved in resistance to the ST antibiotic. Interestingly, plu2236 is also predicted to encode an FAD-dependent oxidoreductase (best BLASTP hit, 31% identity to FAD-dependent oxidoreductase from Rubrobacter xylanophilus DSM 9941). The genes plu2232 and plu2231 are predicted to encode proteins of unknown function with homologues in other enteric bacteria (best BLASTP hit for plu2232, 62% identity with hypothetical protein from Y. pseudotuberculosis (YP_070711); score = 7e−31; best BLASTP hit for plu2231, 70% identity with hypothetical protein from Y. pseudotuberculosis (YP_070710); score = 7e−17). Therefore, plu2231–plu2236 have homologues in Y. pseudotuberculosis (YP_07010–YP_07012), a bacterium that is not reported to produce any stilbene-based molecules, suggesting that these genes are unlikely to be involved in ST production in P. luminescens. Immediately downstream from stlA is a gene predicted to encode a chitinase (plu2235; best BLASTP hit, 49% identity to a predicted chitinase from Burkholderia fungorum; score = e−144). As P. luminescens is an insect pathogen, it is not surprising that this bacterium produces chitinase activity, although it is unlikely that the chitinase is directly involved in ST production. Therefore, from our analysis, it would appear likely that stlA is the only gene encoding a protein with a role in ST production in this region of the P. luminescens genome.

The stlA gene encodes a protein with homology to PAL

In P. luminescens TT01, stlA is predicted to encode a protein with 31.6% identity, at the amino acid level, to Salmonella enterica HAL. HAL converts histidine to trans-urocanate as the first step in the catabolism of this amino acid. In S. enterica, HAL is encoded by hutH, the final gene in the hut (histidine utilization) operon. Using PhotoList, we undertook a BLASTP analysis of the TT01 genome using HAL (encoded by hutH) from S. enterica as the query. In this way, we found two genes predicted to encode proteins with homology to HAL present in the TT01 genome, stlA and plu3192. The gene plu3192 is predicted to encode a protein with 72.8% identity, at the amino acid level, to HAL from S. enterica. Moreover, plu3192 is found in a locus that contains other genes with strong homology to other genes in the hut operon from S. enterica (plu3192–plu3197; http://genolist.pasteur.fr/PhotoList). This strongly suggests that plu3192 encodes the canonical HAL protein of P. luminescens and it is, therefore, unlikely that stlA encodes a protein involved in histidine catabolism.

Recent work in the actinomycete 'Streptomyces maritimus' has identified a protein, EncP, that, despite strong homology with prokaryotic HALs, has been shown to have PAL activity (Xiang & Moore, 2002). Although the active sites of PALS and HALs are reported to be very similar, they are predicted to differ in some key residues (Calabrese et al., 2004; Xiang & Moore, 2002). In particular, Met382 and Gln414 in the HAL of Pseudomonas putida are highly conserved in other HALs but are replaced by Lys and Gln, respectively, in PALS (Calabrese et al., 2004). These residues are predicted to be involved in substrate loading and would be important in distinguishing between L-histidine and L-phenylalanine. Therefore, to determine whether StlA could be a PAL, we compared the amino acid sequences of StlA with the predicted HAL proteins from Pseudomonas putida, S. enterica, Y. pestis and P. luminescens TT01 using the CLUSTAL W algorithm in DNAStar (Fig. 4). All of the key active-site residues shared by PALS and HALs are present in StlA, suggesting that StlA is either a HAL or a PAL (data not shown). However, it is clear from Fig. 4 that StlA is predicted to have a Lys (Lys407) residue in place of Met382 and a Gln (Gln440) in place of Gln414. Therefore, StlA has the sequence signature expected from a protein with PAL activity.

The mutation in BMM901 can be complemented by the addition of cinnamic acid to the growth medium

PAL catalyses the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid and we have shown that stlA in P. luminescens encodes a protein with homology to PAL in key residues. Moreover, we have also shown that stlA is required for the production of the stilbene antibiotic in P. luminescens. Based on these data (and the obvious structural similarities between cinnamic acid and the ST antibiotic; see Fig. 1), we hypothesized that cinnamic acid could be the precursor of the Photorhabdus antibiotic, implying that the antibiotic-minus phenotype of BMM901 would be due to the inability of this strain to produce cinnamic acid. If this hypothesis was correct, we reasoned that it would be possible to restore antibiotic production to BMM901 by supplementing the growth medium with cinnamic acid, as has been shown to be the case with an encP
null mutant in ‘Streptomyces maritimus’ (Kalaitzis et al., 2003). To test this, BMM901 was spotted onto LB agar containing 33 mg cinnamic acid ml\(^{-1}\) and, after 4–5 days growth at 28 °C, the colony was overlaid with \textit{M. luteus} to assay for antibiotic production. It is clear from Fig. 5(a) that when BMM901 is cultured in the presence of cinnamic acid there is a level of antibiotic production that is similar to wild-type levels. Therefore, supplementation of the growth medium with cinnamic acid rescues antibiotic production in BMM901, supporting the hypothesis that \textit{stlA} encodes a bacterial PAL. In addition, we also confirmed, by TLC analysis, that the addition of cinnamic acid to the culture medium restores the production of the ST molecule in the supernatant of \textit{P. luminescens} cultures (Fig. 5b).

**Biochemical evidence that \textit{stlA} encodes PAL**

If PAL activity is required for antibiotic production in \textit{P. luminescens}, it should be possible to detect this activity in total cell extracts. In \textit{P. luminescens}, maximal ST production has been shown to occur during the stationary phase of growth (Hu & Webster, 2000). Therefore, we cultured TT01 and BMM901 at 28 °C for 24 h in LB broth, harvested the cells by centrifugation and produced a total cell extract by sonication (see Methods). To assay for PAL activity, we added 20 mM L-phenylalanine to the cell extract and monitored the production of cinnamic acid by measuring the increase in \(A_{254}\) over time. From these experiments, we could clearly detect PAL activity in TT01 cell extracts and we calculated the specific activity of PAL in TT01 in stationary phase as 318 pmol min\(^{-1}\) (mg cell extract\(^{-1}\)). Interestingly, under similar conditions, we could not detect any PAL activity in BMM901, suggesting that \textit{stlA} does encode PAL.

To confirm that \textit{stlA} encodes PAL, we cloned \textit{stlA} into pTRC99a-His, a derivative of pTRC99a that places a 6\(\times\)His fusion onto the N terminus of the expressed protein, resulting in PBMM902 (see Methods). His-tagged StlA (His–StlA) was overproduced in \textit{E. coli} and purified using Ni-NTA chromatography. The eluted protein was assessed for purity by SDS-PAGE and we observed a single major protein band migrating with a molecular mass of approximately 50 kDa, slightly smaller than the predicted molecular mass of StlA (57–7 kDa) (data not shown). The purified protein was then used in PAL enzyme assays as described in Methods. From these assays, it is clear that His–StlA was able to produce cinnamic acid from L-phenylalanine and, using the direct linear method, we calculated the \(K_m\) (320±35 \(\mu\)M) and the \(v_{\text{max}}\) [13.2±0.02 pmol s\(^{-1}\) (\(\mu\)g protein\(^{-1}\))] of His–StlA. Therefore, the \(k_{\text{cat}}\) for His–StlA is 0.8 s\(^{-1}\), a value that falls within the range of \(k_{\text{cat}}\) values (0.1–3.2 s\(^{-1}\)) recorded for some plant PAL enzymes (Cochrane et al., 2004). It should also be noted that PAL has high sequence identity to tyrosine ammonia-lyase (TAL), an enzyme that produces coumaric acid from tyrosine. However, we could not detect any TAL activity with His–StlA (data not shown).

**DISCUSSION**

In this report, we describe the characterization of \textit{stlA}, a gene encoding PAL in \textit{P. luminescens} TT01. This is the first reported occurrence of PAL activity in a member of the
Proteobacteria. Several lines of evidence support the conclusion that stlA encodes PAL: (i) sequence analysis of StlA reveals the active-site amino acid signatures of PAL (Fig. 4); (ii) a mutation in stlA can be rescued by supplementation of the growth media with cinnamic acid, the product of PAL activity (Fig. 5), and (iii) purified StlA has PAL activity.

PAL activity is ubiquitous in plants and the production of cinnamic acid is the starting point for the biosynthesis of a variety of plant phenylpropanoids, including some phytoalexins shown to have beneficial affects on human health, e.g. resveratrol (Dixon, 2001; Pervaiz, 2003). However, PAL activity is extremely rare in prokaryotes and, in addition to StlA, the only other PAL identified in bacteria is EncP from the actinomycete ‘Streptomyces maritimus’ (Xiang & Moore, 2002). Interestingly, stlA and encP are predicted to encode proteins of similar sizes (532 and 522 amino acids, respectively). Moreover, the proteins encoded by stlA and encP are also predicted to be similar in size to prokaryotic HALs (see Fig. 4). However, StlA and EncP are notably smaller than their eukaryotic PAL counterparts. The model plant Arabidopsis thaliana has genes encoding four isoforms of PAL (PAL1–PAL4) and the predicted size of these proteins ranges from 695 to 723 amino acids (Cochrane et al., 2004). This discrepancy in size suggests that the genes encoding the prokaryotic PALs do not have their origin in plants. Rather, it seems more likely that stlA and encP arose through a duplication of the hutH gene followed by subsequent mutation of the key active-site residues (see Fig. 4).

In ‘Streptomyces maritimus’, encP was shown to be part of a larger gene cluster that contains all of the genes required for the biosynthesis of the antibiotic enterocin (Xiang & Moore, 2002, 2003). However, from our analysis of the stlA locus (Fig. 3), it appears that stlA is not part of a large ST biosynthetic gene cluster, suggesting that the other genes involved in the production of the ST antibiotic are located elsewhere on the TT01 genome. This may also suggest that the hutH duplication occurred independently in P. luminescens and ‘Streptomyces maritimus’.

The unlinked nature of the genes involved in ST production makes it difficult to speculate on the nature of the biochemical pathway involved in the production of this antibiotic. However, the ST antibiotic produced by Photorhabdus is very similar in structure to pinosylvin (3,5-dihydroxystilbene), a phytoalexin produced by pine trees (Raiber et al., 1995; Schanz et al., 1992). The only difference between ST and pinosylvin is the presence of an isopropyl group at position 4 of the stilbene ring of ST (Fig. 1). Biosynthesis of pinosylvin is predicted to occur through the action of a type III polyketide synthase (PKS) called pinosylvin synthase (Schanz et al., 1992). Using a variety of methods, including degenerate PCR, Southern blotting and in silico homology searches, we could not find any gene encoding a protein with significant homology to pinosylvin synthase (or any other type III PKS) in the genome of P. luminescens TT01 (J. S. Williams, unpublished data). Moreover, it has been shown that ST antibiotic production in Photorhabdus requires the activity of a phosphopantetheinyl (P-pant) transferase, NgrA (Ciche et al., 2001).

Type III PKS action is independent of P-pant transferase activity and, therefore, it is unlikely that ST production involves a type III PKS. However, there are two other types of PKS (type I and type II), and both type I and type II PKSs do require P-pant transferase activity. Type I PKSs are large, multifunctional enzymes that contain a set of distinct, non-iteratively acting activities that are required for the production of the polyketide molecule, e.g. acetyltransferase (AT), keto-synthase (KS) (Hopwood, 1997). Type II PKSs, on the other hand, are multi-enzyme complexes that carry a single set of iteratively acting activities (Hopwood, 1997). Because of their large size and modular arrangement, it is relatively straightforward to identify genes encoding type I PKSs. Moreover, as the domains act non-iteratively, it is often possible to predict the molecule being produced by the PKS based on the number and arrangement of the active domains. We have used well-characterized AT and KS domains as queries in BLAST searches of the P. luminescens TT01 genome and we could not find a type I PKS with the arrangement of domains required to synthesize the ST antibiotic (J. S. Williams, unpublished data). Therefore, by default, we suggest that the production of ST requires an as-yet-unidentified type II PKS. This suggests that, although ST production in Photorhabdus involves the same starting molecule as used in plants, the production of the antibiotic probably requires a novel biochemical pathway. We are currently undertaking further genetic studies to identify other genes involved in the biosynthesis of this broad-spectrum antibiotic.

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