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Biosynthesis of Novel Pyoverdines by Domain Substitution in a Nonribosomal Peptide Synthetase of *Pseudomonas aeruginosa*

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Pyoverdine is a fluorescent nonribosomal peptide siderophore made by fluorescent pseudomonads. The *Pseudomonas aeruginosa* nonribosomal peptide synthetase (NRPS) PvdD contains two modules that each incorporate an L-threonine residue at the C-terminal end of pyoverdine. In an attempt to generate modified pyoverdine peptides, we substituted alternative-substrate-specifying adenylation (A) and peptide bond-catalyzing condensation (C) domains into the second module of PvdD. When just the A domain was substituted, the resulting strains produced only wild-type pyoverdine—at high levels if the introduced A domain specified threonine or at trace levels otherwise. The high levels of pyoverdine synthesis observed whenever the introduced A domain specified threonine indicated that these nonnative A domains were able to communicate effectively with the PvdD C domain. Moreover, the unexpected observation that non-threonine-specifying A domains nevertheless incorporated threonine into pyoverdine suggests that the native PvdD C domain exhibited stronger selectivity than these A domains for the incorporated amino acid substrate (i.e., misactivation of a threonine residue by the introduced A domains was more frequent than misincorporation of a nonthreonine residue by the PvdD C domain). In contrast, substitution of both the C and A domains of PvdD generated high yields of rationally modified pyoverdines in two instances, these pyoverdines having either a lysine or a serine residue in place of the terminal threonine. However, C-A domain substitution more commonly yielded a truncated peptide product, likely due to stalling of synthesis on a nonfunctional recombinant NRPS template.

Nonribosomal peptide synthetases (NRPS) are microbial enzymes that can assemble diverse products from a pool of over 500 possible amino acid substrates (1). According to the most recent published report, there are 1,164 different nonribosomal peptides currently known (2), many of which are medically and industrially relevant (e.g., antibiotics, immunosuppressants, and anticancer agents) (3). The diversity of products is possible due to a modular enzymatic assembly line-like mechanism wherein, at least in linear syntheses, each NRPS module selects and incorporates a specific substrate into the final product.

With their complex structures often precluding effective *in vitro* synthesis, many clinical derivatives of natural products are created by semisynthesis, a process whereby the natural product is chemically modified after isolation from a biological source (4–6). However, the modular nature of NRPS enzymes offers enticing prospects for targeted modification of products at a biosynthetic level by altering the substrate specificity of individual modules. This could allow the creation of altered scaffolds for drug development or open up the possibility to create *de novo* NRPS pathways.

During NRPS-mediated peptide synthesis, discrete adenylation (A), thiolation (T), and condensation (C) domains act in substrate activation, transfer, and condensation, respectively (Fig. 1). Additional accessory domains can be present to alter monomers, such as an epimerization (E) domain that can be present between the T and C domains of adjacent modules and that catalyzes epimerization of the donor substrate to a D isomer prior to condensation by the C domain (7). The final product is typically released by a thioesterase (TE) domain associated with the terminal module in the NRPS complex.

Previous efforts to generate novel peptide products *in vivo* by NRPS manipulation have involved either (i) replacement of the A domain by one that activates an alternative substrate, (ii) alteration of the substrate binding pocket of the A domain, or (iii) substitutions that treat C-A domains as inseparable pairs (8). However, in nearly all cases, these manipulations have resulted in little to no product being made. *In vitro* observations have indicated that A domain manipulation strategies may be limited by the upstream C domain exhibiting specificity for the acceptor substrate (Fig. 1B) (9, 10), and it has also been inferred from structural studies that A domain substitutions may disrupt an essential C-A domain interface (11). C-A domain substitutions avoid both of these issues and yet have also enjoyed very little success.

This work uses the enzyme PvdD as a model to better understand constraints to NRPS domain substitution *in vivo*. PvdD from *Pseudomonas aeruginosa* PAO1 is a bimodular NRPS that incorporates the final two threonine residues into pyoverdine (12), a product synthesized from four NRPS enzymes comprising 12 modules (Fig. 2A). Pyoverdine consists of a conserved chromophore attached to a variable peptide chain (13) (Fig. 2C). The

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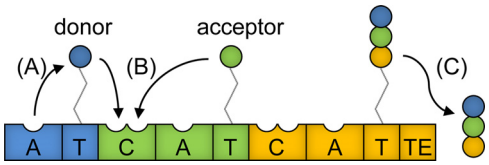

FIG 1 Domain arrangement within a hypothetical three-module NRPS comprised of an initiation (blue), an elongation (green), and a termination (orange) module. Numerous elongation modules may be present, with each incorporating an additional residue into the peptide product. Each module is activated by posttranslational attachment of a 4′-phosphopantetheine (4′-pp) cofactor to the T domain (also referred to as a peptidyl carrier protein [PCP] domain). (A) The first domain to act within each module is an A domain, which activates and tethers a specific monomer via a thioester bond to the 4′-pp prosthetic group of the immediately downstream T domain. (B) Starting at the first elongation module, the C domain condenses the upstream donor substrate onto the downstream acceptor substrate. This reaction breaks the upstream thioester bond, resulting in the newly formed peptide being attached to the downstream T domain, and this peptide serves as the donor substrate for the C domain of the next module. (C) In this way, the growing peptide chain is passed down the modules, and following addition of a final monomer by the termination module, the peptide product is released by a TE domain. Detailed analyses of the structures and functions of individual NRPS domains are provided in several reviews (3, 37–39).

variable peptide chains of pyoverdines are highly diverse between different strains of fluorescent pseudomonads (14), an observation that provided this work with a range of evolutionarily related modules for domain substitutions (Fig. 2A and B). Other major advantages of this model system are that the pyoverdine chromophore is easily detectable *in vivo* due to characteristic absorbance and fluorescence and that, as a siderophore, an absolute requirement for pyoverdine synthesis can be imposed by growing the *P. aeruginosa* host strain in an iron-restricted environment.

Previous domain substitution experiments in the first module of *pvdD* found that, consistent with A domain substitutions being limited by C domain acceptor site specificity, A domain substitutions were successful when the introduced A domain specified threonine but not otherwise (15). In contrast, C-A domain substitutions were all nonfunctional (15). However, the previous work had several limitations, including the facts that the sample size of substituted domains was small, the substituted domains were of both eukaryotic and prokaryotic origin, and the substitutions were introduced into the first module of *pvdD*. With regard to the last point, because this module must interact with the upstream NRPS enzyme in *trans*, it is unknown whether critical interactions between PvdJ and PvdD may have been disrupted by the C-A domain substitution. Another hypothesis for the lack of activity observed with C-A domain substitutions was that C domains might also exhibit some level of donor site specificity, so that the introduced C domain could prove incompatible with the incoming residue supplied by the upstream NRPS module, or that a newly incorporated amino acid substrate might not be recognized at the donor site of the C domain immediately downstream (15).

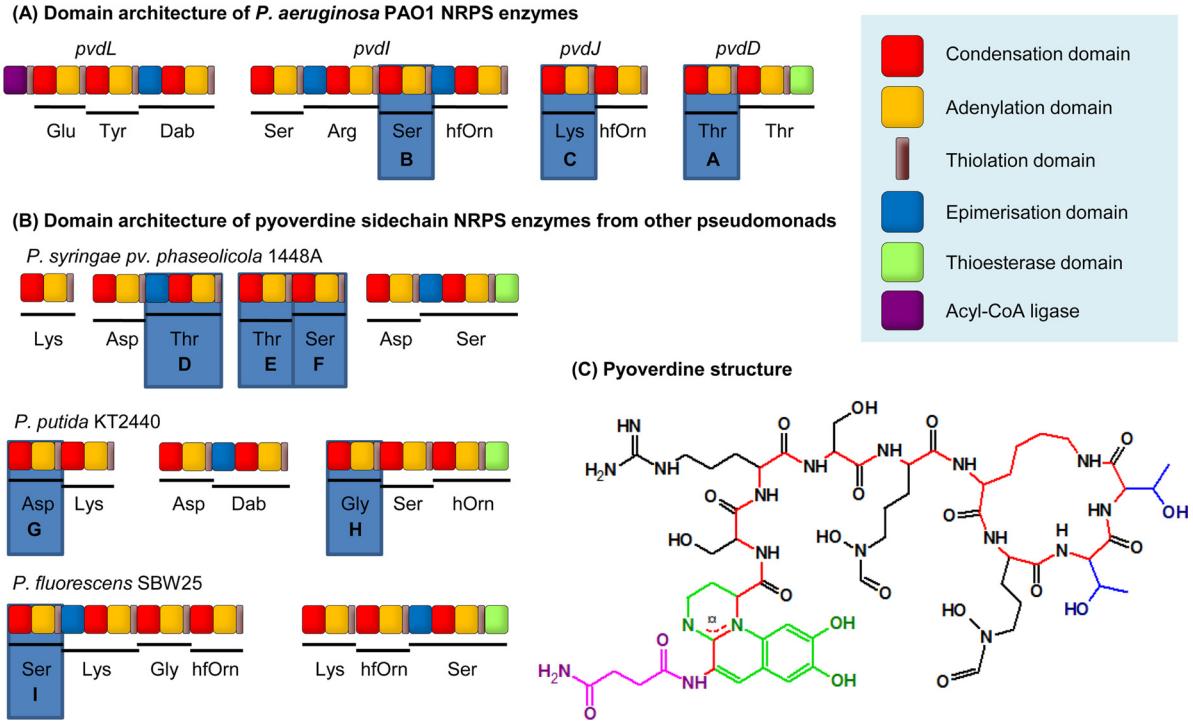

FIG 2 NRPS enzymes, labeled from A to I, with modules used for domain substitutions highlighted. (A) Domain architecture of the four NRPS enzymes involved in *P. aeruginosa* PAO1 pyoverdine synthesis, based on previous annotation (15). (B) Domain architecture of the NRPS enzymes involved in synthesis of the pyoverdine variable peptide chains from three other *Pseudomonas* strains. An additional NRPS, PvdL (as shown in panel A), is conserved in each strain and incorporates the residues that later form the chromophore. The figure is based on previous annotation by Owen and Ackerley (40) (*P. syringae* 1448a), Moon et al. (41) (*P. fluorescens* SBW25), and Ravel and Cornelis (42; adapted with permission of the publisher) (*P. putida* KT2440). (C) Structure of pyoverdine from *P. aeruginosa* PAO1 (43, 44) with the succinimide group highlighted in pink, the chromophore in green, the peptide backbone in red, and the side chains of the threonine residues incorporated by PvdD in blue. Dab, 2,4-diaminobutyric acid; hfOrn, L-N^5-formyl-N^5-hydroxyornithine; hOrn, N^5-hydroxyornithine.

TABLE 1 Oligonucleotide primers used in this study

Function	Name	Sequence (5′→3′)[b]
Primers for pSMC	CATfwd	CCCCGAATTCCATATGCAAGCACTCATAGAGAAGGTG
	CATrev	CCCCActagTCAATCCCTGGGCGAACGC
	TTEFwd	CGCCCAGGGATTGTCTAGAGCGGCCGCGTCGCAGCAGGCCTATCGAGCGC
	TTERev	CCCCGAGCTCTCAGCGCCCGGCACGCTCCAGG
Primers for pSMA	Thr-WT_CAfwd	GGGGTCTAGAACGACGGATGCGGTCTCGACGA
	CATCrev	ATAGGCCTGCTGCGACGCGGCCGCTCTAGAATCAGCGCAACCGCCTGT
Forward primers for C-A domain substitutions	Thr-A_CAfwd	CCCCTCTAGAGGTGTCAATCTCTTCGAG
	Ser-B_CAfwd	CCCTCTAGAGCGGCGAGCGCTGCCCCTGTGCT
	Lys-C_CAfwd	CCCTCTAGATTCGCCCGCCTGCCGATCCCGC
	Thr-D_CAfwd[a]	CCCCTCTAGAACGACGGATGCGGTCTCGACGATACCGCTTGCCGATCGGC AGGAAGATATCTACCCGCTGTCGC
	Thr-E_CAfwd	CCCCTCTAGAAATCTCTACGGGGTCACACCGA
	Ser-F_CAfwd	CCCCTCTAGAGGGCAGGGCAATGCTGCG
	Asp-G_CAfwd	CCCTCTAGAGATTTCGCGCTGTTGCCGATCGCG
	Gly-H_CAfwd	CCTCTAGAAACCTCTACGGGGTGACGCGCAT
	Ser-I_CAfwd	CCCTCTAGAGACTTTTCCCGGTTTCCGATTCC
Forward primers for A domain substitutions	Thr-WT_Afwd	CCCCTCTAGACGAACAACGGTTGAGCTA
	Thr-A_Afwd	CCCCTCTAGACGAACAACGGTTGAGCTA
	Ser-B_Afwd	CCCCTCTAGAGGAACGCCTGGACTACGCCGAG
	Lys-C_Afwd	CCTCTAGAGGGGCAGGCCTTGAGCTACGCC
	Thr-D_Afwd	CCCCTCTAGATGGCGAGCAATTGAGATA
	Thr-E_Afwd	CCCCTCTAGATGGCGAGCAATTGAGCTA
	Ser-F_Afwd	CCCCTCTAGAGGCTGAACAACTGAGCTA
	Asp-G_Afwd	CCCTCTAGACGATGGCTCGCTCAGTTACGGC
	Gly-H_Afwd	CCCTCTAGACGAGCAGACCTTGAGCTACGCCG
	Ser-I_Afwd	CCCTCTAGATGCAACCACGCTGACCTACGCCC
Reverse primers for CA domain and A domain substitutions	Thr-WT_Rev	CCCCGCGGCCGCATCCGGTTGCGGCAACGCCTGC
	Thr-A_Rev	CCCCGCGGCCGCATCCGGTTGCGGCAACGCCTGC
	Ser-B_Rev	CCCCGCGGCCGCTTGCGGTCGCGGCAGCGCCTTG
	Lys-C_Rev	CCCCGCGGCCGCATCCGGCGCGGGCAGCGCCTTG
	Ser-F_Rev	CCCCGCGGCCGCGTCTGGTGTCGGCAGGGC
	Asp-G_Rev	CCCCCGCGGCCGCGTCCGGCAGCGGCAATCTGGCC
	Gly-H_Rev	CCCCGCGGCCGCGTCAGGCGCCGGCAGCGCCTTG
	Ser-I_Rev	CCCCGCGGCCGCATCCGGCAATGGCAAGGCCTTG

[a] Due to lower sequence identity of $^{D}C_{L}$ domains (C domains that receive a D-amino acid at their donor site), a linker of 42 nucleotides was inserted to shift the recombination site further downstream.
[b] Restriction sites are underlined.

In this study, we used instead the second module of PvdD for domain substitution. This approach avoided the possibility that C-A domain substitution in the first module might disrupt critical interactions between PvdD and PvdJ. Moreover, targeting the final module of the NRPS template meant that introduction of a novel amino acid into a growing peptide chain could not cause donor site incompatibility with the C domain immediately downstream. By conducting a large number of carefully selected C-A domain substitutions and analyzing their abilities to generate a pyoverdine product relative to the corresponding A domain substitutions, we were able not only to create rationally modified pyoverdines with a high yield, but also to gain insight into factors that restrict the functionality of NRPS enzymes with domains substituted *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5α, *P. aeruginosa* PAO1, and *Pseudomonas putida* KT2440 were sourced from existing Ackerley laboratory stocks. *Pseudomonas syringae* pv. phaseolicola 1448A was generously provided by John Mansfield (Imperial College, London, United Kingdom) and *Pseudomonas fluorescens* SBW25 by Paul Rainey (Massey University, Auckland, New Zealand). All strains were grown in LB medium with shaking at 200 rpm at 28°C for *P. syringae* pv. phaseolicola 1448A and 37°C for the other strains. For maintenance of plasmids, tetracycline was added to a final concentration of 15 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa* PAO1.

DNA methods. The PCR primers and plasmids used in this study are shown in Tables 1 and 2, respectively. The percent amino acid identity and similarity shared between each introduced A domain and the module 2 A domain of PvdD is listed in Table 3. Primers were designed to amplify pyoverdine NRPS domains from the genomes of fluorescent pseudomonads based on the annotation detailed in Fig. 2, as well as the polyketide synthase (PKS)/NRPS analysis tools available online (http://nrps.igs.umaryland.edu/nrps/; 16). PCRs used Phusion DNA polymerase (Finnzymes, Espoo, Finland). Plasmids were constructed from pSW196 (17) in *E. coli* DH5α and then transformed into a previously characterized *pvdD* deletion mutant (15) for single-copy integration at the *attB* locus (18).

TABLE 2 Plasmids used in this study

Plasmid	Brief description	Reference
pSW196	Integration-proficient vector; P_{BAD} promoter	17
pSMC	pSW196-based staging plasmid allowing the substitution of C-A domains into *pvdD*	This study
pSMC::ThrWT	pSMC with C-A domains from the second module of *pvdD*	This study
pSMC::CAThrA	pSMC with C-A domains from module A	This study
pSMC::CASerB	pSMC with C-A domains from module B	This study
pSMC::CALysC	pSMC with C-A domains from module C	This study
pSMC::CAThrD	pSMC with C-A domains from module D	This study
pSMC::CAThrE	pSMC with C-A domains from module E	This study
pSMC::CASerF	pSMC with C-A domains from module F	This study
pSMC::CAAspG	pSMC with C-A domains from module G	This study
pSMC::CAGlyH	pSMC with C-A domains from module H	This study
pSMC::CASerI	pSMC with C-A domains from module I	This study
pSMA	pSW196-based staging plasmid allowing the substitution of A domains into *pvdD*	This study
pSMA::ThrWT	pSMC with C domain from the second module of *pvdD*	This study
pSMA::ThrA	pSMA with A domain from module A	This study
pSMA::SerB	pSMA with A domain from module B	This study
pSMA::LysC	pSMA with A domain from module C	This study
pSMA::ThrD	pSMA with A domain from module D	This study
pSMA::ThrE	pSMA with A domain from module E	This study
pSMA::SerF	pSMA with A domain from module F	This study
pSMA::AspG	pSMA with A domain from module G	This study
pSMA::GlyH	pSMA with A domain from module H	This study
pSMA::SerI	pSMA with A domain from module I	This study

To generate the substitution plasmid pSMC (Fig. 3B), the first module of *pvdD* was PCR amplified using primers CATfwd and CATrev and ligated into pSW196 (17); then, the T-TE domains from the second module of *pvdD* were amplified using primers TTEFwd and TTERev and inserted downstream of the first module. pSMC contained SpeI and NotI restriction sites between the C-A-T and T-TE domains to enable addition of C-A domains. To create pSMA (Fig. 3A), the C domain from the second module of *pvdD* was amplified using primers Thr-WT_CAfwd and CATCrev and ligated into the SpeI and NotI restriction sites of pSMC using compatible XbaI and NotI restriction sites added by the PCR primers. This destroyed the SpeI restriction site immediately upstream of the C domain so that a new SpeI site added by CATCrev would be downstream of the introduced C domain. A and C-A domains were then amplified from genomic DNA and ligated into the corresponding plasmids pSMA and pSMC using XbaI and NotI restriction sites. Plasmids created in this work were sequence verified by Macrogen Inc. (Seoul, South Korea).

TABLE 3 Amino acid sequence homology of introduced A domains aligned against the module 2 PvdD A domain

Substitution[a]	% identity	% similarity	% gaps
Thr-A	99.6	99.8	0.0
Ser-B	50.6	64.1	7.3
Lys-C	52.3	64.9	5.9
Thr-D	64.7	77.4	2.3
Thr-E	65.1	77.3	3.5
Ser-F	47.6	61.5	5.7
Asp-G	40.7	56.3	5.3
Gly-H	47.1	62.3	6.8
Ser-I	46.8	61.7	6.9
Thr-*snbC* m1	**53.2**	**64.5**	**4.5**
Thr-*syrB* m9	**44.1**	**60.2**	**5.4**
Cys-*acvA* m2	**36.2**	**54.0**	**11.8**
Ser-*pvdI* m1	**51.4**	**64.8**	**5.7**
Val-*acvA* m3	**36.4**	**54.7**	**10.8**

[a] Substitutions created in this study are aligned with the A domain from Thr-WT. Substitutions from previous work (15) are in boldface. The nomenclature for A domain substitutions from the previous work shows the substrate activated by the module followed by the gene the module was sourced from and the number of the module within the named gene (15).

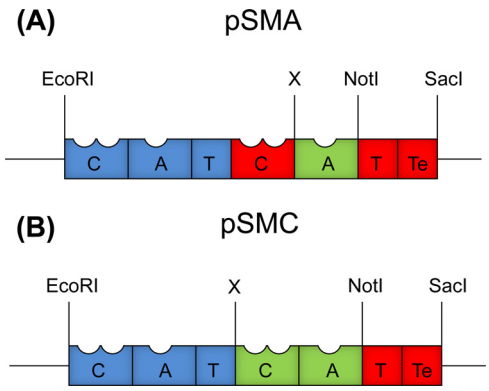

FIG 3 Domain arrangement within substitution plasmids pSMA (A) and pSMC (B) used in this work. The domains colored blue and red were derived from the first and second modules of *pvdD*, respectively. The domains in green were absent in the substitution plasmids and indicate where alternative domains would be added. X refers to a SpeI restriction site that was destroyed when ligating A and C-A domains into the plasmids via a compatible XbaI sticky end.

Measurement of pyoverdine production. To detect pyoverdine production on solid media, strains were inoculated onto iron-limiting King's B agar plates (20 g/liter Bacto peptone, 1.5 g/liter K_2HPO_4, 1% [vol/vol] glycerol, 6.1 mM $MgSO_4$, and 1.5% [wt/vol] agar) in both the presence and absence of the iron chelator ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA; final concentration, 200 μg/ml). In the presence of EDDHA, mutants deficient in pyoverdine production are unable to grow due to their inability to passively take up iron (15). Plates were incubated for 24 h at 37°C, and then growth was assessed visually and the presence or absence of pyoverdine was determined by fluorescence under UV light.

To measure pyoverdine levels in liquid media, strains were grown in 200 μl of LB medium in a 96-well plate for 24 h at 37°C. This starter culture was used to inoculate M9 medium [6 g/liter K_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter $(NH_4)_2SO_4$, and 6.1 mM $MgSO_4$] containing 0.2% (wt/vol) L-arabinose and 4 g/liter succinate (pH 7.0) at a 20× dilution with a total volume of 200 μl. After 24 h of growth, the cultures were centrifuged to pellet bacteria, and then 100 μl of supernatant was transferred to a fresh 96-well plate. The supernatant was diluted 2 times in fresh M9 medium to give a total volume of 200 μl. Fluorescence (excitation, 400 nm; emission, 440 nm) and absorbance (400 nm) were measured using an EnSpire 2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

Mass spectrometry. Samples were mixed with matrix solution (500 μl acetonitrile, 500 μl ultrapure water, 1 μl trifluoroacetic acid, 10 μg α-cyano-4-hydroxycinnamic acid) in a volumetric sample-to-matrix ratio ranging from 1:5 to 1:20. Aliquots of 1 μl from each sample were spotted in duplicate onto an Opti-TOF 384-well matrix-assisted laser desorption ionization (MALDI) plate (Applied Biosystems, Foster City, CA) and allowed to dry at room temperature. The spots were analyzed using a MALDI-time of flight (TOF)/TOF 5800 mass spectrometer (Applied Biosystems) in positive-ion mode. Each spot was externally calibrated using cal2 calibration mixture (Applied Biosystems). Peaks in spectra were labeled in Data Explorer (Applied Biosystems).

RESULTS

Selection of alternative A and C-A domains for substitution experiments. To create domain substitution variants of *pvdD*, A and C-A domains were sourced from the nine modules labeled A to I in Fig. 2A and B. These modules are involved in pyoverdine synthesis by four different fluorescent *Pseudomonas* species. The modules were selected to provide a range of activated substrates (threonine, serine, glycine, aspartate, and lysine), as well as modules that receive peptide chains with different C-terminal amino acids (arginine, N^5-formyl-N^5-hydroxyornithine, aspartate, threonine, and diaminobutyric acid) into the donor site of their C domains. All selected modules, with the exception of module D, receive an L-amino acid at the C domain donor site in their native contexts.

Generation and phenotypic evaluation of A domain substitution strains. Based on previous results (15), we reasoned that substituted A domains might be functional in an autonomous sense but limited by C domain acceptor site specificity within the recombinant NRPS environment. To test this, A domain substitutions were created by individual PCR amplifications of each of the A domains highlighted in Fig. 2, followed by ligation into a module 2 A domain deletion construct of *pvdD* in the plasmid pSMA (a complementation plasmid that integrates into the *P. aeruginosa* chromosome using the integrase ϕCTX [18]) (Fig. 3A). Recombination junctions in pSMA were chosen based on the equivalent location having previously been shown to be permissive of A domain substitutions in the first module of PvdD (15). Once created, A domain substitution variants were transformed into a previously characterized *pvdD* deletion mutant (15). The resulting substitution strains are referred to in terms of the substrate specified by the new A domain followed by the letter of the module designated in Fig. 2; for example, the variant containing the A domain from the first module of *pvdD* is referred to as strain Thr-A. In addition to the A domain substitution variants, a positive-control strain (Thr-WT) complemented by the wild-type *pvdD* module 2 A domain (with introduced restriction sites as shown in Fig. 3A) was generated and demonstrated to synthesize pyoverdine at levels similar to those for wild-type *P. aeruginosa* PAO1. This indicated that the introduced restriction sites did not impair function and that complementation of *pvdD* by a single gene copy expressed from the genome-integrated pSMA plasmid did not reduce pyoverdine production, as had been seen in previous work using a high-copy-number complementation plasmid (15).

As initial qualitative tests for pyoverdine production, the Thr-WT positive control, nine A domain substitution strains, and a pyoverdine negative control (a *pvdD* deletion strain containing the empty pSW196 plasmid from which pSMA was derived) were streaked on iron-limiting King's B agar plates in both the presence and absence of EDDHA. The three threonine-specifying A domain substitution strains (Thr-A, Thr-D, and Thr-E) each exhibited increased levels of fluorescence compared to the pyoverdine negative control and were also viable in the presence of EDDHA (Fig. 4A). The strain Thr-E had substantially reduced fluorescence and viability relative to Thr-A, Thr-D, or Thr-WT, indicating reduced pyoverdine synthesis, but was still clearly viable. In contrast, none of the nonthreonine A domain substitution strains were viable in the presence of EDDHA and all appeared to have levels of fluorescence similar to that of the negative control. However, due to the subjective nature of this test, it was difficult to ascertain whether there might have been small increases in fluorescence for these strains.

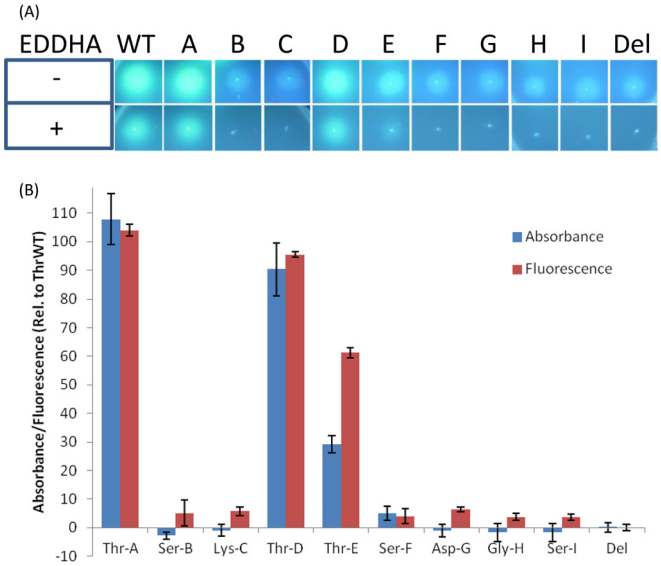

FIG 4 Detection of pyoverdine production by A domain substitution variants. (A) Fluorescence of A domain substitution strains on King's B agar plates in the absence (−) or presence (+) of EDDHA. The plates were inoculated with the positive-control strain Thr-WT (WT), A domain substitution strains A to I (Fig. 2), and the *pvdD* deletion pyoverdine negative control (Del) and then incubated for 24 h at 37°C. The photographs were taken under UV light. (B) Percentages of pyoverdine production from A domain substitution strains grown in liquid media. The values are expressed as percentages of absorbance (400 nm) or fluorescence (excitation, 400 nm; emission, 440 nm) relative to the Thr-WT strain and were zeroed against the background levels recorded for the *pvdD* deletion mutant. The data are the means of 6 independent replicates, and the error bars indicate 1 standard deviation.

To more accurately quantify levels of pyoverdine, all A domain substitution strains were grown in liquid media, and their pyoverdine levels were measured by both absorbance (400 nm) and fluorescence (excitation, 400 nm; emission, 440 nm) of the supernatant (Fig. 4B). Both measurements were used because initial tests of pyoverdine serial dilutions found that absorbance followed a linear trend whereas fluorescence became saturated (see Fig. S1 in the supplemental material). This meant absorbance was generally more accurate in terms of quantifying pyoverdine levels than fluorescence; however, fluorescence was more sensitive and was able to detect pyoverdine at much lower levels.

The three threonine-specifying A domain substitution strains exhibited increased absorbance and fluorescence compared to all the other strains. The two substitution strains that were most viable in the presence of EDDHA (Thr-A and Thr-D) each had levels of fluorescence and absorbance similar to those of the Thr-WT strain. The strain Thr-E had absorbance levels at approximately 29% relative to Thr-WT, confirming lower levels of pyoverdine synthesis. In contrast, the remaining (non-threonine-specifying) A domain substitution strains all had levels of absorbance similar to that of the *pvdD* deletion mutant, with a possible small increase in absorbance observed for strain Ser-F. Surprisingly, each of these non-threonine-specifying A domain substitution strains appeared to have a small increase in fluorescence compared to the *pvdD* deletion strain. This suggested the non-threonine-specify-

ing A domain substitution strains might be producing, at very low levels, an unknown pyoverdine-like product.

Detection of A domain substitution products by mass spectrometry. The synthesis of pyoverdine by the A domain substitution strains was confirmed using mass spectrometry. For each of the A domain substitution strains, an ion was detected with an *m/z* ratio within 0.05% of 1,333.6 (see Fig. S2 in the supplemental material); this was the expected *m/z* for pyoverdine containing a succinimide acyl group (19). This product was confirmed to be wild-type pyoverdine by collision-induced dissociation (CID) (see Fig. S3 and Table S1 in the supplemental material) and was not detected from the pyoverdine-negative *pvdD* deletion strain. These results confirm that both the threonine- and non-threonine-specifying A domain substitution strains synthesized wild-type pyoverdine, the former efficiently and the latter at only trace levels.

Generation and testing of C-A domain substitution strains. Substituting C-A domain pairs together bypasses the potential restrictions of C domain acceptor site specificity and avoids disruption of the C-A domain interface. However, four C-A domain substitutions attempted previously in the first module of *pvdD* all yielded nonfunctional hybrid enzymes (15). To more rigorously test whether C-A domain substitutions could provide a route to new pyoverdines, nine C-A domain substitutions corresponding to each of the highlighted modules in Fig. 2 were created by ligation of PCR-amplified C-A domains into a *pvdD* module 2 C-A domain deletion construct in the plasmid pSMC (Fig. 3B). The recombination junction between the T and C domains in pSMC was selected to be 37 amino acid residues downstream of the conserved serine residue of the T domain, a linker region shown to be tolerant of modifications in other NRPS enzymes (20, 21). Complementation strains were generated by integration of these C-A domain substitution constructs into the *pvdD* mutant and were named according to the convention used for the A domain substitution strains but with a CA prefix (for example, the variant containing the C-A domains from the first module of *pvdD* is referred to as strain CAThr-A). The positive-control strain, CAThr-WT, showed no reduction in pyoverdine synthesis compared to wild-type *P. aeruginosa*, indicating that the introduction of restriction sites had not impaired function.

When the C-A domain substitution strains were grown on solid King's B medium in the absence of EDDHA, only strains CAThr-A and CALys-C had levels of fluorescence that were visibly higher than the background of the pyoverdine negative control (Fig. 5A). However, in the presence of EDDHA, CAThr-A, CALys-C, and CASer-F all yielded visible levels of growth (Fig. 5A). Interestingly, in contrast to the A domain substitutions, only one threonine-specifying C-A domain substitution was functional. Of the two nonfunctional threonine-specifying C-A domain substitutions, strain CAThr-D contained a C domain that receives a D-amino acid from the upstream module in its native context; due to the stereospecificity of C domain donor sites (10), it was not expected to be able to accommodate the L-threonine at the C terminus of the peptide chain received from the upstream module of PvdD. However, strain CAThr-E, which contained not only an A domain that was functional in the A domain substitution experiments, but also a C domain that "expects" in its native context to receive L-threonine at its donor site, also failed to produce detectable levels of fluorescence.

When the pyoverdine absorbances of the supernatants of C-A domain substitution strains grown in liquid media were measured (Fig. 5B, blue bars), strains CAThr-A and CALys-C gave high levels of absorbance at 83% and 76% of the levels of the CAThr-WT positive control. Strain CASer-F, which exhibited low levels of growth on solid King's B medium containing EDDHA (Fig. 5A), had much lower absorbance at 18% of CAThr-WT levels. The other C-A domain substitution strains all had much lower levels of absorbance. However, when the fluorescence of the supernatant was measured (Fig. 5B, red bars), all C-A domain substitution strains except strain CAAsp-G still had elevated levels of fluorescence relative to the negative control.

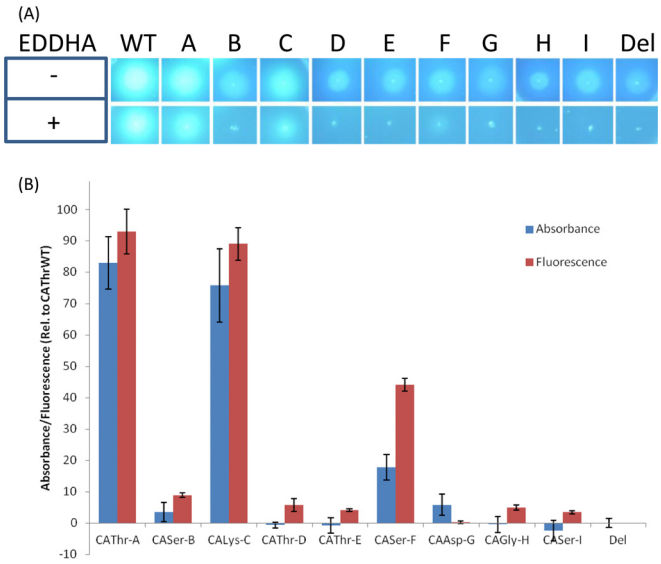

FIG 5 Detection of pyoverdine production by C-A domain substitution variants. (A) Fluorescence of C-A domain substitutions strains on King's B agar plates in the absence (−) or presence (+) of EDDHA. The plates were inoculated with the positive-control strain Thr-WT (WT), C-A domain substitution strains A to I (Fig. 2), and the *pvdD* deletion pyoverdine negative control (Del) and then incubated for 24 h at 37°C. The photographs were taken under UV light. (B) Percentages of pyoverdine production from C-A domain substitution strains grown in liquid media. The values are expressed as percentages of absorbance (400 nm) or fluorescence (excitation, 400 nm; emission, 440 nm) relative to the CAThr-WT strain and were zeroed against the background levels recorded for the *pvdD* deletion mutant. The data are the means of 6 independent replicates, and the error bars indicate 1 standard deviation.

Detection of pyoverdine from C-A domain substitution strains by mass spectrometry. The synthesis of wild-type pyoverdine by strain CAThr-A and incorporation of lysine and serine into pyoverdine by strains CALys-C and CASer-F were confirmed by mass spectrometry. Assuming that cyclization of the peptide was still possible, replacement of the terminal threonine of pyoverdine with lysine or serine was expected to change the mass of pyoverdine by +27 Da and −14 Da, respectively. Consistent with this, products of *m/z* 1,333.6 from CAThr-A, 1,360.7 from CALys-C, and 1,319.6 from CASer-F were detected by mass spectrometry (see Fig. S4 in the supplemental material). From the CID spectra of the *m/z* 1,360.7 and 1,319.6 pyoverdine species (see Fig. S5A and Table S2 in the supplemental material), ions expected to contain the new lysine and serine residues had mass changes consistent with the presence of the altered residue. These results confirm that the CALys-C and CASer-F domain substitution strains incorporated new residues into pyoverdine.

In contrast, no full-length pyoverdine was detected from the other C-A domain substitution strains. Instead, a product of only *m/z* 991.5 was detected from all these strains except strain CAAsp-G, for which no pyoverdine was detected (see Fig. S4 in the supplemental material). The *m/z* 991.5 product was consistent with pyoverdine missing the three terminal residues and has been detected previously as a breakdown product when the final L-threonine residue was not incorporated onto pyoverdine (15). Ions produced by CID were also consistent with this product (see Fig. S5B and Table S3 in the supplemental material). Interestingly, the *m/z* 991.5 product was also detected as a minor peak during mass spectrometry of the CAThr-A, CALys-C, and CASer-F C-A domain substitution strains (see Fig. S5B and Table S3 in the supplemental material). This product was not detected for any of the A domain substitution strains above the signal/noise ratio of 20 set for CID, although a smaller peak did appear to be present for the strain Lys-C and may have been present but buried in the spectra for other A domain substitution strains (see Fig. S2 in the supplemental material).

DISCUSSION

In this study, the *pvdD* mutant strains expressing PvdD variants with A domain substitutions that specify threonine ($n = 3$) all synthesized pyoverdine relatively effectively. In contrast, non-threonine-specifying A domains ($n = 6$) yielded only trace levels of pyoverdine. It is possible that higher levels of sequence identity shared between the introduced threonine-specifying A domains and the PvdD module 2 A domain contributed in part to this successful outcome. The PvdD module 1 A domain in strain Thr-A shares 99.6% amino acid identity with the module 2 A domain, so it is not at all surprising that it proved a successful replacement, while the A domains substituted into Thr-D and Thr-E shared on average 17.3% greater amino acid identity with the native module 2 A domain than did the six inactive, non-threonine-specifying A domains (Table 3). Nonetheless, the observation that 3/3 threonine-specifying A domains were active while 6/6 non-threonine-specifying A domains were inactive provides strong evidence that substrate specificity plays a major role in determining whether a construct with an A domain substitution will be active.

This observation is consistent with an earlier pilot study conducted in the first module of *pvdD*, where 2/2 threonine-specifying A domains were active (despite being derived from different bacterial genera and sharing substantially lower levels of amino acid identity than the A domains used in this study), whereas 3/3 non-threonine-specifying A domains were inactive (15). Collectively, these results provide strong evidence in support of the hypothesis that C domain acceptor site substrate specificity plays a major role in determining whether an introduced A domain will be active in a nonnative context (9, 10), whereas the disruption of a specialized C-A domain interface that evolution has optimized (hypothesized to be a potential limiting factor [11]) was not a major factor restricting A domain substitution in our system.

The C domain acceptor site specificity hypothesis receives additional support from our novel observation that the non-threonine-specifying A domain substitution strains nevertheless produced low levels of wild-type pyoverdine. A likely scenario to explain the production of wild-type pyoverdine by these recombinant PvdD enzymes is low-level "leaky" activation of noncognate substrates, including threonine, followed by exclusive selection for threonine within the C domain acceptor site. Low-level promiscuous activities have frequently been observed for A domains *in vitro* (22–26). However, our result was particularly interesting in that it allowed a direct comparison of the relative strengths of the substrate specificities of the A domain and the acceptor site of the C domain. If the substrate specificities of all the domains were approximately equivalent, then both modified and wild-type pyoverdines should be produced (that is, the alternative substrate preferred by the introduced A domain would be accepted at low levels by a leaky C domain). As no modified pyoverdine species were observed, it can be concluded that the C domain proofreading is very stringent in PvdD. It has been suggested that strong specificity within the C domain acceptor site in conjunction with release of incorrect amino acids by type II thioesterases is of great value in ensuring correct peptide assembly, as it is more energetically favorable to release monomers before condensation than to produce an entire nonfunctional peptide chain (27).

Further support for stringent C domain acceptor site proofreading was provided by the C-A domain substitutions, which confirmed that at least two of the non-threonine-specifying A domains had the potential to be active in the PvdD context. When substituted along with their native C domain partners, the lysine- and serine-specifying A domains from PvdJ of *P. aeruginosa* and an unnamed *P. syringae* pyoverdine NRPS exhibited high-level activation of their natural substrates. This successful rational introduction of new amino acid residues into pyoverdine confirms that novel pyoverdine peptides can be potentially generated at a high yield via C-A domain substitution.

In contrast, two of the A domains that had previously been functional in the A domain substitution strains Thr-D and Thr-E no longer yielded a full-length pyoverdine when substituted as a C-A domain pairing, suggesting that the newly introduced C domain impaired function. We considered that this might have been due to selectivity constraints at the donor site of the C domain. In contrast with the evidence that C domains exhibit stereospecificity but not side chain specificity at the donor site (9, 10), Stein et al. found that the second C domain of the tyrocidine NRPS system showed a degree of side chain specificity for the terminal amino acid of dipeptides artificially loaded onto the upstream T domain (28). However, our results were not consistent with donor site side chain specificity being a major limiting factor. If this were the case, then the C domain introduced into strain CAThr-E should have been an effective replacement for the native C domain of PvdD module 2, as both C domains receive L-threonine at their donor and acceptor sites in their native contexts. More significantly, neither C domain in the functional C-A substitution strains CAThr-A and CA-LysC should have been able to tolerate L-threonine, as each receives L-N^5-formyl-N^5-hydroxyornithine as the C-terminal residue at the donor site in its native context. Finally, strain CASer-F, containing a substituted C domain that does normally receive L-threonine at the donor site, was the least active of the functional C-A domain substitution strains.

Instead, the detection of only truncated pyoverdine resulting from the nonfunctional C-A domain substitutions suggests that the inability to catalyze an effective condensation reaction causes peptide synthesis to stall. One possible explanation for this is that C domains in a new context may be unable to receive some incoming peptides due to steric constraints. The crystal structures of C domains show the catalytic center covered by a lid region within a channel between two subdomains (11, 29–31), and these features

may contribute to some incoming peptide chains being physically blocked from reaching the catalytic center. It is also important to note that the previous *in vitro* studies of C domain donor site specificity focused on single residues (9, 10), or at most a tetrapeptide (28), rather than the longer peptide chains that substituted C domains further down an NRPS assembly line might encounter. Further tests of C domain donor site specificity using peptides rather than single amino acids are needed to more thoroughly interrogate the tolerance of C domain donor sites for alternative peptide substrates.

An alternative reason for the loss of activity observed with most C-A domain substitutions is that introduced C-A domains may not communicate appropriately with the neighboring PvdD T domains. T domains function to transfer substrates between multiple domains during a catalytic cycle. For example, the T domain from the first module of PvdD receives a threonine residue from the A domain and must pass it between the upstream and downstream C domains. Structural studies have shown that T domains adopt multiple distinct conformational states, and this conformational plasticity is hypothesized to aid in interacting with different domains (11, 32), which may themselves be highly dynamic (33, 34). Although our work here has shown that the PvdD T domains are generally able to communicate effectively with a new A domain introduced immediately upstream, in other work, we and others have seen that T domains can show some specificity for the type of domain immediately downstream; in particular, directed evolution has been required in several instances to enable an introduced T domain to communicate effectively with a downstream TE domain (35, 36; J. G. Owen, M. J. Calcott, K. J. Robins, and D. F. Ackerley, unpublished data). It may be that, in analogous fashion, a newly introduced C domain is often unable to communicate effectively with the T domain immediately upstream. This issue could potentially be resolved by treating NRPS modules as inseparable T-C-A domain units and substituting them accordingly. There is at least one example where dipeptides were created via T-C-A domain substitution (22). In ongoing work, we are testing T-C-A domain substitution in the PvdD model with the aim of keeping T-C domain communication intact, as well as examining how generally promiscuous T domains are in their interactions with other domains, in particular C domains, and whether this may be a primary factor inhibiting the success of C-A domain substitution strategies.

The pyoverdine NRPS system is not only ideally suited to targeted domain recombination studies, it also holds great potential for directed evolution, in particular of C domain regions. Excitingly, our discovery that certain C-A domain substitutions were active while the corresponding A domain substitutions were not opens the way for directed-evolution studies targeting the acceptor site of the native PvdD C domain. Relaxation of the stringent proofreading requirements of the acceptor site would open the door to generation of novel peptides via simple A domain substitution or recoding. The ability of pyoverdine to provide conditional viability selection (e.g., only pyoverdine-synthesizing strains will grow on media containing EDDHA) will enable high-throughput analysis of gene libraries derived from variants of *pvdD* with A domain substitutions to achieve this goal.

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