Paerucumarin, a New Metabolite Produced by the *pvc* Gene Cluster from *Pseudomonas aeruginosa*[▽][†]

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The *pvc* gene cluster from *Pseudomonas aeruginosa* has been linked to the biosynthesis of both the pyoverdine chromophore and pseudoverdine. Our reinvestigation of the role this gene cluster plays in *P. aeruginosa* secondary metabolite biosynthesis shows that its major product is actually paerucumarin, a novel isonitrile functionalized cumarin.

One of the most extensively studied families of secondary metabolites in pseudomonads is the pyoverdines, cyclic peptide siderophores that are important virulence factors in *Pseudomonas aeruginosa* (16, 19, 24, 25). Pyoverdines contain both a variable cyclic peptide moiety and a conserved tricyclic chromophore. In *P. aeruginosa*, the biosynthesis of the pyoverdine chromophore has been attributed to two different loci: a non-ribosomal peptide synthase known as *pvdL* and a four-gene operon known as the *pvc* gene cluster (Fig. 1) (18, 22, 23). The *pvc* gene cluster is unique in that it is also reported to be involved in the biosynthesis of pseudoverdine, a fluorescent bicyclic metabolite that resembles the pyoverdine chromophore (15). Our recent observation that many homologs of *pvcA* are actually isonitrile synthases led us to reinvestigate the role that the *pvc* gene cluster plays in *P. aeruginosa* secondary metabolite biosynthesis (1, 2). Here we report the characterization of paerucumarin, an isonitrile functionalized cumarin that is produced by the *pvc* gene cluster, and demonstrate that the *pvc* genes are not required for pyoverdine production.

To better understand the role of the *pvc* cluster in *P. aeruginosa* secondary metabolite production, the *pvc* operon was PCR amplified and cloned into the broad-host-range expression vector pMMB67, and the resulting construct, pPVCAD, was conjugated into *P. aeruginosa* PAK (8, 13). A comparison of the ethyl acetate extracts derived from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced and uninduced cultures by silica gel thin-layer chromatography indicated that the overexpression of the *pvc* gene cluster led to the production of a single organic extractable metabolite. This compound was purified by silica gel flash chromatography, and the structure was elucidated by using a combination of mass spectrometry and nuclear magnetic resonance experiments (1). The final structure was confirmed by single crystal X-ray diffraction (Fig. 2) and found to be a novel isonitrile-functionalized dihydroxycumarin, which we have given the trivial name paerucumarin (for *P. aeruginosa* cumarin).

The biosynthesis of paerucumarin can be easily rationalized from the general function predictions for each of the enzymes found in the *pvc* gene cluster (Fig. 1). PvcA is related to known isonitrile synthases (2), PvcB is related to amino acid oxidizing enzymes (2), and PvcC and D are related to HpaB- and HpaC-like two-component flavin adenine dinucleotide-dependent monooxygenases that have been shown to oxidize phenols to both dihydroxy phenols and catechols (9, 26). In the biosynthetic scheme proposed in Fig. 1b, PvcA generates an isonitrile-functionalized tyrosine (compound 3) that is oxidized by PvcB to give the unsaturated intermediate compound 4. Oxidation of intermediate compound 4 to a catechol (compound 5) by PvcC/D, followed by intermolecular cyclization of the catechol and the carboxylate, would give paerucumarin (compound 1).

In our initial thin-layer chromatography analyses of the extracts derived from *P. aeruginosa* cultures that overexpress the *pvc* gene cluster, we did not detect pseudoverdine, the *N*-formyl adduct of paerucumarin that was previously reported to arise from the overexpression of the *pvc* gene cluster (15). However, upon closer inspection of these extracts by liquid chromatography-mass spectrometry (LC-MS), we were able to identify a minor IPTG-inducible peak that corresponds to the predicted mass for pseudoverdine (molecular weight of 221) (Fig. 2). In *Escherichia coli*-based studies with other *pvcA* containing gene clusters, both isonitrile and *N*-formyl functionalized metabolites have also been observed, and in at least one case a *pvcA* homolog containing gene cluster was found to produce exclusively an *N*-formyl adduct (1). Isonitrile and N-formyl functional groups are very closely related, differing by only the addition or loss of water. The isolation of both isonitrile and *N*-formyl functionalized metabolites from cultures that express different *pvcA* containing gene clusters suggests that individual members of this family of enzymes may actually produce mixtures of these two functionalities. Although it is possible that paerucumarin was not found in earlier studies on the *pvc* gene cluster because isonitrile functionalized metabolites are unstable and often rapidly decompose during purification, it is also possible that different *pvc* gene clusters produce different ratios of isonitrile and N-formyl functionalized metabolites.

It is known that the *pvc* gene cluster is positively regulated by PtxR, a LysR transcription factor that also controls exotoxin A

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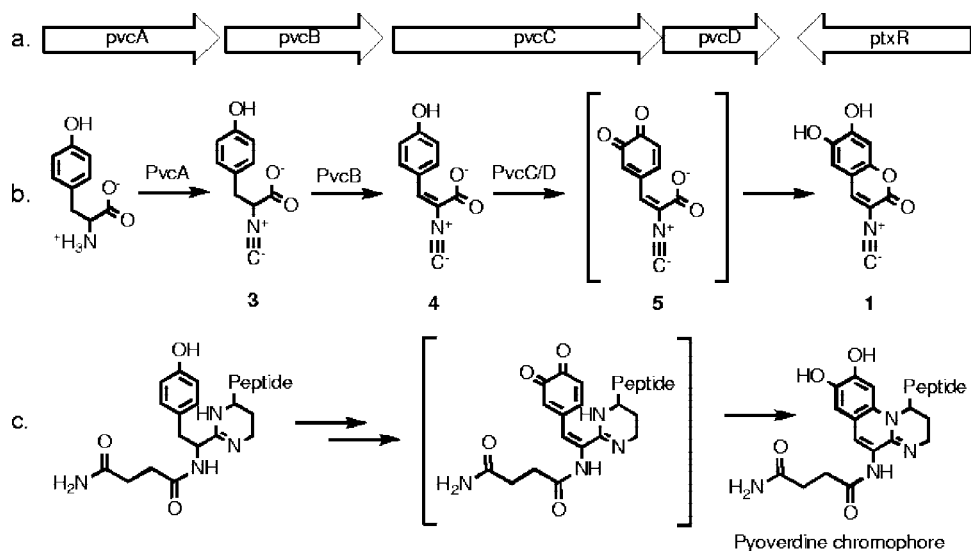
FIG. 1. (a) *pvc* gene cluster. (b) Proposed biosynthetic scheme for paerucumarin. (c) Proposed biosynthetic scheme for the pyoverdine chromophore (6).

production through *regA* and homoserine lactone production through *lasI* (3–5, 7, 10, 21, 23). To investigate the products of *pvc* gene clusters from different *P. aeruginosa* strains, we over-expressed PtxR in PAO1, PAK, and PA14 strains. Culture broth extracts from each of these strains were then examined by LC-MS (for methods, see the supplemental material). The LC-MS analysis showed that each strain produced a different ratio of paerucumarin to pseudoverdine (Fig. 3). PAK and
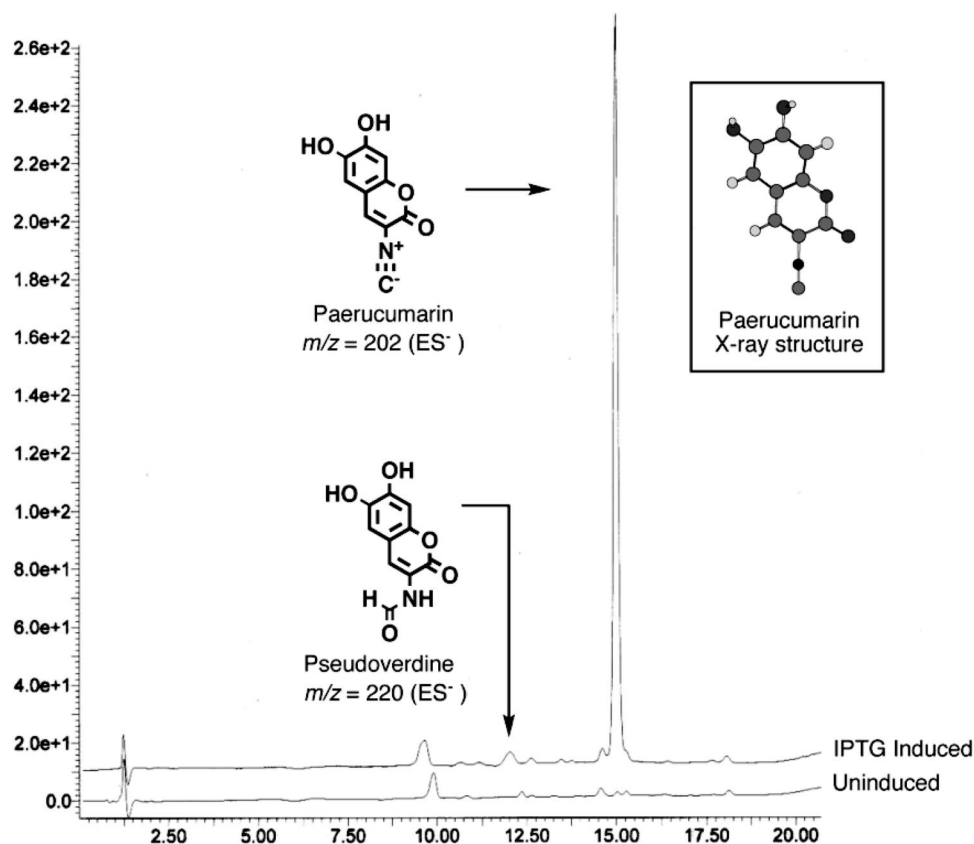

FIG. 2. LC-MS (negative-ion electrospray ionization) traces of the ethyl acetate extracts derived from IPTG-induced and uninduced cultures of *P. aeruginosa* transformed with pPVCAD are shown.
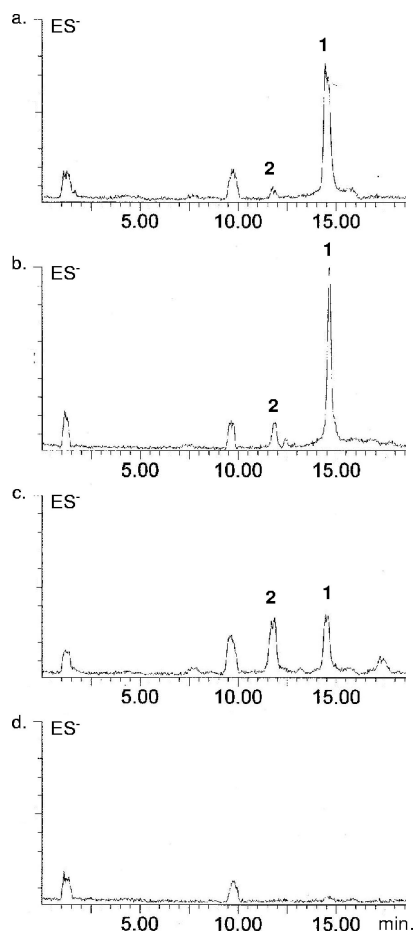
FIG. 3. LC-MS (negative-ion electrospray ionization) traces of ethyl acetate extracts from cultures of PAK (a), PAO1 (b), and PA14 (c) strains transformed with pPTXR and strain PAK (d) transformed with a vector control. Peaks for paerucumarin (peak 1) and pseudoverdine (peak 2) are marked.

TABLE 1. Pyoverdine and paerucumarin production by PAK strains

Strain (genotype)/plasmid	Production[a] of:	
	Pyoverdine	Paerucumarin
PAK	+	−
PAK/pPVCAD	+	+
PAK/pPTXR	+	+
PAK (ΔpvcA)	+	−
PAK (ΔpvcA-ptxR)	+	−
PAK (ΔpvcA)/pPTXR	+	−
PAK2 (ΔpvdI)	−	−
PAK2 (ΔpvdI)/pPTXR	−	+
PAK2 (ΔpvdI)/pPVCAD	−	+

[a] Production of pyoverdine was monitored in SM medium. Production of paerucumarin was monitored in LB medium.

dine biosynthesis transposon knockouts (12, 20). Under the culture conditions we examined (Luria-Bertani broth, succinate minimal medium [SM] [17], and SM supplemented with 1% Casamino Acids) *pvc* gene cluster knockouts continued to produced pyoverdines (Table 1 and Fig. 4) (14). Although similar reactions may be used in the biosynthesis of both paerucumarin and the pyoverdine chromophore, it appears that only paerucumarin and pseudoverdine are produced by the *pvc* gene cluster.

PvcA and PvcB homologs are found in a number of different bacteria, including *Frankia* sp. strain CcI3, *Erwinia carotovora*, *Photorhabdus luminescens*, *Burkholderia mallei*, *Bdellovibrio bacteriovorus*, *Legionella pneumophila*, and *Vibrio cholerae*. Even though many of these bacteria are well-studied pathogens, no functions have yet been assigned to any of the metabolites that are produced by *pvcA/B*-containing gene clusters. In *L. pneumophila* the *pvcA/B* homologs are regulated in sessile (biofilm) cells with respect to iron, and in *P. aeruginosa* the *pvc* gene cluster expression is controlled by the same transcription factor that controls at least two other important pathogenicity determinants (3, 10, 11). Although the functional significance of the strain-to-strain differences in paerucumarin and pseudoverdine production by *P. aeruginosa* is still unknown, the assignment of a well-defined function to the *pvc* gene cluster should make it possible to more easily decipher the various roles that *pvc*-like biosynthetic clusters play in bacteria.
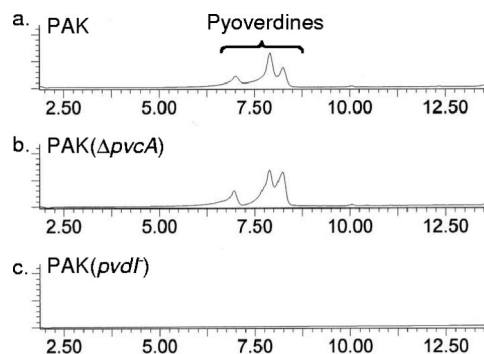
PAO1 produce almost exclusively paerucumarin, while PA14 produces approximately a one-to-one mixture of paerucumarin and pseudoverdine. Neither paerucumarin nor pseudoverdine was detected in extracts from strains that did not overexpress PtxR, nor was either compound seen in extracts from *pvcA* knockouts that overexpressed PtxR. *pvcA* genes from different sequenced *P. aeruginosa* strains contain a small number of point mutations. Whether these mutations result in the strain-to-strain difference we observed, or whether other factors contribute to the observed differences will likely require a detailed structural analysis of at least one PvcA homolog.

In addition to its role in the biosynthesis of pseudoverdine and now paerucumarin the *pvc* gene cluster has been reported to be involved in the biosynthesis of pyoverdine (22, 23). The catechol formation and intermolecular cyclization steps in the proposed biosynthetic scheme for paerucumarin are very similar to the biosynthetic steps that have been proposed for the formation of the pyoverdine chromophore (Fig. 2c) (6). To explore the possibility that the *pvc* gene cluster might play a role in pyoverdine biosynthesis, we generated both *pvc* gene cluster deletion mutants (ΔpvcA and ΔpvcA-ptxR) and pyover-

FIG. 4. High-pressure liquid chromatography (UV) traces of the material extracted by solid-phase extraction from cultures of strains PAK (a), PAK (ΔpvcA) (b), and PAK2 (ΔpvdI) (c).

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