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Short communication

Pseudomonas syringae pv. *syringae* B728a hydrolyses indole-3-acetonitrile to the plant hormone indole-3-acetic acid

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SUMMARY

Nitrilase enzymes catalyse the hydrolysis of nitrile compounds to the corresponding carboxylic acid and ammonia, and have been identified in plants, bacteria and fungi. There is mounting evidence to support a role for nitrilases in plant–microbe interactions, but the activity of these enzymes in plant pathogenic bacteria remains unexplored. The genomes of the plant pathogenic bacteria *Pseudomonas syringae* pv. *syringae* B728a and *Pseudomonas syringae* pv. *tomato* DC3000 contain nitrilase genes with high similarity to characterized bacterial arylacetonitrilases. In this study, we show that the nitrilase of *P. syringae* pv. *syringae* B728a is an arylacetonitrilase, which is capable of hydrolysing indole-3-acetonitrile to the plant hormone indole-3-acetic acid, and allows *P. syringae* pv. *syringae* B728a to use indole-3-acetonitrile as a nitrogen source. This enzyme may represent an additional mechanism for indole-3-acetic acid biosynthesis by *P. syringae* pv. *syringae* B728a, or may be used to degrade and assimilate aldoximes and nitriles produced during plant secondary metabolism. Nitrilase activity was not detected in *P. syringae* pv. *tomato* DC3000, despite the presence of a homologous nitrilase gene. This raises the interesting question of why nitrilase activity has been retained in *P. syringae* pv. *syringae* B728a and not in *P. syringae* pv. *tomato* DC3000.

INTRODUCTION

Pseudomonads are found colonizing a range of terrestrial and aquatic habitats, as well as plant and animal tissues (Gupta, 2000). Within this genus is the plant pathogenic bacterium *Pseudomonas syringae*, which is capable of infecting a wide variety of plant hosts, although individual pathovars often show a high degree of host specificity (Hirano and Upper, 2000, Sarkar and Guttman, 2004). Five *P. syringae* genomes have been published to date. *Pseudomonas syringae* pv. *syringae* B728a (*P.s.*B728a) and *P. syringae* pv. *phaseolicola* 1448A (*P.s.*1448A) are pathogens of bean plants, and the causal agents of bacterial brown spot disease and halo blight, respectively. *Pseudomonas syringae* pv. *tomato* DC3000 (*P.s.*DC3000) and T1 (*P.s.*T1) are causal agents of bacterial speck of tomato, and *P. syringae* pv. *oryzae* 1-6 (*P.s.*1-6) is the causal agent of halo blight of rice (Almeida *et al.*, 2009; Boureau *et al.*, 2002; Buell *et al.*, 2003; Feil *et al.*, 2005; Joardar *et al.*, 2005; Preston, 2000; Reinhardt *et al.*, 2009). *P.s.*B728a has a broad host range and is an effective epiphyte, whereas *P.s.*DC3000 has a narrow host range and is a good endophyte (Boureau *et al.*, 2002; Feil *et al.*, 2005; Hirano and Upper, 2000). Genome sequencing of *P.s.*B728a and *P.s.*DC3000 has revealed that both pathogens possess a gene encoding a putative nitrilase enzyme, which was speculated to act on indole-3-acetonitrile (IAN), a precursor of indole-3-acetic acid (IAA) (Feil *et al.*, 2005). However, until now, the nitrile hydrolysing activity of these bacteria has not been investigated.

Nitrilase enzymes (nitrilases) catalyse the hydrolysis of nitrile compounds (RCN) to the corresponding carboxylic acid and ammonia (O'Reilly and Turner, 2003; Pace and Brenner, 2001). Nitrilases have been characterized in a number of bacteria found colonizing the plant and soil environment, including *Pseudomonas fluorescens* SBW25 (Howden *et al.*, 2009), *P. fluorescens* EBC191 (Kiziak *et al.*, 2005), *Alcaligenes faecalis* JM3 (Kobayashi *et al.*, 1993) and *Pseudomonas putida* MTCC (Banerjee *et al.*, 2009). Bacteria may use nitrilases for the purpose of nitrogen utilization and nitrile detoxification, and for the modulation of plant, bacterial and fungal hormone signalling and gene regulation (Howden and Preston, 2009; Leveau and Preston, 2008; Liu and Nester, 2006; Yang *et al.*, 2007). The aim of this study was to determine whether the putative nitrilase genes Psyr_0007 of *P.s.*B728a and PSPTO_0189 of *P.s.*DC3000 encode active nitrilases, and, if so, whether these enzymes are capable of hydrolysing nitrile compounds found in the plant environment.

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METHODS AND RESULTS

Previous studies have shown that phylogenetically related nitrilases frequently hydrolyse similar substrates (Podar *et al*., 2005; Robertson *et al*., 2004). Psyr_0007 and PSPTO_0189 were found to group with characterized bacterial arylacetonitrilase enzymes in phylogenetic analyses (Fig. 1). Arylacetonitrilases have been extensively studied in plants, especially in *Arabidopsis thaliana* (reviewed by Piotrowski, 2008), in which they have been shown to catalyse the hydrolysis of arylacetonitriles synthesized during glucosinolate metabolism and to hydrolyse IAN to IAA (Vorwerk *et al*., 2001). Arylacetonitrilase enzymes have also been identified in a range of bacterial species (Banerjee *et al*., 2009; Kato *et al*., 2000; Kiziak *et al*., 2005; Kobayashi *et al*., 1993; Layh and Willetts, 1998). Many of the bacterial arylacetonitrilases studied to date have been hypothesized to hydrolyse plant-derived arylacetonitriles (Kiziak *et al*., 2005; Kobayashi *et al*., 1993).

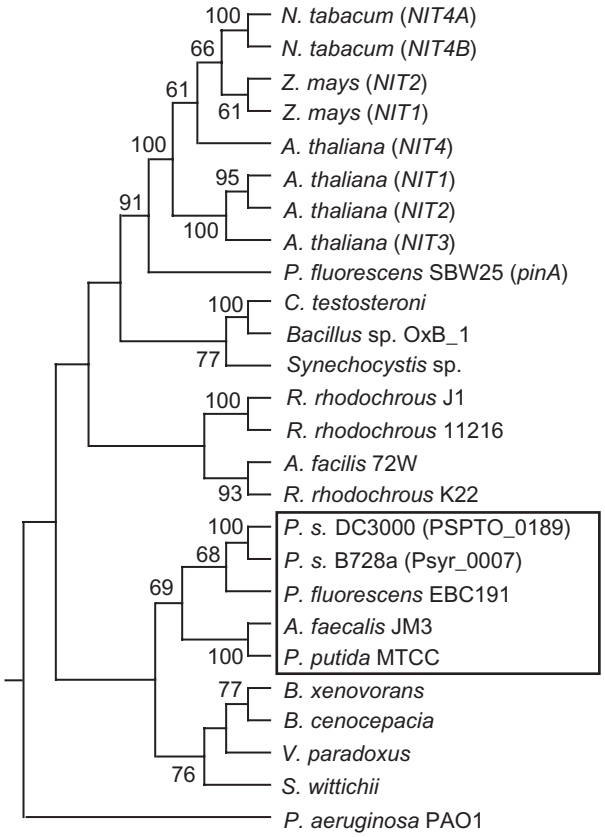

Fig. 1 Nitrilase genes from *Pseudomonas syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 group with genes encoding arylacetonitrilase enzymes. Amino acid sequences for Psyr_0007, PSPTO_0189 and a representative set of plant and bacterial nitrilases, including nitrilases representing each class of enzyme (aliphatic, aromatic and arylacetonitrile) were aligned using Se-Al (Rambaut, 1996), and a cladogram depicting the predicted phylogeny was generated by parsimony analysis using PAUP 4.0 (Sinauer Associates, Sunderland, MA, USA). Statistical support was provided using the bootstrap test with 500 replicates. Psyr_0007 and PSPTO_0189 group with enzymes predicted to have arylacetonitrilase activity, as indicated by the box. Full names of the organisms and GENBANK accession numbers are provided in Table S1 (see Supporting Information).

Examination of the genomic region surrounding a nitrilase gene can provide further insight into substrate preference and enzyme activity. For example, the nitrilase of *P. fluorescens* EBC191 has been shown to hydrolyse mandelonitrile and is located in close proximity to genes predicted to encode enzymes involved in the mandelate pathway (Kiziak *et al*., 2005). The nitrilase region of *Bacillus* sp. OxB-1 contains a phenylacetaldoxime (PAOx) dehydratase, which has been shown to dehydrate PAOx and indoleacetaldoxime (IAOx) to phenylacetonitrile and IAN, respectively (Asano and Kato, 1998; Kato *et al*., 2000). Aldoxime dehydration and nitrilase activity are linked, as the activity of PAOx dehydratase produces a nitrile compound that can subsequently be hydrolysed by nitrilase activity (Asano and Kato, 1998).

The nitrilase regions of *P.s*.B728a and *P.s*.DC3000 are highly conserved, as can be seen in Fig. 2. Of particular interest are the genes on either side of Psyr_0007, which encode a putative PAOx dehydratase (Psyr_0006) (previously reported by Feil *et al*., 2005) and a putative positive transcriptional regulator belonging to the AraC family (Psyr_0008). An AraC-type transcriptional regulator (*nitR*) has also been found in the nitrilase regions of *Rhodococcus rhodochrous* J1 and *Bacillus* sp. OxB-1, and has been shown in *R. rhodochrous* to be essential for nitrilase activity (Kato *et al*., 2000; Komeda *et al*., 1996). This suggests that Psyr_0008 may regulate nitrilase activity. The putative PAOx dehydratase Psyr_0006 may enable *P.s*.B728a to metabolize aldoximes and to dehydrate IAOx to IAN, which can then be converted to IAA by the activity of Psyr_0007. The final gene in the putative nitrilase operon, Psyr_0005, is similar to *Acinetobacter* sp. ADP1 ACIAD1619 (which is also flanked by genes predicted to be involved in aldoxime and nitrile metabolism), and to genes located in gene clusters involved in phenol and alcohol metabolism, such as the *qbdB* gene of *Pseudomonas putida* and the *chnX* gene of *Acinetobacter* sp. SE19 (Cheng *et al*., 2000; Toyama *et al*., 2003). Like other members of this gene family, Psyr_0005 has a putative signal peptide, indicating that it may be secreted. However, although *qbdB* has been shown to be co-regulated with the quinohaemoprotein alcohol dehydrogenase *qbdA* in a substrate-dependent manner (Promden *et al*., 2009), it has not yet been shown to be involved in phenol catabolism, and a *chnX* mutant of *Acinetobacter* sp. SE19 has been shown to retain the ability to degrade cyclohexanol (Cheng *et al*., 2000). Therefore, although the genomic context suggests that Psyr_0005 could have a role in aldoxime or nitrile metabolism, the function of this gene remains unknown.

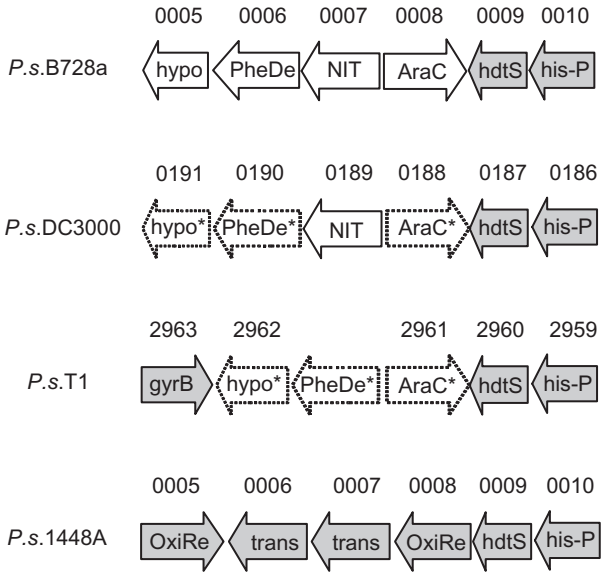

Fig. 2 The nitrilase region of *Pseudomonas syringae* pathovars. The nitrilase regions of *P. syringae* pv. *syringae* B728a (*P.s.*B728a), *P. syringae* pv. *tomato* DC3000 (*P.s.*DC3000), *P. syringae* pv. *tomato* T1 (*P.s.*T1) and the homologous region in *P. syringae* pv. *phaseolicola* 1448A (*P.s.*1448A) (defined by flanking sequences) were annotated using the *Pseudomonas* database (http://www.pseudomonas.com), PseudoDB (http://xbase.bham.ac.uk/pseudodb/) and the National Center for Biotechnology Information (NCBI) genome database (http://www.ncbi.nlm.nih.gov). Gene annotations are as follows: AraC, AraC-type transcriptional regulator; gyrB, DNA gyrase B; hdtS, hdtS-type protein; his-P, histidinol-phosphate phosphatase family protein; hypo, hypothetical protein; NIT, nitrilase; OxiRe, oxidoreductase; PheDe, phenylacetaldoxime dehydratase; trans, transposase. * indicates a predicted pseudogene. Genes outside the nitrilase region are shaded with a grey background. Genes are not drawn to scale. Gene numbers are shown above each gene without organism identifiers.

Interestingly, although the nitrilase flanking regions in *P.s.*B728a and *P.s.*DC3000 are highly conserved, all three of the nitrilase flanking genes in *P.s.*DC3000 appear to be pseudogenes, with the sequences terminated by premature stop codons relative to the full-length sequences in *P.s.*B728a. The nitrilase region is also partially conserved in *P.s.*T1 (Almeida *et al.*, 2009), which lacks the nitrilase, but contains pseudogenes corresponding to the AraC-type regulator, Psyr_0005, and the putative PAOx dehydratase (Fig. 2). In *P.s.*1448A, *P.s.*1-6 and the draft genome of *P. syringae* pv. *tabaci* ATCC11528 (*P.s.*11528, GENBANK ACHU00000000), the region extending from Psyr_0005 to Psyr_0008 is completely missing.

To investigate the nitrilase activity in *P. syringae*, *P.s.*B728a, *P.s.*DC3000 and *P.s.*1448A were screened for growth in modified M9 minimal medium (Sambrook and Russell, 2001) with 50 mM succinic acid as the carbon source and the sole nitrogen source a nitrile compound. Succinate was also employed as the carbon source in all subsequent experiments using M9. Under these conditions, bacteria will only grow if they can hydrolyse the nitrile compound to the corresponding carboxylic acid and ammonia, and use this ammonia as a nitrogen source (method described by Howden *et al.*, 2009). Nine different nitrile compounds were tested, representing each class of nitrile: aromatic (benzonitrile, *m*-toluonitrile, 2-cyanopyridine), aliphatic (β-cyano-L-alanine, acetonitrile, propionitrile) and arylacetonitriles (IAN, phenylpropionitrile (PPN), mandelonitrile). Each nitrile was tested at a concentration of 2 mM, except for IAN, which was used at a concentration of 1 mM because of the toxicity of this compound. Bacterial growth was also examined in M9 supplemented with nitrile compounds, and with ammonia as an alternative nitrogen source, to confirm that nitrile compounds were not toxic to bacteria at the concentrations used, and that bacteria were able to grow in M9 (data not shown). Nitrilase activity was only detected in *P.s.*B728a, which was able to use IAN and PPN, and which grew to an optical density at 600 nm (OD_{600}) of 0.349 ± 0.024 and 0.339 ± 0.026 in 1 mM IAN and 2 mM PPN, respectively, 24 h after inoculation. *P.s.*DC3000 and *P.s.*1448A were unable to use IAN or PPN as a nitrogen source. None of the three strains was able to use IAA as a carbon or nitrogen source (data not shown), which indicates that the final products of the nitrilase pathway in *P.s.*B728a are likely to be ammonia and IAA (or other arylacetonitrile-derived carboxylic acids, such as phenylpropionic acid).

In order to confirm that the nitrile hydrolysing activity of *P.s.*B728a was caused by Psyr_0007, this gene was mutated by insertional mutagenesis using a homologous recombination strategy. A 500-bp internal fragment from Psyr_0007 was amplified using the primers nitdeletion-F (5′-TGGTGCTGG ACAGTGTTCAG-3′) and nitdeletion-R (5′-AGCAGACCTTCCT CATGTTC-3′), cloned into the vector pCR2.1 using a TA Cloning Kit (Invitrogen, Paisley, Renfrewshire, UK), and transformed into electrocompetent *P.s.*B728a cells. A single homologous recombination event would result in the internal fragment inserting within the nitrilase gene, thus disrupting gene function and conferring kanamycin resistance. As Psyr_0007 is predicted to be upstream of Psyr_0006 and Psyr_0005 in a putative operon (Fig. 2), it is also possible that plasmid insertion will have a polar effect on the expression of these two genes. Site-specific recombination of the internal fragment in kanamycin-resistant transformants was confirmed by polymerase chain reaction (PCR) and sequencing. The resulting nitrilase mutant was named *P.s.*B728a*nit**. The nitrilase mutation was complemented using Psyr_0007 cloned into the broad host range expression vector pBroadgate. Psyr_0007 was amplified using the primers nit-F (5′-CACCGTCAGGAAAGCTCATAG-3′) and nit-R (5′-TGT TCAGGAATCGCTGAGTG-3′) and cloned into pENTR-D/TOPO by TOPO cloning (Invitrogen). The clone was checked by sequencing before being transferred to pBroadgate by Gateway cloning

(Invitrogen) to create pBroad*nit*. The full set of bacterial strains and plasmids used in this study is described in Table S2 (see Supporting Information).

Wild-type *P.s*.B728a, *P.s*.B728a*nit** and the complemented strain *P.s*.B728a*nit** (pBroad*nit*) were tested for their ability to use IAN and PPN as sole nitrogen sources. *P.s*.B728a*nit** was unable to grow in medium in which the sole nitrogen source was either IAN or PPN (Fig. 3), but growth could be rescued by complementation with pBroad*nit*, confirming that Psyr_0007 was responsible for the nitrilase activity observed in *P.s*.B728a. Interestingly, the growth of *P.s*.B728a*nit** was also moderately reduced in M9 with ammonia as the nitrogen source, and partially rescued by complementation with pBroad*nit* (Fig. S1, see Supporting Information). One possible explanation for this phenotype is that the disruption of Psyr_0007 results in the accumulation of endogenous nitriles that can no longer be degraded and which inhibit bacterial growth. The observation that complementation with Psyr_0007 only partially restores growth to the Psyr_0007 mutant may indicate that growth inhibition is also linked to the polar effect of Psyr_0007 disruption on expression of Psyr_0006. The disruption of PAOx dehydratase activity may cause the accumulation of aldoximes that can be toxic to bacteria (Asano and Kato, 1998).

Having shown that *P.s*.B728a can use IAN and PPN as a nitrogen source, and that Psyr_0007 is required for growth on IAN and PPN, we then carried out experiments to confirm that IAN was being converted to IAA as a result of nitrilase activity. IAN and IAA can be measured in solution using an established high-performance liquid chromatography (HPLC) method (Tsavkelova *et al*., 2007). The hydrolysis of IAN by nitrilase activity should result in the depletion of IAN in growth medium and the accumulation of IAA. Wild-type *P.s*.B728a, *P.s*.B728a*nit** and *P.s*.B728a*nit** (pBroad*nit*) were grown in M9 in which the sole nitrogen source was 1 mM IAN. After 24 h, IAN was virtually undetectable in cultures of *P.s*.B728a and *P.s*.B728a*nit** (pBroad*nit*), but remained at its initial concentration in medium inoculated with *P.s*.B728a*nit**. No IAA could be detected at 0 h, but, after 24 h, 0.73 mM and 0.69 mM IAA were detected in cultures of *P.s*.B728a and *P.s*.B728a*nit** (pBroad*nit*), respectively (Table 1). This verifies that Psyr_0007 is a nitrilase capable of hydrolysing IAN to IAA. Representative images from the complete HPLC dataset are shown in Fig. S2 (see Supporting Information).

The absence of detectable nitrilase activity in *P.s*.DC3000 indicates that the nitrilase gene is not expressed, or that the enzyme it encodes is unable to hydrolyse IAN. The nitrilase from *P.s*.B728a was heterologously expressed in *P.s*.DC3000 to determine whether the constitutive expression of Psyr_0007 could confer nitrile hydrolysing activity. *P.s*.DC3000 was transformed with pBroad*nit*, and a control strain was generated by transformation with empty vector (pBroad). When inoculated into medium with IAN or PPN as the sole nitrogen source, neither

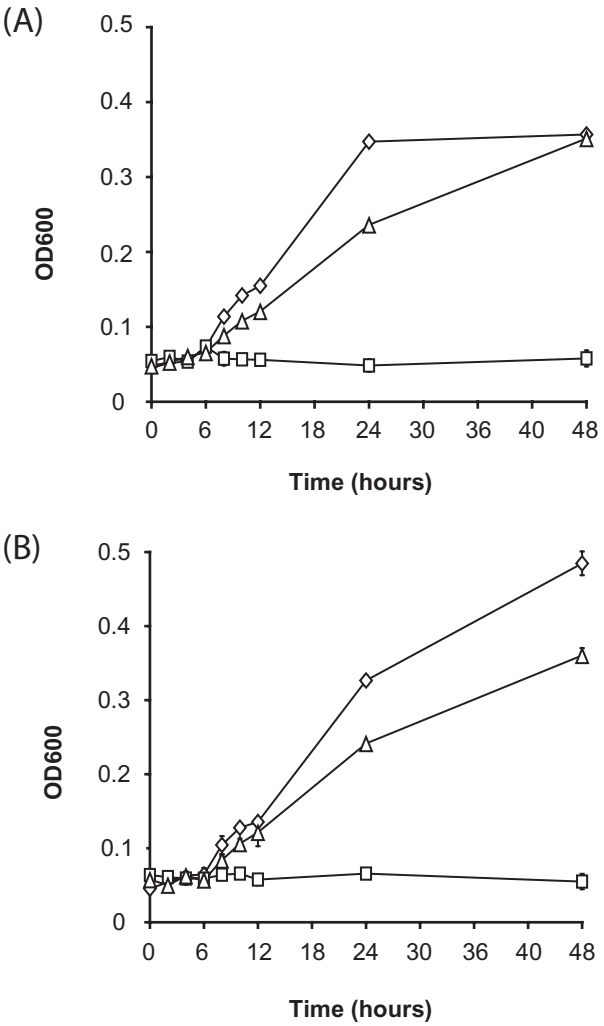

Fig. 3 *Pseudomonas syringae* pv. *syringae* B728a has arylacetonitrilase activity and is capable of hydrolysing indole-3-acetonitrile (IAN) and phenylpropionitrile (PPN). *Pseudomonas syringae* pv. *syringae* B728a (*P.s*.B728a) (◇), *P. syringae* pv. *syringae* B728a*nit** (*P.s*.B728a*nit**) (□) and *P. syringae* pv. *syringae* B728a*nit** (pBroad*nit*) (Δ) were tested for their ability to use IAN and PPN as a nitrogen source. Strains were grown in M9 minimal medium, and growth was monitored over 48 h by measuring the optical density at 600 nm (OD_{600}) of cell cultures. (A) Bacterial growth in M9 lacking ammonia, supplemented with 1 mM IAN as the sole nitrogen source. (B) Bacterial growth in M9 lacking ammonia, supplemented with 2 mM PPN as the sole nitrogen source. Each value represents the average of three replicates from one experiment ± standard deviation. The experiment was carried out three times, producing similar results.

P.s.DC3000 (pBroad) nor *P.s*.DC3000 (pBroad*nit*) showed a significant increase in population density after 24 h (data not shown). However, HPLC analysis revealed that *P.s*.DC3000 (pBroad*nit*) hydrolysed a small quantity of IAN to IAA during this time period, whereas *P.s*.DC3000 (pBroad) failed to hydrolyse IAN (Table 1; Fig. S3, see Supporting Information). As

Table 1 Conversion of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) by *Pseudomonas syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000.

	0 h		24 h	
	IAN (mM)	IAA (mM)	IAN (mM)	IAA (mM)
*P.s.*B728a	1.08 ± 0.06*	0	0	0.73 ± 0.05
*nit**	1.06 ± 0.16	0	1.17 ± 0.03	0.01 ± 0.001
*nit** (pBroad*nit*)	1.10 ± 0.09	0	0.03 ± 0.04	0.69 ± 0.16
*P.s.*DC3000 (pBroad)	1.17 ± 0.04	0	1.12 ± 0.09	0
*P.s.*DC3000 (pBroad*nit*)	1.13 ± 0.10	0	0.84 ± 0.03	0.21 ± 0.04

*Strains were grown in M9 with 1 mM IAN as the sole nitrogen source. Samples were taken at 0 and 24 h and filtered using a Millex-HV 0.45-μm syringe filter unit (Millipore, Watford, UK). Ten microlitre samples were injected on to a reverse-phase C18 column (Agilent, West Lothian, UK) and analysed using a Beckman (Beckman, High Wycombe, UK) Coulter PF2D fractionation system. Samples were run in a mobile phase of water–acetonitrile–acetic acid (40 : 60 : 1, v/v) pH 2.5, at a flow rate of 1 mL/min, and the absorbance was measured at a wavelength of 254 nm. Peak retention times were compared with IAN and IAA standards run according to the same procedure. IAN had a retention time of 7.10 min and IAA had a retention time of 4.55 min. The concentrations of IAN and IAA were estimated using standard curves generated using known concentrations of these chemicals. Each value represents the average of three replicates, and the standard deviation is shown. The experiment was carried out twice, producing similar results.

*P.s.*DC3000 (pBroad) and *P.s.*DC3000 (pBroad*nit*) were able to grow in complete medium supplemented with 2 mM IAA or 1 mM IAN (data not shown), it seems likely that the inability of *P.s.*DC3000 (pBroad*nit*) to grow using IAN as a nitrogen source is a result of a low rate of nitrile uptake or nitrilase activity relative to *P.s.*B728a and *P.s.*B728a*nit** (pBroad*nit*), rather than inhibition by IAN or IAA. This, in turn, suggests that *P.s.*B728a contains genes in addition to Psyr_0007 that promote nitrile uptake or nitrilase activity. These may include the genes adjacent to Psyr_0007, which are pseudogenes in *P.s.*DC3000, or additional genes that have yet to be identified.

DISCUSSION

Nitrilase enzymes have previously been identified in plants, animals, bacteria and fungi. However, this is the first demonstration of nitrilase activity in *P. syringae*. We have shown that the Psyr_0007 gene of *P.s.*B728a encodes an arylacetonitrilase, which is capable of hydrolysing IAN and PPN. The location of this nitrilase gene next to a putative PAOx dehydratase (Psyr_0006) suggests a role for this nitrilase in aldoxime metabolism, nutrient assimilation, auxin synthesis or tolerance to plant secondary metabolites. Interestingly, the analysis of available genome sequence data, including draft genome sequence data, shows that genes similar to Psyr_0006 and Psyr_0007 are present in a wide range of plant pathogenic microorganisms, and are particularly common in members of the Ascomycota, including plant pathogens such as *Gibberella moniliformis*, *Fusarium oxysporum* f. sp. *lycopersici*, *Botryotinea fuckeliana*, *Sclerotinia sclerotiorum*, *Pyrenophora tritici-repentis*, *Alternaria brassicicola* and *Verticillium dahliae* (Tables S3 and S4, see Supporting Information). However, our analyses have also provided evidence that nitrilase and PAOx dehydratase enzymes have been lost in many agriculturally important lineages of *P. syringae*, particularly when viewed in relation to current models of *P. syringae* evolution (Sarkar and Guttman, 2004). This raises an interesting question. Why has the nitrilase gene, or nitrilase activity, been lost in some strains of *P. syringae*, such as *P.s.*DC3000 and *P.s.*1448A, but retained in *P.s.*B728a?

There are several hypotheses that could explain why *P.s.*B728a might benefit from possessing genes for IAA synthesis from IAOx and IAN. IAA has previously been shown to be an important pathogenicity or virulence factor for a number of pathogens, enabling them to subvert plant defence mechanisms and enhance the colonization of the plant environment (Ding *et al*., 2008; Kazan and Manners, 2009; Robinette and Matthysse, 1990; Spoel and Dong, 2008; Wang *et al*., 2007). Furthermore, increased IAA levels may cause modifications to plant physiology, such as alterations in nutrient trafficking, cell expansion and cell wall loosening, which would, in turn, lead to an increase in available nutrients (Catala *et al*., 2000; Lindow and Brandl, 2003).

However, if IAA synthesis is beneficial for plant colonization, why is IAN nitrilase activity present in *P.s.*B728a and not in the other strains examined? There are several possible explanations. Firstly, it is possible that the IAN pathway is of particular value for epiphytic growth, where IAA synthesis may help stimulate the release of nutrients to the plant surface (Brandl and Lindow, 1998; Lindow and Brandl, 2003; Manulis *et al*., 1998). *P.s.*B728a has been described as a more effective epiphyte than *P.s.*DC3000, and has been reported to reach 100-fold higher population densities than *P.s.*DC3000 on plant leaves (Feil *et al*., 2005). It is conceivable that the IAN pathway has specific advantages in an epiphytic context, because it uses different endogenous or plant-derived substrates from other IAA synthesis pathways. Interestingly, strains of *P. syringae* pv. *syringae* are

among the few plant pathogenic bacteria reported to contain both tryptophan-dependent and tryptophan-independent pathways for IAA synthesis (Fett *et al*., 1987; Glickmann *et al*., 1998), and a mutant of *P. syringae* pv. *syringae* Y30 lacking the tryptophan-2-monooxygenase (IaaM) IAA synthesis pathway has been reported to show little or no reduction in epiphytic growth on bean (Mazzola and White, 1994).

A second explanation for the presence of nitrilase activity in *P.s.*B728a and the absence of nitrilase activity in other *P. syringae* strains could be that the primary role of this enzyme, and the adjacent PAOx dehydratase, is not to produce IAA and other auxins, but rather to metabolize toxic aldoximes and nitriles to nontoxic carboxylic acids. The importance of PAOx dehydratase and nitrilase activity would then be expected to vary according to the quantity and chemical nature of the aldoximes and nitriles produced by host plants, and the extent to which bacteria are exposed to these compounds during host colonization. Aldoximes and nitriles are produced during the synthesis and degradation of plant defence molecules, such as glucosinolates, cyanogenic glycosides and phytoalexins (Glawischnig, 2007; Halkier and Gershenzon, 2006; Nafisi *et al*., 2007; Vetter, 2000). However, glucosinolates and cyanogenic glycosides are generally sequestered in plant vacuoles (Halkier and Gershenzon, 2006; Vetter, 2000), whereas *P. syringae* multiplies biotrophically in the intercellular spaces between plant cells; therefore, *P. syringae* may not encounter high levels of these compounds and their derivatives unless cell death occurs. Consistent with this, *P.s.*DC3000 is able to colonize the glucosinolate-producing plant *Arabidopsis thaliana* (Buell *et al*., 2003; Preston, 2000), despite its inability to degrade nitriles *in vitro*. However, our results do not exclude the possibility that the nitrilase gene present in *P.s.*DC3000 is specifically induced during growth in plant tissues.

In considering whether there is likely to be a link between nitrilase activity and host specificity, it may be worth noting that, although *P. syringae* pv. *syringae* has been reported to have a broad host range, the four nitrilase-deficient pathovars, *P. syringae* pv. *tomato*, pv. *tabaci*, pv. *phaseolicola* and pv. *oryzae*, colonize tomato, tobacco, bean and rice, respectively, all of which may produce relatively low levels of aldoximes and nitriles compared with some plant species. They produce terpenoid and flavonoid phytoalexins derived from the shikimate, acetate-malonate and acetate-mevalonate pathways rather than the aldoxime pathway (Hammerschmidt, 1999), do not produce glucosinolates (Halkier and Gershenzon, 2006) and, in the case of tomato and tobacco, lack the ability to produce cyanogenic glycosides (Eich, 2008; Vetter, 2000); therefore, it is possible that *P. syringae* strains that colonize these hosts have relatively little need to use PAOx dehydratase and nitrilase activity for protection or nutrient assimilation. In addition, all four species appear to lack the IAOx/IAN pathway for auxin biosynthesis (Cohen *et al*., 2003; Dohmoto *et al*., 2000; Piotrowski, 2008; Schneider and Wightman, 1974); therefore, *P. syringae* is unlikely to encounter plant-derived IAN that can be converted to IAA when colonizing these species.

Finally, it is possible that the absence of nitrilase genes or nitrilase activity in several genome-sequenced *P. syringae* strains reflects the fact that other virulence factors produced by these strains are able to perform an equivalent role in terms of the up-regulation of expression of IAA-responsive genes in order to modulate host physiology and suppress host defences, thereby reducing the need to produce IAA using nitrilase activity. For example, the jasmonate-mimicking toxin coronatine, produced by *P.s.*DC3000, and the type III-secreted effector protein AvrRpt2, produced by *P.s.*T1, have both been shown to cause increased expression of IAA-responsive genes during pathogenesis (Chen *et al*., 2007; Kazan and Manners, 2009; Uppalapati *et al*., 2005). Furthermore, IAA synthesis by IAN-independent pathways is relatively common in *P. syringae* (Glickmann *et al*., 1998), which could result in functional redundancy in terms of IAA synthesis.

To summarize, this study has shown that *P.s.*B728a possesses a functional arylacetonitrilase capable of hydrolysing IAN to IAA. Although a homologue of the *P.s.*B728a nitrilase gene is present in *P.s.*DC3000, *P.s.*DC3000 appears to be incapable of hydrolysing IAN, and only gains weak nitrilase activity when transformed with the *P.s.*B728a nitrilase gene, suggesting that additional genes are required for efficient nitrile uptake or full nitrilase activity. Further work must now be conducted in order to fully understand the evolution and function of nitrilase activity in *P. syringae*. Research priorities include: (i) the identification of the genes that act upstream of Psyr_0007 to generate nitrile substrates; (ii) the determination of whether the AraC-like regulator Psyr_0008 regulates the expression of the nitrilase operon; (iii) the establishment of whether the nitrile region is used for the endogenous synthesis of IAA; and (iv) the investigation of the expression and role of nitrilase activity during plant colonization and pathogenesis. Preliminary investigations using the *P.s.*B728a nitrilase mutant described in this paper have revealed that the mutant is impaired in the colonization of bean pods (A. Rico and G. M. Preston, unpublished work), but it was not possible to distinguish whether this was caused by the role played by nitrilase in plant interactions, or by the general growth defect observed for the mutant *in vitro*, as *P.s.*B728a*nit** showed significantly reduced growth relative to *P.s.*B728a in both M9 (Fig. S1) and in media such as *hrp*-inducing minimal medium (HIM; Huynh *et al*., 1989) and mannitol–glutamate medium (MG; Keane *et al*., 1970), which are thought to more closely mimic the plant environment (data not shown).

The observation that PAOx dehydratase and nitrilase-like genes are widespread in plant pathogenic microorganisms clearly indicates that it will be worth extending studies of PAOx dehydratase and IAN nitrilase activity to other plant–pathogen

systems. Future studies will be able to take full advantage of the increasing amount of genome sequence data available for *P. syringae* and other plant pathogens to further explore the evolution and ecology of these intriguing enzymes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Growth of *Pseudomonas syringae* pv. *syringae* B728a (*P.s.*B728a) (◇), *P. syringae* pv. *syringae* B728a*nit**

(*P.s*.B728a*nit**) (□) and *P. syringae* pv. *syringae* B728a*nit** (pBroad*nit*) (Δ) in M9 minimal medium with ammonia as the nitrogen source. *P.s*.B728a, *P.s*.B728a*nit** and pBroad*nit* were grown in M9 minimal medium to assess whether the nitrilase mutation confers a general fitness effect. Growth was monitored over 48 h. The data show the average of three replicates ± standard deviation. The experiment was carried out twice, producing similar results.

Fig. S2 High-performance liquid chromatography (HPLC) spectra showing the nitrilase-dependent production of indole-3-acetic acid (IAA) from indole-3-acetonitrile (IAN) by *Pseudomonas syringae* pv. *syringae* B728a. *Pseudomonas syringae* pv. *syringae* B728a (*P.s*.B728a), *P. syringae* pv. *syringae* B728a*nit** (*P.s*.B728a*nit**) and *P. syringae* pv. *syringae* B728a*nit** (pBroad-*nit*) were grown in M9 minimal medium with 1 mM IAN as the sole nitrogen source. Hydrolysis of IAN by nitrilase activity results in the depletion of IAN in the growth medium and the synthesis of IAA. Samples were taken at 0 and 24 h and analysed by HPLC. Representative spectra for each strain at each time point are shown and are labelled as follows: *P.s*.B728a at 0 h (A) and 24 h (B); *P.s*.B728a*nit** at 0 h (C) and 24 h (D); and *P.s*.B728a*nit** (pBroad*nit*) at 0 h (E) and 24 h (F).

Fig. S3 Heterologous expression of Psyr_0007 in *Pseudomonas syringae* pv. *tomato* DC3000 confers indole-3-acetonitrile (IAN) hydrolysing activity. *Pseudomonas syringae* pv. *tomato* DC3000 strains transformed with pBroadgate (A) or pBroadgate carrying Psyr_0007 (pBroad*nit*) (B) were grown in M9 minimal medium with 1 mM IAN as the sole nitrogen source. Samples were taken from the bacterial cultures after 0 and 24 h for the measurement of IAN and IAA by high-performance liquid chromatography (HPLC). Representative HPLC spectra at 24 h are shown.

Table S1 GenBank accession numbers for the protein sequences used in Fig. 1.

Table S2 Bacterial strains and plasmids used in this study.

Table S3 TBLASTN hits to Psyr_0007 (nitrilase) in whole-genome shotgun sequences.

Table S4 TBLASTN hits to Psyr_0006 (phenylacetaldoxime dehydratase) in whole-genome shotgun sequences.

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