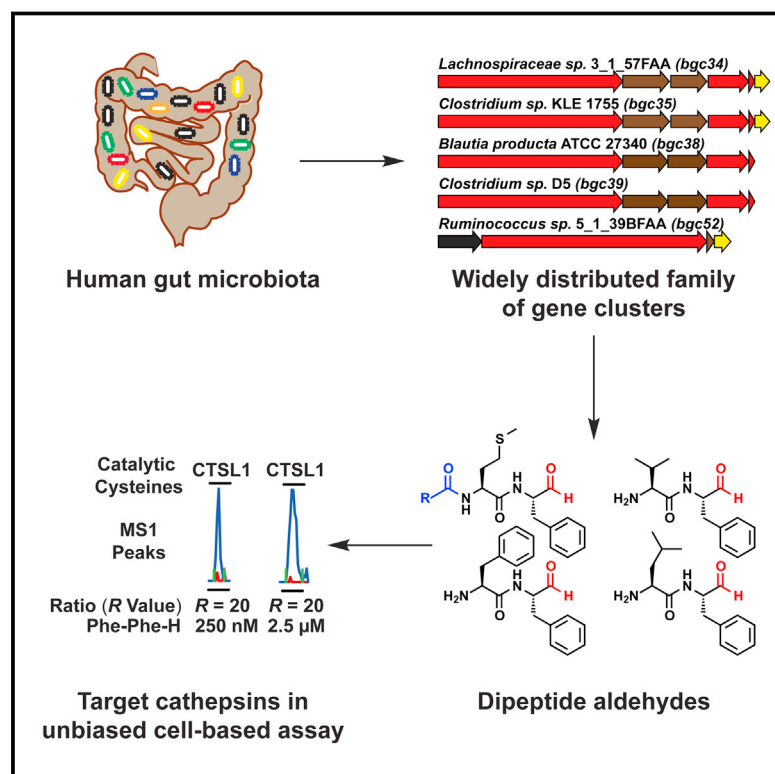


Discovery of Reactive Microbiota-Derived Metabolites that Inhibit Host Proteases

Graphical Abstract



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In Brief

Guo et al. report the discovery of a new family of microbial metabolites that are widely produced by the microbes of the human gut and that chemically modify host cellular proteins.

Highlights

- A family of gene clusters in gut bacteria encodes dipeptide aldehydes
- Present in 88% of humans and transcribed under conditions of host colonization
- One compound, Phe-Phe-H, targets cathepsins in an unbiased cell-based assay
- Uncovers a possible role for lysosomal proteases in microbiota-host interactions



Discovery of Reactive Microbiota-Derived Metabolites that Inhibit Host Proteases

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SUMMARY

The gut microbiota modulate host biology in numerous ways, but little is known about the molecular mediators of these interactions. Previously, we found a widely distributed family of nonribosomal peptide synthetase gene clusters in gut bacteria. Here, by expressing a subset of these clusters in *Escherichia coli* or *Bacillus subtilis*, we show that they encode pyrazinones and dihydropyrazinones. At least one of the 47 clusters is present in 88% of the National Institutes of Health Human Microbiome Project (NIH HMP) stool samples, and they are transcribed under conditions of host colonization. We present evidence that the active form of these molecules is the initially released peptide aldehyde, which bears potent protease inhibitory activity and selectively targets a subset of cathepsins in human cell proteomes. Our findings show that an approach combining bioinformatics, synthetic biology, and heterologous gene cluster expression can rapidly expand our knowledge of the metabolic potential of the microbiota while avoiding the challenges of cultivating fastidious commensals.

INTRODUCTION

The microbiota influence host biology in numerous ways, very few of which are understood at the level of molecular mechanism (Donia and Fischbach, 2015; Lee and Hase, 2014; Nicholson et al., 2012). In a previous survey of biosynthetic gene clusters from the human microbiome, we reported the presence of thousands of biosynthetic loci of unknown function, including large families that are present in >50% of the subjects from the

National Institutes of Health (NIH) Human Microbiome Project (Donia et al., 2014). The small-molecule products of these genetic elements represent large gaps in our knowledge of what the microbiota are capable of producing and constitute an enticing opportunity to discover new mediators of interspecies interactions.

As a test case for expanding our knowledge of the biosynthetic capacity of the microbiota, we set out to characterize a family of nonribosomal peptide synthetase (NRPS) gene clusters that are found in a variety of gut bacterial genome sequences (Donia et al., 2014). These clusters attracted our attention for two reasons. First, they are present in >90% of the stool samples from the Human Microbiome Project (HMP), suggesting that the metabolites they encode are widely distributed among healthy humans. Second, they reside almost exclusively in gut bacterial genome sequences; only a few environmental isolates harbor a cluster in the family, raising the possibility that their small-molecule products play a role in interspecies signaling in the gut.

RESULTS

Computational Analysis of the Gut NRPS Cluster Family

We began by performing a multi-gene BLAST search (Medema et al., 2013) to identify new clusters in the family from genome sequences that had been deposited since our previous analysis. This search yielded 19 new clusters at a threshold of 30% average sequence identity, increasing the size of the family to 47 (Figure 1). The resulting reanalysis shows a family that has the following characteristics. (1) It consists of four clades: one features a three-module NRPS (e.g., *bgc52*), another a two-module NRPS and a loading module on a separate protein (e.g., *bgc35*), a third consists solely of a two-module NRPS (e.g., *bgc26*), and a fourth contains NRPSs of variable domain architecture. In every case, the NRPS features a terminal reductase (R) domain. (2) Almost all of the gene clusters are found in anaerobic Firmicutes from the class Clostridia, although a few

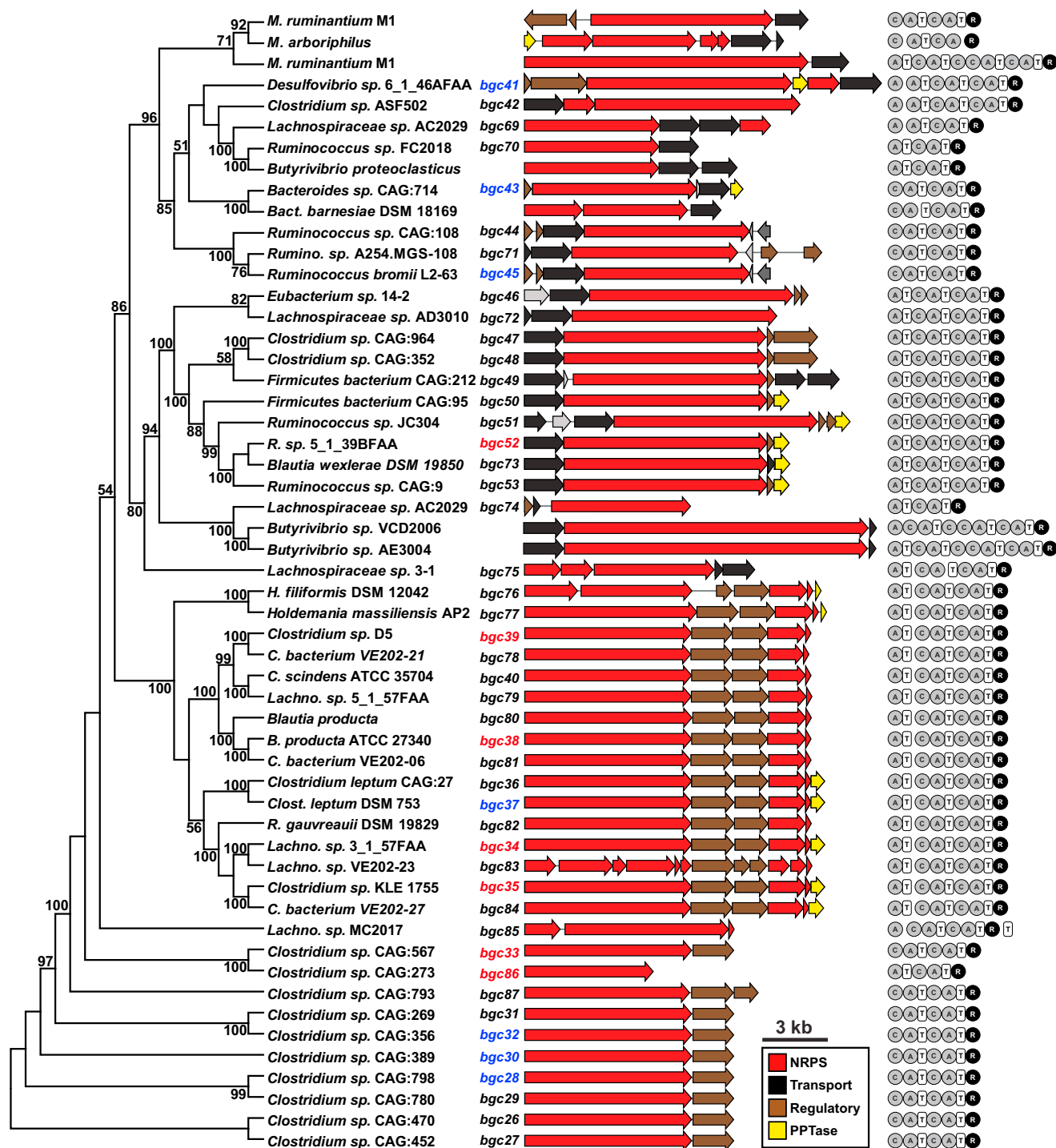


Figure 1. Phylogenetic Analysis of a Family of NRPS Biosynthetic Gene Clusters Found Exclusively in Gut Isolates

Shown on the left is a phylogenetic tree (maximum parsimony, MEGA6) based on the large NRPS gene of the 47 BGCs in the family. Numbers next to the branches represent the percentage of replicate trees in which this topology was reached using a bootstrap test of 1,000 replicates. The names of BGCs characterized experimentally are colored red (products obtained) or blue (no products observed). The domain organization of the NRPS enzyme(s) is shown to the right of each cluster (A, adenylation domain; C, condensation domain; T, thiolation domain; R, reductase domain). BGCs without an index number were discovered from non-human (e.g., rumen) gut bacterial isolates.

See also [Figures S1, S4, and S7](#) and [Tables S1 and S2](#).

of the clusters are found in Gram-negative organisms (*Bacteroides* and *Desulfovibrio*). (3) Nearly all of the clusters reside in isolates from the human gut and that of other mammals (Figure 1). Very few of the clusters are found in relatives of these organisms that are free-living or inhabit a non-intestinal host-associated niche, implying a function that is relevant to the biology of host colonization. (4) Each clade contains clusters from hosts that have never been isolated; genome sequences of the hosts from which these clusters derive were assembled from metagenomic samples in a recent study (Nielsen et al., 2014). As such, the only way to access these clusters is to synthesize them, a problem of increasing importance as the volume of metagenomic sequence data increases and tools are developed for assembling short-read metagenomic data into cluster-size fragments.

We selected 14 of these clusters for analysis (colored red or blue in Figure 1). Clusters were chosen to represent the diversity of sequences and domain architectures from the four clades of the family. Since none of the host organisms have been manipulated genetically and most are from a bacterial class (Clostridia) that is largely refractory to genetic manipulation, we decided not to make targeted genetic deletions in any of the native host strains. Instead, we expressed gene clusters heterologously in two commonly used laboratory hosts, *Escherichia coli* and *Bacillus subtilis*. The host organisms of three of the clusters (*bgc34*, *bgc35*, and *bgc52*) were available from laboratories or culture collections; clusters from these hosts were cloned in their native form (omitting regulatory genes) into *E. coli* or *B. subtilis* vectors in which expression was driven by a strong promoter (see Figure S1 for more details). The remaining 11 clusters were either from organisms that could not be obtained or from metagenomic sequence data, so the host organism was never isolated. These clusters were synthesized directly from primary genome or metagenome sequence with optimized codons (leaving out regulatory genes) and cloned into *E. coli* or *B. subtilis* expression vectors under the control of a strong promoter. Cluster-harboring strains were cultivated at 5 mL scale for 1–2 days to determine whether they produce a pathway-specific metabolite.

Experimental Analysis of the Gut NRPS Gene Clusters

Liquid chromatography-mass spectrometry (LC-MS) analysis of culture fluid extract from *E. coli* strains harboring *bgc35* and *bgc52* showed evidence of seven and eight new peaks, respectively (Figure 2), which is notable, since both clusters are from Gram-positive hosts. From 4 L of culture fluid, we purified multi-milligram quantities of each compound (Figure 2). Three lines of evidence reveal that these molecules are a family of pyrazinones and dihydropyrazinones: (1) the purified compounds have UV absorption maxima at 220 and 300 nm, consistent with a pyrazinone core; (2) high-resolution LC-MS analysis of the compounds yields masses and empirical formulae consistent with a series of α -amino-acid-derived pyrazinones and dihydropyrazinones with variable side chains at both positions (Table S4); and (3) 1D and 2D nuclear magnetic resonance (NMR) experiments show chemical shifts and correlations characteristic of pyrazinones (Data S1) (MacDonald et al., 1976). An *E. coli* strain harboring *bgc34* produced a subset of the *bgc35* prod-

ucts, but at a level so low it would not have been observed without a targeted mass ion search.

Results from two other clusters showed additional modes of diversity in the family. When *bgc38* was expressed in *B. subtilis*, we observed one new peak corresponding to a pyrazinone with ethyl and methylindole side chains, indicating that α -aminobutyrate (or an unknown precursor) can be a native monomer; *bgc39* was a low-level producer of the *bgc38* products. *bgc33* and *bgc86* were of particular interest; these clusters were discovered from a metagenomic sample, so their host organisms were never isolated (Figure 1). An *E. coli* strain carrying a synthetic, codon-optimized version of *bgc33* yielded two classes of molecules: a pyrazinone that derives from methionine and valine (15) and a corresponding set of *N*-acylated dipeptide aldehydes, including one that bears an *N*-octanoyl acyl chain (16) (Figure 2; Table S5). In comparison, only the pyrazinone product (15) can be identified from the *E. coli* strain harboring *bgc86*.

In addition to the 16 molecules that were produced at a titer sufficient to purify milligram quantities for NMR experiments, we identified 16 additional pathway-dependent molecules from *bgc35* and *bgc52*. These metabolites are produced at a lower titer and their structures are proposed on the basis of diagnostic high-resolution tandem mass spectrometry (MS/MS) fragmentation patterns (Figure S2; Table S4). In addition to the seven clusters from which we observed products, seven additional clusters were synthesized and expressed in *E. coli* DH10 β , *E. coli* BAP1, or *B. subtilis* 168 (colored blue in Figure 1); no high-titer products were observed from any of these BGCs using an LC-MS trace comparison. In total, from 7 of 14 heterologously expressed clusters, we discovered 32 compounds, of which 28 are previously unknown molecules (Figure 2; Table S4).

The Same Molecules Are Produced by a Native Strain and in a Biochemical Reconstitution

The results from our heterologous expression experiments raise an important question: Are the molecules we isolated the native products of the cluster or artifacts of expression in *E. coli* or *B. subtilis*? To address this question, we used two complementary approaches. First, we cultivated the *bgc52* producer, *Ruminococcus* sp. 5_1_39BFAA, in 12 different culture media in an effort to find a condition under which we could observe production of the *bgc52* products. LC-MS analysis of cell-free culture extracts from one of the media, M17, revealed peaks identical to five of the most prominent compounds produced under conditions of heterologous expression in *E. coli* (Figure S3), suggesting that these pyrazinones are the native products of *bgc52* (it is unlikely these compounds derive from a different biosynthetic pathway in the same organism, since *Ruminococcus* sp. 5_1_39BFAA harbors only one additional nonribosomal peptide synthetase in its genome, a condensation-thiolation di-domain protein).

Using a similar approach, we could not find a condition under which the *bgc35* producer, *Clostridium* sp. KLE 1755, produced the molecules we observed from *E. coli*-*bgc35*. As an alternative approach, we overexpressed the 280-kDa, two-module *bgc35* NRPS in *E. coli* and purified it as an N-terminal His₆ fusion protein. We then attempted to reconstitute the biosynthetic pathway by incubating the NRPS with Sfp and coenzyme A

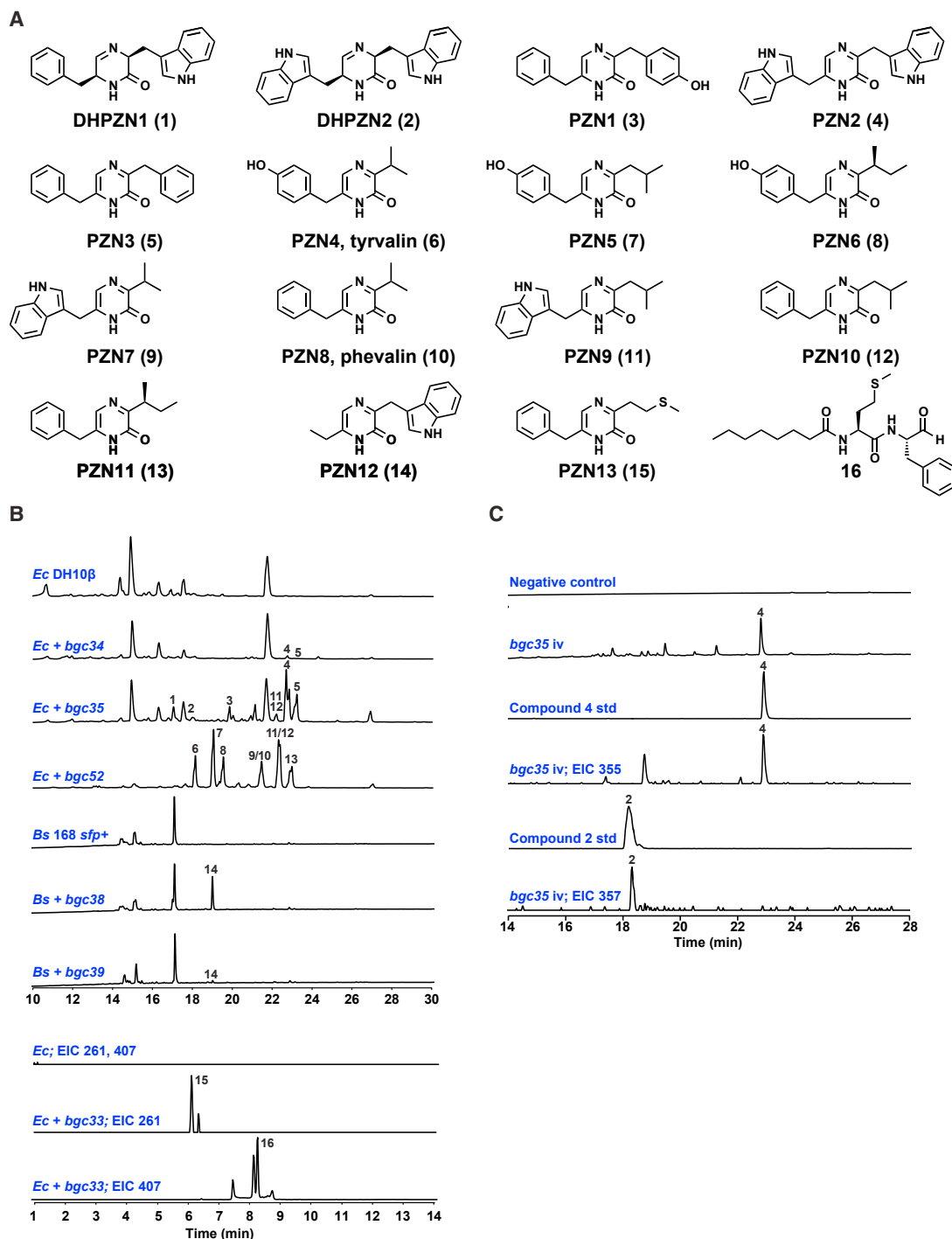


Figure 2. Chemical and Biochemical Analysis of the Gut NRPS BGCs

(A) Chemical structures of the small-molecule products of the gut NRPS gene clusters.

(B) High-performance liquid chromatography (HPLC) or LC-MS profiles showing the production of each molecule in *Escherichia coli* or *Bacillus subtilis*. From top left: *E. coli* DH10β (*Ec*); and *Ec* expressing *bgc34*, *bgc35*, and *bgc52*, as detected by UV absorption at 300 nm. *B. subtilis* 168 *sfp+* (*Bs*); and *Bs* expressing *bgc38* and *bgc39*, as detected by UV absorption at 360 nm. *E. coli* BAP1 (*Ec*) and *Ec* expressing *bgc33*; extracted ion chromatograms for the indicated masses are shown.

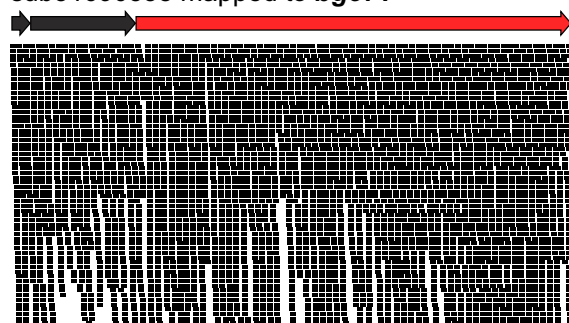
(C) In vitro reconstitution of the *bgc35* NRPS. From top right: HPLC profiles of organic extracts of the reaction without adding the enzyme (negative control) and the complete in vitro reaction (*bgc35* iv), as detected by UV absorption at 300 nm. Below are authentic standards of compounds 4 and 2, and extracted ion chromatograms at the indicated masses showing production of compounds 4 and 2 in the reaction. The numbering of the peaks in (B) and (C) corresponds to the small molecules shown in (A).

See also Figures S1, S2, S3, and S7 and Tables S1, S2, S4, and S5.

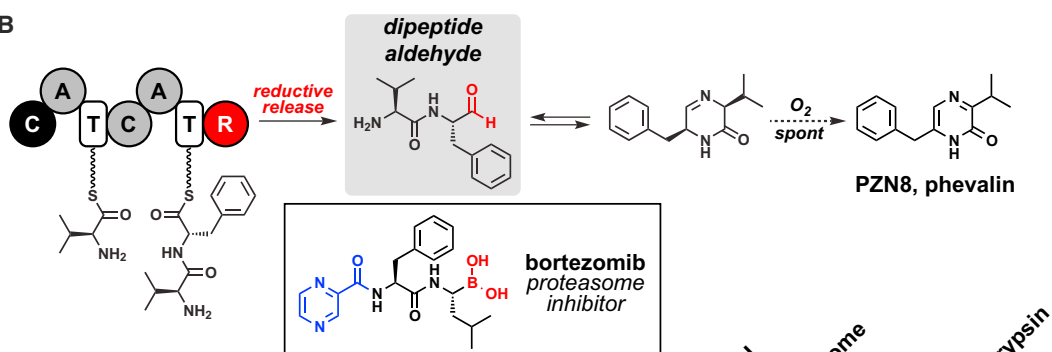
A sub316701492 mapped to *bgc52*



sub31690558 mapped to *bgc71*



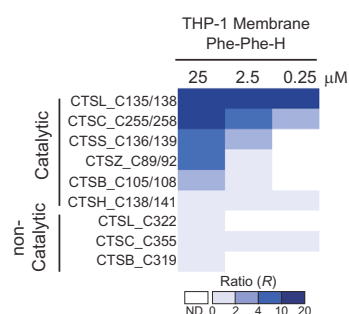
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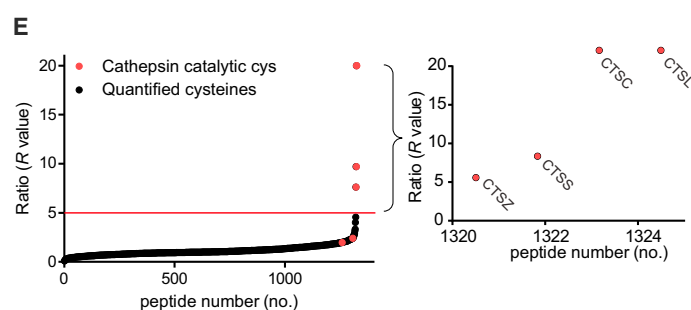
C

| | Cat B | Cat L | Cat C | Cat S | Calpain I | Proteasome | Trypsin | Chymotrypsin |
|-----------|-------|-------|-------|-------|-----------|------------|---------|--------------|
| Peptide 1 | 2.8 | 0.006 | 0.5 | 2 | 1.0 | >30 | N/O | N/O |
| Peptide 2 | 46.0 | 0.006 | 8 | 0.9 | 1.5 | >30 | N/O | N/O |
| Peptide 3 | 9.4 | 0.005 | >20 | 0.6 | N/O | >30 | N/O | N/O |
| Peptide 4 | 0.38 | N/O | 0.37 | 0.013 | 1.3 | 2.8 | N/O | >30 |

D



E



(legend on next page)

(to 4'-phosphopantetheinylate both thiolation domains), amino acid substrates (either all 20 proteinogenic amino acids or aromatic amino acids only), ATP for monomer activation, and NADH as a cofactor for the terminal reductase domain. LC-MS analysis of a time course of this reaction showed the formation of two products identical to compounds **2** and **4**, suggesting that these are native products of *bgc35* (Figure 2).

The Gut NRPS Family Is Widely Distributed in Healthy Humans

Having identified the small-molecule products of a subset of the gut NRPS family, we next turned to the question of how widely distributed this cluster family is in the human population. In previous work, we showed that of the smaller subset of 28 clusters that were known at the time, at least one cluster was present in >90% of the ~100 stool metagenomic samples from HMP phase 1. These data were derived from a global analysis that involved mapping metagenomic reads to proteins from 14,000 biosynthetic gene clusters (BGCs) using the fast, metagenomics-optimized algorithm mBLASTx (Donia et al., 2014). Here, we used two complementary methods to determine the abundance of the gut NRPS family in publicly available metagenomic datasets. First, since we had a smaller set of BGCs to map, we developed a highly sensitive and specific analytical method in which we used BLASTn to map quality-filtered metagenomic reads from the 149 metagenomic stool samples from HMP phase 1 to the large NRPS gene in each BGC. Using this analysis, we found that at least one of the clusters is present in >88% of the 149 stool samples. Second, we used a recently developed approach that leverages a large gene catalog of >9 million gut microbiome genes (Li et al., 2014; Nayfach et al., 2015) and found that at least one of the clusters was present in >93% of 1,267 publicly available stool samples (minimum abundance = 1 copy/1,000 cells). Together, these results confirm that this gene cluster family is widely distributed in healthy human subjects.

Gut NRPS Clusters Are Actively Transcribed under Conditions of Host Colonization

A cluster might be present in a metagenomic sample, but not expressed in the gut; indeed, many metabolic pathways present in metagenomic samples are expressed at very low levels in corresponding metatranscriptomic datasets (Franzosa et al., 2014; Gosalbes et al., 2011). To address whether the gut NRPS clus-

ters are transcribed under conditions of native host colonization, we recruited reads from publicly available RNA-sequencing (RNA-seq) datasets from the stool samples of eight healthy subjects (Franzosa et al., 2014). Illumina reads from several runs on each sample were combined and used to construct a BLAST database, which we then searched using the 47 full-length gut NRPS BGCs as query sequences. Seven of the eight samples (87.5%) harbored at least one actively transcribed gene cluster from this family, and the robust level of transcription observed in most samples is notable, given that the anaerobic Firmicutes from which the gut NRPS clusters derive are often lower-abundance members of the community (Figures 3 and S4).

The Active Gut NRPS Product May Be the Initially Released Dipeptide Aldehydes

The NRPSs in this family harbor a C-terminal reductase domain that catalyzes the chain-terminating release of a C-terminal aldehyde (Figures 1 and 3). The newly liberated dipeptide aldehydes exist in equilibrium with the cyclic imine; in the presence of oxygen, this dihydropyrazinone oxidizes spontaneously and irreversibly to the fully aromatic pyrazinone. Three lines of evidence suggest that the active form of the gut NRPS product is the initially released dipeptide aldehyde:

First, under physiological conditions, the peptide aldehydes are stable for long enough to be active. We measured the half-life of oxidation for three compounds (the peptide aldehyde versions of 5, 10, and 12) in vitro at physiological pH; they ranged from 3 to 28 hr (Figure S5), which would provide sufficient time for systemic distribution in the host. Indeed, compounds 1 and 2 are stable enough that we isolate milligram quantities of the cyclic imine after >24 hr of aerobic *E. coli* culture. Moreover, these molecules are produced in the gut, which is anaerobic, so the slow process of spontaneous oxidation would not begin until the compounds encounter oxygenated host tissues. Notably, the major product of *bgc33*, *N*-octanoyl-Met-Phe-H (16), is *N*-acylated, preventing it from cyclizing and oxidizing to a pyrazinone. These findings raise the possibility that peptide aldehydes are the predominant active product of every cluster in the family.

Second, peptide aldehydes have a long history in the literature of being highly potent, cell-permeable protease inhibitors. Starting with the discovery of the leupeptins from soil isolates of *Streptomyces* almost four decades ago (Aoyagi et al., 1969a,

Figure 3. Functional Analyses of the Gut NRPS Cluster and Its Small-Molecule Products

(A) Seven of eight publicly available gut metatranscriptomic datasets harbored an actively transcribed gene cluster from the gut NRPS family. Typical cluster tiling is shown for *bgc52*; a sample with unusually robust transcription of *bgc71* is also shown (see Figure S4 for the remaining sample tilings). (B) A biosynthetic scheme for the pathways encoded by the gut NRPS clusters. A C-terminal reductase (R) domain catalyzes nicotinamide-dependent reduction of the thioester, releasing a free dipeptide aldehyde that has a half-life of hours under physiological conditions and exists in equilibrium with the cyclic imine. In the presence of oxygen, this dihydropyrazinone oxidizes irreversibly to the pyrazinone. The chemical structure of the proteasome inhibitor bortezomib, which has a scaffold derived from dipeptide aldehydes, is shown in the box. (C) Results from a panel of in vitro protease inhibition assays using free-amino and *N*-acylated dipeptide aldehydes discovered in this study. IC₅₀ values are shown in μ M. Cat, cathepsin; N/O, no inhibition observed. Data for the corresponding *N*-Boc protected dipeptide aldehydes and pyrazinones are shown in Figure S5. (D and E) Competitive isoTOP-ABPP identifies CTSL as a target of the *bgc35* product Phe-Phe-H. The heatmap (D) shows all cathepsin cysteines, including both catalytic and non-catalytic detected in isoTOP-ABPP experiments where the THP-1 membrane fraction was subjected to the indicated concentrations of the Phe-Phe-H aldehyde. Note that cysteines on the same tryptic peptide cannot be differentiated and are indicated together, e.g., C135/138. The graphs (E) show the MS1 chromatographic peak ratios (*R* values) for all peptides identified from the THP1 membrane fraction treated with 25 μ M Phe-Phe-H. The red dots mark catalytic cysteine residues of cathepsin proteases, and the red line indicates the *R* value > 5 threshold. See also Figures S4, S5, and S6 and Table S1.

1969b), numerous peptide aldehydes—mostly *N*-carboxybenzyl (Cbz) protected di-, tri-, and tetrapeptide aldehydes—have been synthesized and shown in vitro and in vivo to have potent inhibitory activity against serine and cysteine proteases and the proteasome (Lee and Goldberg, 1998; Otto and Schirmeister, 1997; Thompson, 1973; Westerik and Wolfenden, 1972). Dipeptide aldehydes were the starting point for the development of the clinically used dipeptide boronate proteasome inhibitor bortezomib (Figure 3) (Adams et al., 1998). Cbz-protected versions of multiple gut NRPS products, including Cbz-Val-Phe-H, have been synthesized and shown to inhibit various cysteine proteases (Mehdi et al., 1988; Woo et al., 1995).

Third, since dipeptide aldehydes with a free amino group have not been tested as protease inhibitors, we assessed the activity of three of the *bgc52* and *bgc35* products (Val-Phe-H, Leu-Phe-H, and Phe-Phe-H) and the *bgc33*-derived compound 16 against a panel of proteases in vitro, comparing them to the corresponding pyrazinones and *N*-tert-butyloxycarbonyl (*N*-Boc) protected dipeptide aldehydes (Figures 3 and S5). Consistent with previous reports on *N*-Cbz-protected dipeptide aldehydes (Mehdi et al., 1988; Woo et al., 1995), the *N*-Boc protected dipeptide aldehydes were active at low- to mid-nanomolar against the lysosomal cysteine proteases cathepsins B and L. The free-amino dipeptide aldehydes had similarly potent (single-digit nanomolar) activity against cathepsin L but greatly reduced activity against cathepsin B, showing that they are capable of highly potent inhibitory activity and exhibit selectivity not seen in their *N*-Boc protected counterparts (Figures 3 and S5). This difference in selectivity was also seen in compound 16, which had undetectable activity against cathepsin L but 13 nM activity against cathepsin S. None of the compounds tested here had quantifiable activity against trypsin or chymotrypsin at the concentrations tested. Overall, these data suggest that the dipeptide aldehydes harbor potent and selective protease inhibitory activity, as assessed in vitro.

The proteases we assayed were chosen based on literature precedent, so the enzyme inhibition data do not point to a specific target. To determine the target of the peptide aldehydes in an unbiased way, we applied a quantitative chemical proteomic method, termed isotopic tandem orthogonal proteolysis-activity-based protein profiling (isoTOP-ABPP) (Weerapana et al., 2010; Backus et al., 2016), to measure the global interactions of a representative dipeptide aldehyde, *bgc35* product Phe-Phe-H, with cysteine residues in the human cell lysates. We treated membrane preparations of the human innate immune (monocytic) cell line THP-1 with the *bgc35* product Phe-Phe-H or vehicle, and then these samples were treated with a cysteine reactive iodacetamide-alkyne (IA-alkyne) probe and conjugated to an isotopically differentiated (light or heavy, respectively) TEV protease-cleavable biotin tag using copper-catalyzed azide-alkyne cycloaddition (CuAAC or click) chemistry. Vehicle- and peptide-treated samples were then combined, enriched, subjected to sequential trypsin and TEV digests, and evaluated by LC-MS/MS. Site-specific blockade of IA-alkyne labeling as measured by quantitation of heavy/light MS1 chromatographic peaks designates cysteine residues that are targeted by Phe-Phe-H (heavy/light ratios, or *R* values > 5, red line; Figures 3D, 3E, and S6). Treatment of THP-1 membranes with 250 nM

Phe-Phe-H, the lowest concentration tested, fully blocked IA-alkyne labeling of the catalytic cysteine of cathepsin L (CTSL1; Cys138, *R* > 20) while showing only partial (cathepsin C [CTSC] *R* = 2.9 for catalytic cysteine Cys258) or negligible (*R* < 2.0; cathepsins B [CTSB], H [CTSH], S [CTSS], and Z [CTSZ]) cross-reactivity with other cathepsins (Figure 3D). Phe-Phe-H also interacted with a subset of additional cathepsin targets when tested at higher concentrations (25 μ M; *R* > 5 for CTSC, CTSS, and CTSZ) in THP-1 (Figures 3D and 3E) and other human cell proteomes (Ramos, H1975; Data S2). Phe-Phe-H showed remarkable selectivity for cathepsins, exhibiting no additional targets across the more than 3,500 total quantified cysteines across three different human cell proteomes (*R* < 2.0; Data S2). Additional experiments at the characteristic acidic pH of the lysosome (pH 5 or 5.5), where cathepsins reside, produced similar results (Data S2). Together, these data designate the cathepsins (specifically cathepsin L) as principal targets of the gut NRPS product Phe-Phe-H.

DISCUSSION

We have found 32 compounds that represent a subset of the molecular output from this family of NRPSs. Gene clusters in this family are widely distributed in the human gut microbiome, and they are transcribed robustly under conditions of host colonization. The discovery approach we used (to express cloned or synthetic BGCs in *E. coli* or *B. subtilis*) revealed two unanticipated findings that may be relevant to similar discovery efforts in the future. First, *bgc35* and *bgc52* were functional in *E. coli* in their native form (driven by an *E. coli* promoter); this is notable, given that these clusters are from a Gram-positive host. *E. coli* might therefore be an appropriate heterologous host for a broader range of gene clusters than previously imagined. Second, the *E. coli* strain harboring *bgc34* produced a subset of the *bgc35* products, but at a level so low it would not have been observed without a targeted mass ion search; likewise, *bgc39* was a low-level producer of the *bgc38* products. Importantly, the amino acid sequences of the NRPS genes from the *bgc34-bgc35* and *bgc38-bgc39* pairs share 70% and 51% identity, respectively, pointing to the potential importance of subtle changes in primary sequence that alter expression level as a determinant of whether an NRPS will work in a heterologous host.

Notably, of the 4 out of 32 molecules that were previously known, three are produced by an unrelated NRPS conserved across all known skin-associated species of *Staphylococcus*. Thus, the gut and *Staphylococcus* NRPSs are an example of convergent evolution toward a common scaffold, suggesting that the same compounds might play a biological role in more than one host-associated niche (Wyatt and Magarvey, 2013; Wyatt et al., 2012; Zimmermann and Fischbach, 2010).

We presented three lines of evidence suggesting that the active small-molecule products are peptide aldehydes: (1) the free amino dipeptide aldehydes have a half-life of hours, and some compounds in the family are stabilized by *N*-acylation; (2) peptide aldehydes have a long history in the literature as potent, cell-permeable protease inhibitors; and (3) four of our compounds exhibit potent and selective protease inhibitory activity in vitro. From studies of peptide aldehydes and other

C-terminally modified peptide protease inhibitors, it has become clear that the side chains in the inhibitor help occupy the P1 and P2 pockets (Siklos et al., 2015) and a free amino terminus can form specific charge contacts in the active site (Katunuma, 2011; Laine and Busch-Petersen, 2010). Thus, side chain identity and *N*-terminal acylation state are key determinants of selectivity, potentially helping to explain the breadth of chemical diversity in this family. Mutational experiments with *bgc35* show that both adenylation domains participate in generating side-chain chemical diversity (Figure S7). Likewise, an analysis of products from *bgc86*, a version of *bgc33* with a truncated NRPS system, suggests that the starting condensation domain of *bgc33* NRPS is responsible for *N*-acylation (Figure S7).

Our quantitative, unbiased chemical proteomics experiments suggest that one of the dipeptide aldehydes, Phe-Phe-H, targets the catalytic cysteines of multiple cathepsins, showing the highest potency for cathepsin L. Our substrate assay data with recombinant proteases generally matched our proteomic data, with the exception of CTSC, which was more potently inhibited in proteomes by Phe-Phe-H. This result could indicate that endogenously expressed CTSC is post-translationally regulated to create a form of the protease that is more sensitive to peptide aldehyde inhibition. Our chemical proteomic studies also revealed that Phe-Phe-H exhibits very high selectivity for cathepsins, as we did not detect any additional cysteines targeted by this peptide aldehyde in human cell lysates. Although these data do not prove a mammalian (rather than bacterial) target for the peptide aldehydes, they raise the possibility that the gut NRPS product acts in the host lysosome. Further support for this premise comes from a recently reported screen for *Staphylococcus aureus* genes required for survival in and escape from the phagosome (Blättner et al., 2016). Among the top hits was the *Staphylococcus* NRPS described above (Wyatt and Magarvey, 2013; Wyatt et al., 2012; Zimmermann and Fischbach, 2010), which is unrelated to the gut NRPS enzymes but produces two of the same compounds. Although the authors were unaware that the active form of the NRPS product is likely a dipeptide aldehyde rather than a pyrazinone, these data provide independent evidence that the dipeptide aldehydes and the NRPS genes that encode them might play a role in an intracellular niche for bacteria in the phagolysosome.

Taken together, these two lines of evidence raise the intriguing possibility of a previously unknown interaction between the commensal gut microbiota and a cysteine protease system in the host lysosome. Since cathepsins play an important role in antigen processing and presentation in intestinal epithelial cells (Hershberg et al., 1997) and TLR9 activation in macrophages and dendritic cells (Matsumoto et al., 2008; Park et al., 2008), their inhibition by dipeptide aldehydes might block adaptive or innate immune recognition of a subset of anaerobic Firmicutes in the gut.

Another possibility is that dipeptide aldehyde-mediated cathepsin inhibition enables gut mutualists to stably occupy and emerge from a niche in the phagolysosome. Intracellular pathogens commonly inhabit the phagolysosome (Rosenberger and Finlay, 2003), and a subset of Gram-negative pathobionts, including *Alcaligenes*, are found in Peyer's patches and other gut lymphoid tissues (Fung et al., 2014; Obata et al., 2010). Our

data raise the possibility that dipeptide aldehydes enables a broad set of mutualistic Gram-positive species to reside in gut epithelial or immune cells. If borne out by subsequent studies, either possibility (cathepsin inhibition by extracellular or intracellular mutualists) would represent a previously undescribed form of immune modulation by the gut microbiota. In addition, since the small molecules produced by this cluster family harbor a simple and general scaffold (small peptide aldehydes, some of which are *N*-acylated), it remains possible that some of them exert biological activities distinct from protease inhibition.

Our findings show that an approach combining bioinformatics, synthetic biology, and heterologous gene cluster expression can rapidly expand our knowledge of the metabolic potential of the microbiota while avoiding the challenges of cultivating fastidious commensals. Given the large number of biosynthetic gene clusters of unknown function in the human microbiome, such an approach holds great potential for discovering, in a scalable fashion, small-molecule mediators of microbe-host and microbe-microbe interactions relevant to the biology of the microbiome.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Protease Inhibition Assays
 - Target Identification by Chemical Proteomics
 - Detailed Structural Characterization of Purified Compounds Isolated in This Study
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- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, five tables, and two data files and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2016.12.021>.

AUTHOR CONTRIBUTIONS

Conceptualization, C.-J.G. and M.A.F.; Investigation, C.-J.G., F.-Y.C., T.P.W., K.M.B., T.M.A., M.F., M.T., M.S.D., and S.N.; Writing – Original Draft, C.-J.G. and M.A.F.; Writing – Review & Editing, F.-Y.C., T.P.W., T.M.A., K.M.B., M.F., M.T., M.S.D., S.N., K.S.P., C.S.C., B.F.C., J.C., and C.A.V.; Funding Acquisition and Supervision, M.S.D., K.S.P., C.S.C., B.F.C., J.C., C.A.V., and M.A.F.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---------------------------------|--------------------------------|
| Antibodies | | |
| Anti-His antibody | Invitrogen | Cat# 1819587; RRID: AB_2153866 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Acetone- d_6 | Cambridge Isotope Laboratories | N/A |
| 2-amino-3-phenyl-1-propanol | Sigma-Aldrich | N/A |
| Anaerobic Basal Broth | HiMedia | Cat# M1636 |
| Adenosine 5'-triphosphate disodium salt hydrate (ATP) | Sigma-Aldrich | N/A |
| Bacto Agar | BD | Ref# 214010 |
| Bacto Brain Heart Infusion Broth | BD | Ref# 237200 |
| Bacto Protease Peptone No. 3 | BD | Ref# 211693 |
| Boc-IEGR-AMC | Bachem | N/A |
| Boc-Leu-OH | Sigma-Aldrich | Cat# 15450 |
| Boc-Phe-OH | Sigma-Aldrich | Cat# 15480 |
| Boc-Val-OH | Sigma-Aldrich | Cat# 15528 |
| Calpain I | Sigma-Aldrich/Abcam | N/A |
| Carbenicillin | Teknova | C2105 |
| Carfilzomib | Onyx Pharmaceuticals | N/A |
| Casman Broth Base | HiMedia | Cat# M766 |
| Cathepsin B | R&D Systems | N/A |
| Cathepsin C | R&D Systems | N/A |
| Cathepsin L | R&D Systems | N/A |
| Cathepsin S | R&D Systems | N/A |
| Chymostatin | Research Products International | N/A |
| Chymotrypsin | Sigma-Aldrich | N/A |
| Cooked Meat Medium | HiMedia | Cat# M1495 |
| Dess-Martin periodinane | Sigma-Aldrich | Cat# 274623 |
| Dextrose | Sigma-Aldrich | Cat# D9434 |
| Difco Columbia Broth | BD | Ref# 294420 |
| Difco M17 Broth | BD | Ref# 218561 |
| Difco Marine Broth | BD | Ref# 27910 |
| Difco Nutrient Basal Broth | BD | Ref# 234000 |
| Difco Reinforced Clostridium Media | BD | Ref# 218081 |
| Difco Wilkins Chalgren Anaerobic Base Broth | HiMedia | Cat# M863 |
| Difco Yeast extract | BD | Ref# 210929 |
| Dithiothreitol (DTT) | All Star | Cat# 610-005 |
| DMEM medium | Mediatech | Cat# 15-013-CV |
| DMSO- d_6 | Cambridge Isotope Laboratories | N/A |
| EDTA-free Protease Inhibitors | Roche | N/A |
| EtN(iPr) ₂ | Sigma-Aldrich | N/A |
| Fetal bovine serum | Omega Scientific | Cat# FB-11 |
| GR-AMC | Bachem | N/A |
| HATU | AK Scientific | N/A |
| Hemin | Sigma-Aldrich | Cat# 51280 |
| Defibrinated Horse Blood | Hemo Stat Laboratories | Cat# DHB500 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------------------|-------------------------------------|----------------|
| Human 20 s proteasome | Boston Biochem | N/A |
| Imidazole | Sigma-Aldrich | N/A |
| Iodoacetamide-alkyne | Backus et al., 2016 | N/A |
| Kanamycin monosulfate | Alfa Aesar | Cat# J61272 |
| L-Alanine | Sigma-Aldrich | Cat# 05129 |
| L-Arginine | Sigma-Aldrich | Cat# A5006 |
| L-Asparagine | Sigma-Aldrich | Cat# A4159 |
| L-Aspartate | Sigma-Aldrich | Cat# A8949 |
| L-Cysteine | Sigma-Aldrich | Cat# 168149 |
| L-Cysteine·HCl·H ₂ O | Sigma-Aldrich | Cat# C1276 |
| L-Cystine | Sigma-Aldrich | Cat# C8755 |
| L-Glutamate | Sigma-Aldrich | Cat# 49449 |
| L-Glutamine | Sigma-Aldrich | Cat# 49419 |
| L-Glycine | Sigma-Aldrich | Cat# 50046 |
| L-Histidine | Sigma-Aldrich | Cat# H6034 |
| L-Isoleucine | Sigma-Aldrich | Cat# I2752 |
| L-Leucine | TCI | Cat# L0029 |
| L-Lysine dihydrochloride | Sigma-Aldrich | Cat# L5751 |
| L-Methionine | Sigma-Aldrich | Cat# 64319 |
| L-Phenylalanine | Sigma-Aldrich | Cat# P5482 |
| L-Proline | Sigma-Aldrich | Cat# 81709 |
| L-Serine | Sigma-Aldrich | Cat# S4500 |
| L-Threonine | Sigma-Aldrich | Cat# 89179 |
| L-Tryptophan | Sigma-Aldrich | Cat# 93659 |
| L-Tyrosine | Sigma-Aldrich | Cat# 93829 |
| L-Valine | Sigma-Aldrich | Cat# 94619 |
| Lab Lemco Powder | OXOID | Cat# LP0029 |
| Leupeptin | Research Products International | N/A |
| Luria-Bertani (LB) broth | BD | Ref# 244610 |
| MeOH- <i>d</i> ₄ | Cambridge Isotope Laboratories | N/A |
| NADPH | Sigma-Aldrich | N/A |
| Pefabloc | Sigma-Aldrich | N/A |
| Phorbol 12-myristate 13-acetate | Sigma-Aldrich | Cat# P8139-5MG |
| RPMI medium | Mediatech | Cat# 15-040-CV |
| Sodium sulfate | Sigma-Aldrich | N/A |
| Sodium thioglycolate | Spectrum | Cat# SO209 |
| Soluble starch | Sigma-Aldrich | Cat# S9765 |
| Spectinomycin | Alfa Aesar | Cat# J61820 |
| Streptavidin resin | Pierce | Cat# 20349 |
| Streptomycin | GE Healthcare Life Sciences | Cat# SV30010 |
| Suc-ALPF-AMC | Bachem | N/A |
| Suc-LLVY-AMC | Anaspec | N/A |
| Trifluoroacetic acid | Fisher | Cat# A116 |
| Tris base | Fisher | Cat# 153666 |
| Tryptone | Fisher | Cat# BP1421 |
| Trypsin | Sigma-Aldrich | N/A |
| Tryptic Soy Broth | BD | Ref# 211825 |
| Tween 80 | Fisher | Cat# BP338 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|-------------------------------------|---------------|
| z-FR-AMC | Bachem | N/A |
| β -mercaptoethanol | Bio-Rad | Cat# 1610710 |
| Critical Commercial Assays | | |
| Bio-Rad DC protein assay kit | Bio-Rad | N/A |
| Circular Polymerase Extension Cloning (CPEC) | Quan and Tian, 2009 | N/A |
| Slide-a-lyzer Dialysis Cassette G2 20k MWCO | Pierce | N/A |
| DNA Clean and Concentrator | Zymo Research | Cat# D4003 |
| Gibson Assembly Cloning Kit | NEB | Cat# E5510S |
| Golden Gate Assembly | NEB | N/A |
| In-Fusion HD Cloning Kit | Clontech Laboratories | N/A |
| Q5 High-Fidelity DNA Polymerase | NEB | Cat# M0491L |
| Q5 Site-Directed Mutagenesis Kit | NEB | Cat# E0554S |
| T4 DNA Ligase (HC) | Promega | Cat# M1794 |
| ZR Fungal/Bacterial DNA MiniPrep | Zymo Research | Cat# D6005 |
| Experimental Models: Cell Lines | | |
| H1975 cells | ATCC | CRL5908 |
| Ramos cells | ATCC | CRL1596 |
| THP-1 cells | ATCC | TIB-202 |
| Experimental Models: Organisms/Strains | | |
| <i>Bacillus subtilis</i> 168 | Lab stock | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> + | Lab stock | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc28-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc30-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc32-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc37-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc38-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc39-spec</i> | This paper | N/A |
| <i>Bacillus subtilis</i> 168 <i>sfp</i> +, <i>amyE</i> :: <i>pspac(p)-bgc45-spec</i> | This paper | N/A |
| <i>Clostridium</i> sp. KLE1755 | Kim Lewis and Phil Strandwitz | N/A |
| <i>Escherichia coli</i> BAP1 | Lab stock | N/A |
| <i>Escherichia coli</i> BAP1 harboring pET28a- <i>bgc33</i> | This paper | N/A |
| <i>Escherichia coli</i> BAP1 harboring pET28a- <i>bgc86</i> | This paper | N/A |
| <i>Escherichia coli</i> BL21 | Lab stock | N/A |
| <i>Escherichia coli</i> BL21 harboring pET28a- <i>sfp</i> | Lab stock | N/A |
| <i>Escherichia coli</i> BL21 harboring pET28a- <i>bgc35</i> -NRPS | This paper | N/A |
| <i>Escherichia coli</i> BL21 harboring pET28a- <i>bgc41</i> -NRPS | This paper | N/A |
| <i>Escherichia coli</i> DH10 β competent cells | Invitrogen | Cat# 18290015 |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc34</i> | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> | This paper | N/A |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|-------------------|---|
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> _NRPS_D686A | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> _NRPS_D1713A | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> _NRPS_D686A_D1713A | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> _NRPS_TCA ₂ TR | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc35</i> _NRPS_CA ₂ TR | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc41</i> | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc43</i> | This paper | N/A |
| <i>Escherichia coli</i> DH10 β harboring pGFPuv- <i>bgc52</i> | This paper | N/A |
| <i>Lachnospiraceae</i> sp. 3_1_57FAA | Emma Allen-Vercos | N/A |
| <i>Ruminococcus</i> sp. 5_1_39BFAA | Emma Allen-Vercos | N/A |
| Recombinant DNA | | |
| pET28a | Lab stock | N/A |
| pET28a- <i>bgc35</i> -NRPS | This paper | N/A |
| pET28a- <i>bgc41</i> -NRPS | This paper | N/A |
| pET28a- <i>bgc33</i> | This paper | N/A |
| pET28a- <i>bgc86</i> | This paper | N/A |
| pET28a- <i>sfp</i> | Lab stock | N/A |
| pGFPuv | Clontech | Cat# 632370 |
| pMSD | Lab stock | N/A |
| pGFPuv- <i>bgc34</i> | This paper | N/A |
| pGFPuv- <i>bgc35</i> | This paper | N/A |
| pGFPuv- <i>bgc35</i> _NRPS_D686A | This paper | N/A |
| pGFPuv- <i>bgc35</i> _NRPS_D1713A | This paper | N/A |
| pGFPuv- <i>bgc35</i> _NRPS_D686A_D1713A | This paper | N/A |
| pGFPuv- <i>bgc35</i> _NRPS_TCA ₂ TR | This paper | N/A |
| pGFPuv- <i>bgc35</i> _NRPS_CA ₂ TR | This paper | N/A |
| pGFPuv- <i>bgc41</i> | This paper | Synthesized by GenScript |
| pGFPuv- <i>bgc43</i> | This paper | Synthesized by GenScript |
| pGFPuv- <i>bgc52</i> | This paper | N/A |
| pMSD- <i>bgc28</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc30</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc32</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc37</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc38</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc39</i> | This paper | Synthesized by GenScript |
| pMSD- <i>bgc45</i> | This paper | Synthesized by GenScript |
| Sequence-Based Reagents | | |
| Primers used in this study | This paper | Table S3 |
| Software and Algorithms | | |
| Adobe Illustrator | Adobe | N/A |
| Agilent Masshunter Workstation | Agilent | N/A |
| Agilent Qualitative analysis | Agilent | N/A |
| Blast | NCBI | https://blast.ncbi.nlm.nih.gov/Blast.cgi |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|-------------------------------------|---|
| ChemDraw Professional 15.1 | PerkinElmer | |
| Geneious | Geneious | http://www.geneious.com/ |
| GeneOptimizer | ThermoFisher Scientific | |
| GraphPad Prism version 5.0 | GraphPad | www.graphpad.com |
| Mega 6 | Mega | http://www.megasoftware.net/ |
| MestreNova 10.0 | Mestrelab Research | http://mestrelab.com |
| Microsoft excel | Microsoft | N/A |
| Microsoft word | Microsoft | N/A |
| MultiGeneBlast | Medema et al., 2013 | http://multigeneblast.sourceforge.net/ |
| NEBaseChanger | NEB | http://nebasechanger.neb.com/ |
| Scifinder | The American Chemical Society | https://scifinder.cas.org/ |
| SnapGene Viewer | SnapGene | http://www.snapgene.com/products/snapgene_viewer/ |
| XCMS online | The Scripps Research Institute | https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage |
| Xcalibur Software | ThermoFisher Scientific | N/A |
| Other | | |
| Agilent 6120 quadrupole mass spectrometer | Agilent | N/A |
| Agilent 6530 Q-TOF LC/MS equipment | Agilent | N/A |
| Agilent Zorbax SB-C18 3.0 mm by 100 mm, 1.8-Micron, 600 Bar | Agilent | N/A |
| Anaerobic chamber | Coy Laboratory Products | N/A |
| Bsal | NEB | N/A |
| Bruker Avance DRX500 | Bruker | N/A |
| Bruker AvanceIII 600-I | Bruker | N/A |
| Cadenza CD-C18 column (75 × 4.6 mm, 3 μm) | Imtakt | Cat# CD003 |
| Emulsiflex homogenizer | Avestin | N/A |
| In-Fusion HD Cloning Kit | Clontech Laboratories | N/A |
| Thermo Scientific Nanodrop 2000 | Thermo Scientific | N/A |
| Ni-NTA beads | QIAGEN | N/A |
| Nutator | Thermo Scientific | N/A |
| Phenomenex Kinetex Biphenyl C18 (250 × 10 mm, 5 μm) | Phenomenex | N/A |
| Phenomenex Kinetex EVO C18 (100 × 2.1 mm, 2.6 μm) | Phenomenex | N/A |
| Phenomenex Luna 5 μm C18 (250 × 10 mm, 5 μm) | Phenomenex | N/A |
| QIAGEN Miniprep Kits | QIAGEN | N/A |
| VWR V-1200 Spectrophotometer | VWR | N/A |
| Thermo Q-exactive Orbitrap Velos MS | ThermoFisher Scientific | N/A |

CONTACT FOR REAGENT AND RESOURCE SHARING

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METHOD DETAILS

A general scheme of all the experiments and analyses performed in this study can be found in [Table S1](#). Details of the characterized NRPSs, including their host organisms, putative substrates, and products can be found in [Table S2](#). Primers used in this study are listed in [Table S3](#).

EXPERIMENTAL DESIGN

Detailed Information About Refactoring and Synthesizing BGCs in This Study

Since the bacterial species shown in Figure 1 have not been manipulated genetically, we decided not to use a targeted gene deletion strategy. Instead, we expressed these BGCs heterologously in hosts whose genetic systems have been well developed. Each BGC contains a core biosynthetic gene encoding an NRPS. Two extra genes, which putatively encode an A domain and a T domain (e.g., *bgc35*) are in close proximity to their core NRPS genes. In addition, a 4'-phosphopantetheinyl transferase (PPTase) gene is located at 3' end of some of the BGCs. PPTase enzymes, such as Sfp from *B. subtilis*, catalyze an essential posttranslational modification in nonribosomal peptide biosynthesis (Reuter et al., 1999). The targeted BGCs were reconstructed by excising regulatory genes which, we presumed, are not directly involved in the biochemical steps of the biosynthetic pathway. Other genes, including those that encode the core NRPS, the extra A and T domains, the PPTase, and some transporter genes, remain intact in our assembled BGCs.

For molecular cloning experiments, BGCs are divided into two categories. BGCs from hosts we could obtain were assembled in the pGFPuv vector and heterologously expressed in *E. coli* DH10 β (Figure S1). BGCs from hosts that were not accessible were synthesized by GenScript. For any BGCs without a PPTase gene, we included a PPTase at the 3' end of the assembled cluster. The synthesized clusters were codon-optimized for their expression host (either *E. coli* or *B. subtilis*) and assembled into their respective vectors for heterologous expression.

Strains and Molecular Genetic Manipulations

BGCs expressed in *E. coli* (Figure 1, colored red or blue) were regulated by the *lac* promoter except *bgc33* (T7 promoter) (Figure S1). BGCs expressed in *B. subtilis* 168 are regulated by the hyper-*Pspac* promoter. *bgc34*, *bgc35*, *bgc41*, *bgc43*, and *bgc52* were assembled in the vector pGFPuv and verified by sequencing (Figure S1). Synthetic, codon-optimized versions of *bgc33* and *bgc86* were assembled in the vector pET28a and verified by sequencing. Synthetic, codon-optimized versions of *bgc28*, *bgc30*, *bgc32*, *bgc37*, *bgc38*, *bgc39*, and *bgc45* were synthesized in pMSD and verified by sequencing. To verify that clusters integrated properly into the chromosome of *B. subtilis*, we performed diagnostic PCR according to a scheme detailed in Figure S1.

1. Assembling *bgc34*, *bgc35*, and *bgc52* with pGFPuv vector

Due to the relatively large size of these BGCs, regulatory components in these BGCs were omitted. The genes essential for biosynthesis were PCR amplified using Q5 hot start high-fidelity DNA polymerase (See Table S3 and Figure S1). Three fragments for each BGC were synthesized and assembled with pGFPuv vector using either In-Fusion cloning kit (*bgc34* and *bgc35*) or Gibson assembly kit (*bgc52*). The assembled reaction mixture was further purified using Zymo DNA Clean and Concentrator kit and transformed into commercial *E. coli* DH10 β competent cells (Invitrogen). Upon transformation, transformants that are resistant to carbenicillin (carb) were first streaked on LB agar supplemented with carb (100 μ g/ml) followed by diagnostic PCR using primer set F5 (*Bgc34_F3* for *bgc34*) and pGFP-Diag-R (Table S3). Positive hits (mutants carrying assembled plasmids) were then cultivated in 10 mL LB + carb at 30°C, 225 rpm for 24 hr for plasmid extraction. The assembled plasmids were extracted using QIAGEN Miniprep Kits and further verified by sequencing.

2. Heterologous expression (HE) of *bgc28*, *bgc30*, *bgc32*, *bgc37*, *bgc38*, *bgc39*, and *bgc45* in *B. subtilis* and transformation of *bgc41* and *bgc43* in *E. coli*

These BGCs were synthesized and codon optimized for their specific expression strains by GenScript. For transformation of *B. subtilis*, the synthesized BGCs were assembled into the pMSD vector (see Figure S1) and around 80 ng of total DNA was added into 1 mL of protoplast solution for each individual transformation. The transformants were then further verified by diagnostic PCR using primer set BS_amyE_F and BS_amyE_R (Table S3). The transformant with correct single insertion at *amyE* locus should amplify a much larger fragment (~10 kb) in comparison to that of the host strain without an insertion (Figure S1). For transformation of *bgc41* and *bgc43*, the procedures are similar to those described above.

3. Assembling *bgc33* and *bgc86* with pET28a vector and HE in *E. coli* BAP1

The coding sequence (CDS) of the *bgc33* and *bgc86* NRPS biosynthetic genes were synthesized with BsaI flanking sites by GeneArt (Thermo Fisher Scientific) with *E. coli* codon optimization using the GeneOptimizer algorithm. The synthesized gene was cloned by Golden Gate assembly into *E. coli* expression vector under T7 promoter control. In brief, PCR fragment of expression vector pET28a (Novagen) was generated with Q5 High-Fidelity DNA Polymerase (New England Biolabs) following manufacturer protocol with primers pET28a-BsaI-F and pET28a-BsaI-R (Table S3). PCR fragment of pET28a was ligated to synthesized *bgc33* or *bgc86* construct in a 5 μ L one-pot digestion/ligation reaction mix consisting of 10 fmol of *bgc33* construct, 10 fmol of pET28a PCR product, 2.5 u of BsaI (New England Biolabs) and 2.5 u of T4 DNA ligase HC (Promega). Reaction conditions: 1 cycle of 37°C for 5 hr; 10 cycles of 37°C for 2 min, 16°C for 5 min; 1 cycle of 50°C for 15 min; 1 cycle of 80°C for 15 min; 4°C hold. The resulting construct *bgc33*-pET28a was verified by complete plasmid sequencing service (Massachusetts General Hospital DNA core facility). *bgc33*-pET28a and *bgc86*-pET28a were transformed into *E. coli* BAP1 containing T7 DNA polymerase and *sfp* phosphopantetheinyl transferase.

4. Assembling *bgc35* point mutation and NRPS truncation vector with pGFPuv vector

The point mutation mutants of the first adenylation domain (A_1) and the second adenylation domain (A_2) of the *bgc35* NRPS was generated using the Q5 Site-Directed Mutagenesis Kit (NEB). The primers used in this experiment (Table S3) was designed using NEBaseChanger (NEB). The vectors were verified by sequencing and transformed into *E. coli* DH10 β cells.

Detailed Procedure for Fermentation, LCMS, HRMS and HRMS-MS Fragmentation Analyses

1. Bacterial strain fermentation and LCMS sample preparation

A single colony of each mutant strain was used to inoculate a 5 mL LB broth culture (10 g tryptone, 5 g yeast extract, 10 g NaCl, 100 µg/mL of the corresponding antibiotic), which was incubated at 30°C with shaking at 225 rpm. After 48 hr, the culture supernatant was extracted with 5 mL ethyl acetate (EA). The EA layer was evaporated in vacuo and re-dissolved in 200 µL of 20% DMSO/MeOH, 10 µL of which was examined by LC-MS analysis. For HR-ESI-MS analysis of *bgc35*, *bgc38*, and *bgc52*, the DMSO/MeOH extract was diluted to ~1 ng/µL, 10 µL of which was used for an MS-MS fragmentation analysis (Thermo Orbitrap Velos).

2. LCMS analysis using an Agilent 6120 quadrupole mass spectrometer

Solvent system: A: 100% H₂O with 0.1% formic acid; B: MeCN with 0.1% formic acid. For ethyl acetate (EA) extraction of bacterial culture, the gradient for HPLC-MS analysis is 0-5 min 100% A, 5-35 min 100%–0% A, 35-37 min 0% A, 37-39 min 0%–100% A, 39-41 min 100% A at a flow rate of 0.4 mL/min. For analysis of the purified Boc-protected aldehydes and estimation of the stability of those deprotected peptidyl aldehydes, the gradient is 0-8 min 95%–5% A, 8-10 min 5% A at a flow rate of 1.0 mL/min using a Cadenza CD-C18 column (75 × 4.6 mm, 3 µm).

3. HRMS and HRMS-MS analyses

The analysis of pathway dependent molecules from *bgc33*, *bgc35*, *bgc38*, *bgc52*, and *bgc86* was performed on an Agilent 6530 Q-TOF LC/MS equipment and a C18 column. The HPLC gradient for *bgc35*, *bgc38*, and *bgc52* is 0-1 min 99.9% A, 1-7 min 99.9%–50% A, 7-11 min 50%–15% A, 11-13 min 15%–0.1% A, 13-25 min 0.1% A, 25-25.5 min 0.1%–99.9% A, 25.5-28 min 99.9% A at a flow rate of 0.4 mL/min. The gradient for *bgc33* and *bgc86* is 0-1 min 90% A; 1-12 min 90%–0% A; 12-14 min 100% B at a flow rate of 0.3 mL/min. The column was a Phenomenex Kinetex EVO C18 (2.6 µm, 100 × 2.1 mm). (Solvent A: 100% H₂O with 0.1% formic acid; B: MeCN with 0.1% formic acid).

The HRMS-MS fragmentation analysis for pathway dependent molecules was performed on a Thermo Q-exactive Orbitrap Velos MS equipped with a nanospray ESI source using the following gradient: 0-5 min 100% A, 5-35 min 100%–0% A, 35-37 min 0% A, 37-39 min 0%–100% A, 39-41 min 100% A. Pathway dependent molecules were analyzed in auto MS/MS mode with a collision energy of 35 eV.

The HRMS and HRMS-MS analyses for examining the deprotection reaction of boc-protected peptidyl aldehydes were carried out on an Agilent 6530 Accurate-Mass Q-TOF LC/MS. We used the following gradient: 0-8 min 95%–5% A, 8-10 min 5% A at a flow rate of 0.4 mL/min using a C18 column (Agilent Zorbax SB-C18 3.0 mm by 100 mm, 1.8-Micron, 600 Bar). The MS-MS analysis was performed in auto MS-MS mode with a collision energy of 20 eV.

Isolation and Characterization of Secondary Metabolites

For structure elucidation, each mutant strain was cultivated in 4 × 1 L LB medium (with the exception of *bgc33*, which was cultivated on 16 L scale) containing 100 µg/mL of the corresponding antibiotic and incubated at 30°C (25°C for *bgc33*) with shaking at 225 rpm. After 48 hr (28 hr for *bgc33*), the culture supernatant was extracted 2x with an equal volume of EA, and the solvent was removed from the combined extracts by rotary evaporation. The dried material was dissolved in 80% MeOH/DMSO and separated by reverse-phase HPLC (Agilent 1200 series) for small molecule purification. NMR spectra were collected on either a Bruker Avance DRX500 or a Bruker Avance III 600-I spectrometer. Purification of EA fraction was carried on by gradient HPLC on a C18 reverse phase column (Phenomenex Luna 5 µm C18 (2), 250 × 10 mm) with a flow rate of 5.0 mL/min. The gradient system was MeCN (solvent B) and H₂O (solvent A).

1. Purification of compounds 1-5 from *bgc35*

Compounds 1 to 5 were identified in the metabolite profiles of the *E. coli* mutant strains heterologously expressing *bgc35*. The gradient condition for semi-preparative HPLC separation of the crude of the *bgc35* heterologous expressing strain was 0-5 min 100% A, 5-29 min 100%–20% A, 29-30 min 20%–0% A, 30-31 min 0%–100% A, 31-32 min 100% A. Compound 3 (1.68 mg/L of culture) was eluted at 20.11 min. Compounds 1 (0.45 mg/L of culture) and 2 (1.20 mg/L of culture) are in a mixed fraction which was further purified using gradient 0-2 min 100% A, 2-3 min 100%–67% A, 3-21 min 67% A, 21-22 min 67%–0% A, 22-23 min 0% A, 23-24 min 100% A. Compounds 4 and 5 were eluted at 14.0 and 15.7 min, respectively. Compounds 4 and 5 are mixed and the gradient for further purification is 0-2 min 100% A, 2-3 min 100%–62.5% A, 3-20 min 62.5% A, 20-21 min 62.5%–0% A, 21-22 min 0%–100% A. Compounds 4 (2.05 mg/L of culture) and 5 (2.78 mg/L of culture) were eluted at 18.09 and 19.59 min, respectively.

2. Purification of compounds 6-13 from *bgc52*

The gradient for purifying compounds from *bgc52* was the same as that was used for *bgc35*. Compound 6 (1.00 mg/L of culture) was eluted at 18.71 min. Compound 7 (3.13 mg/L of culture) was eluted at 19.59 min. Compound 8 (1.64 mg/L of culture) was eluted at 20.11 min. Compound 13 was eluted at 23.60 min. Compounds 9 and 10 were eluted in the same fraction which was further purified using gradient 0-3 min 100% A, 3-5 min 100%–44% A, 5-10 min 44%–43% A, 10-11 min 0% A, 11-12 min 0%–100% A, 12-13 min 100% A. The same gradient was used to purify fraction containing compounds 11 and 12. The four compounds [9 (1.13 mg/L of culture), 10 (1.19 mg/L of culture), 11 (1.12 mg/L of culture), 12 (1.25 mg/L of culture)] were eluted at 8.90, 9.12, 9.35, 9.70 min, respectively.

3. Purification of compound 14 from *bgc38*

The gradient purifying the crude from the mutant strains carrying *bgc38* is 0–5 min 100% A, 5–23 min 100%–40% A, 23–24 min 40%–0% A, 24–25 min 0%–100% A, 25–26 min 100% A. Compound 14 (1.23 mg/L of culture) was eluted at 20.10 min.

4. Purification of compounds 15 and 16 from *bgc33*

Sixteen L of *bgc33* were extracted with EA and dried by rotary evaporation. The EA extract was purified by reversed-phase preparative HPLC using a gradient of 10% acetonitrile/90% H₂O containing 0.1% acetic acid to 100% acetonitrile in 24 min (10 mL/min). Fractions containing 15 and 16 were purified by reversed-phase HPLC (Phenomenex Luna C18, 250 × 10 mm, 5 μm) using a gradient of 10% methanol/90% H₂O containing 0.1% acetic acid to 40%/60% in 3 min, followed by a gradient to 100% methanol in 22 min. Fractions containing 15 and 16 were subjected to additional reversed-phase HPLC (Phenomenex Kinetex Biphenyl C18, 250 × 10 mm, 5 μm) using a gradient of 10% acetonitrile/90% H₂O containing 0.1% acetic acid to 70%/30% in 25 min, followed by 100% acetonitrile in 1 min, yielding 15 (RT 18.5 min, 0.09 mg/L of culture) and 16 (RT 22.6 min, 0.31 mg/L of culture).

Detailed Procedure for Biochemical Reconstitution of *bgc35*

1. Cloning of *bgc35* NRPS into pET28a

Clostridium sp. KLE1755 was grown in an anaerobic chamber at 37°C in Brain Heart Infusion agar with 0.1% cysteine, 0.5% yeast extract, and 15 mg/L hemin, pH 7.0. Genomic DNA was extracted from the bacteria using ZR bacterial DNA miniprep kit (Zymo). C.sp. KLE_NRPS1_pET28_fwd and rev primers were used to amplify the NRPS gene from genomic DNA and pET28_Sall_fwd and pET28_NdeI_rev were used to amplify the vector (Table S3). The gene was assembled into pET28a with an N terminus His tag using Circular Polymerase Extension Cloning (CPEC) and transformed into *E. coli* BL21 (Quan and Tian, 2009).

2. Purification of *bgc35* NRPS

E. coli BL21 harboring *bgc35* NRPS in pET28a was grown in 20 mL of LB + 50 μg/mL Kanamycin at 30°C overnight and diluted to fresh 1 L of LB + 50 μg/mL Kanamycin the next morning until early log phase (OD 600 ~0.4). The diluted culture was moved to 16°C incubator and shaken overnight without IPTG induction. The next day, cells were pelleted at 6000 g for 10 min, and resuspended in Lysis Buffer (300 mM NaCl, 10 mM Imidazole, 50 mM NaH₂PO₄, pH 8.0) with EDTA-free Protease Inhibitors (Roche). Cells were lysed using the EmulsiFlex (~10 min continuous flow, ~15,000 psi). Lysed cells were centrifuged at 16,000 rpm for 20 min. The supernatant was added to pre-equilibrated Ni-NTA beads (QIAGEN) and rocked on the Nutator at 4°C for 1 hr. The beads were spun down at 1000 rpm for 3 min. 20 mL of Wash Buffer (300 mM NaCl, 20 mM Imidazole, 100 mM NaH₂PO₄) was added and the mixture was transferred to an equilibrated column. Three 20 mL washes were performed and finally eluted in 4 mL of Elution Buffer (300 mM NaCl, 250 mM Imidazole, 50 mM NaH₂PO₄). The eluted protein was dialyzed using a Dialysis Cassette (20K MWCO, Pierce) against a Dialysis Buffer (25 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol).

When we performed SDS-PAGE on the eluent, we noticed that along with the expected full size band at 280 kDa, there were other lower bands present. We performed a western blot with anti-His antibody and found that these lower bands also bound to anti-His antibody, suggesting they were degradation products of the full-length *bgc35* NRPS. In order to verify that the full-length NRPS was present in the eluent, we cut out the highest band that ran above the 212 kDa ladder in the SDS-PAGE gel and submitted it for MS analysis. The MS results showed a tryptic peptide that matched the N terminus beginning and the C terminus end of the amino acid sequence of the NRPS. Therefore we concluded that the full-length NRPS is present in the eluent, and proceeded with the in vitro reconstitution using this full-length and degraded NRPS mixture.

3. In vitro reconstitution of *bgc35*

The activity of *bgc35* NRPS was assayed by comparing the LC-MS profiles of the reaction with and without the biosynthetic enzymes. The in vitro reconstitution reaction was set up as follows: 75 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 0.1 mM CoA, 1 mM Amino Acid Mixture (or 1 mM aromatic amino acids only), 1 mM NADPH, 10 μM NRPS enzyme, 0.1 μM sfp and 5 mM ATP in a total volume of 200 μL. The entire reaction excluding ATP was incubated at 37°C for 30 min before adding the ATP. After addition of ATP, the reaction was incubated at 37°C overnight. The next day, the reaction was quenched by addition 200 μL EA and mixing vigorously on the vortex. The mixture was spun at 10,000 × g for 5 min, and the top layer (EA) was collected and removed by rotary evaporation. The dried crude was resuspended in 40 μL of 20% DMSO in 80% MeOH and spun at 21,000 g for 10 min on the microcentrifuge. A 10 μL aliquot was examined by LC-MS using the same conditions used for analyzing the metabolite profile of the *E. coli* strain expressing *bgc35*.

Identification of Compounds from the Native Organism Harboring the Gene Cluster

Glycerol stocks containing *Clostridium* sp. KLE1755 and *Ruminococcus* sp. 5_1_39BFAA were streaked on pre-reduced EG blood agar plates (Recipe for 1 L: 2.8 g Lab Lemco Powder, 10 g Protease Peptone No. 3, 5 g Yeast Extract, 4 g Na₂HPO₄, 1.5 g D(+)-Glucose, 0.5 g Soluble Starch, 0.2 g L-cystine, 0.5 g L-cysteine·HCl·H₂O, 0.5 g Tween 80, 16 g Bacto Agar, 5% Horse Blood, pH 7.6–7.8) and grown for 2 days at 37°C in an anaerobic chamber. After 2 days, the resulting colonies were inoculated into 6 mL of twelve different pre-reduced liquid media (Anaerobic Basal Broth, BHI Broth, Casman Broth Base, Columbia Broth, Cooked Meat Medium, M17 Broth, Marine Broth, Nutrient Basal Broth, Reinforced Clostridium Media, Tryptic Soy Broth, TYG broth, Wilkins Chalgren Anaerobic Base Broth) and incubated for another 2 days at 37°C anaerobically. Media without the reducing agent L-cysteine in the ingredients were supplemented with L-cysteine for a final concentration of 0.05% (w/v). After another 2 days, some of the liquid cultures showed turbidity (not all media resulted in growth): *Clostridium* sp. KLE1755 grew in TYG, TSB, RCM and M17. *Ruminococcus* sp. 5_1_39BFAA grew in RCM, TYG, ABB, Columbia, M17, WCABB, Casman and TSB. Cultures in which

the bacterial species grew were centrifuged at 3200 *g* for 5 min, and 5 mL of the supernatant was extracted with 5 mL of EA. This mixture was spun down for 10 min at 3200 *g*. The top layer was transferred to 5 mL glass vials and solvent was removed by rotary evaporation. The crude was resuspended in 100 μ L 20% DMSO in MeOH. The resuspended extract was centrifuged at 21,000 *g* for 10 min on the microcentrifuge and a 10 μ L portion of the supernatant was injected for LC-MS analysis.

Analysis of Metatranscriptomic Data

We recruited reads from publicly available RNA sequencing (RNA-seq) datasets from the stool samples of eight healthy subjects (Franzosa et al., 2014). Illumina reads from several runs on each sample were combined, and used to construct a BLAST database that was then searched using the 47 full-length gut NRPS BGCs as query sequences. For this search, we used BLASTn using the default parameters to identify all reads recruited to the BGCs, then used the following parameters to map them back to individual BGCs (minimum number of reads: 100, minimum overlap: 50 bp, minimum percent identity at overlap: 90%, and maximum percentage of mismatch per read: 20%), and finally displayed them using Geneious. The BGCs identified were *bgc41*, *bgc44*, *bgc45/48/71*, and *bgc52/53/73* (the latter two sets are too similar in amino acid sequence to be differentiated in this analysis).

Synthesis, Deprotection of Boc-Val-Phe-H, Boc-Leu-Phe-H, and Boc-Phe-Phe-H and Stability Measurement of Their Deprotected Peptide Aldehydes

1. Synthesis of Boc-Val-Phe-H, Boc-Leu-Phe-H, and Boc-Phe-Phe-H

Boc-Val-OH (286.7 mg, 1.32 mmol, 1.0 equiv.) and 2-amino-3-phenyl-1-propanol (200 mg, 1.32 mmol, 1.0 equiv.) were dissolved in DMF (10 mL) and then EtN(iPr)₂ (0.5 mL, 2.9 mmol, 2.2 equiv.) and HATU (500 mg, 1.32 mmol, 1 equiv.) were added. The reaction was stirred for 2 hr at room temperature. 50 mL ddH₂O was added to quench the reaction and the mixture was extracted with equal amount of EA, twice. The EA layer was washed by brine, dried by adding anhydrous Na₂SO₄, followed by concentration using rotary evaporation. The crude was purified by flash column chromatography on silica gel to give Boc-Val-Phe-OH (256.5 mg). Boc-Val-Phe-OH (99 mg, 0.28 mmol, 1.0 equiv.) was dissolved in DMF (2 mL) and Dess-Martin periodinane (360 mg, 0.85 mmol, 3.0 equiv.) was added. The reaction was stirred for 3 hr at room temperature. 50 mL water was added to quench the reaction and the mixture was extracted with equal volume of EA twice. The EA layer was washed by brine and dried by anhydrous Na₂SO₄. The concentrated EA crude was further purified by HPLC using Phenomenex Luna C18 (250 \times 10 mm, 5 μ m). The HPLC gradient for purification is 0–3 min 80% A, 3–10 min 80%–5% A, 10–13 min 5%–80% A at a flow rate of 5 mL/min (Solvent A: H₂O; Solvent B: MeCN). Fractions were collected in a time-based manner and individual fraction was examined by LCMS. Fractions containing targeted aldehyde compounds were collected and concentrated via freeze-drying to yield Boc-Val-Phe-H (white powder, 21.0 mg).

The aforementioned procedure was applied to synthesize and purify Boc-Leu-Phe-H and Boc-Phe-Phe-H. Boc-Leu-OH (305.3 mg, 1.32 mmol, 1.0 equiv.) was used as a starting material to give 267.1 mg Boc-Leu-Phe-OH. Then 100 mg of Boc-Leu-Phe-OH was oxidized to give Boc-Leu-Phe-H (white powder, 75.3 mg). For the synthesis of Boc-Phe-Phe-H, Boc-Phe-OH (350.2 mg, 1.32 mmol, 1.0 equiv.) was used as a starting material and 197.9 mg of Boc-Phe-Phe-OH was obtained. 100 mg of Boc-Phe-Phe-OH was then oxidized to give Boc-Phe-Phe-H (white powder, 66.5 mg).

2. Deprotection of Boc-protected dipeptide aldehyde compounds

All the deprotection experiments were performed in an anaerobic chamber. Trifluoroacetic acid (TFA) was reduced and added to the purified Boc-protected dipeptidyl aldehydes. 1 μ L of the reaction was added to 100 μ L 80% DMSO in ddH₂O and a 0.5 μ L portion of the solution was examined by an Agilent 6530 qTOF LC/MS. For preparing dipeptide aldehyde solution for protease inhibition assays, the deprotection reaction was left at 30°C for 15 min. Then ddH₂O was added to quench the reaction and give a 2.5 mM peptidyl aldehydes in 1% TFA solution. Some side products like isobutylene and their corresponding imine and pyrazinone products have poor water solubility. The imine and pyrazinone compounds will precipitate in the solution. The reaction was centrifuged for 5 min at 16,000 *g* and the supernatant was given for protease assay immediately. Water containing only 1% TFA was also tested for protease inhibition activity as a negative control.

3. HRMS analyses of peptidyl aldehydes after deprotection (Figure S5)

- (1). Val-Phe-H (aldehyde): HRMS [M + H]⁺ *m/z* found 249.1609, calcd for C₁₄H₂₁N₂O₂ 249.1603; Val-Phe-DHPZN (imine): HRMS [M + H]⁺ *m/z* found 231.1491, calcd for C₁₄H₁₉N₂O 231.1497; Val-Phe-PZN (pyrazinone, compound **10**): HRMS [M + H]⁺ *m/z* found 229.1348, calcd for C₁₄H₁₇N₂O 229.1341.
- (2). Leu-Phe-H: HRMS [M + H]⁺ *m/z* found 263.1787, calcd for C₁₅H₂₃N₂O₂ 263.1760; Leu-Phe-DHPZN (imine): HRMS [M + H]⁺ *m/z* found 245.1679, calcd for C₁₅H₂₁N₂O 245.1654; Leu-Phe-PZN (compound **12**): HRMS [M + H]⁺ *m/z* found 243.1518, calcd for C₁₅H₁₉N₂O 243.1497.
- (3). Phe-Phe-H: HRMS [M + H]⁺ *m/z* found 297.1600, calcd for C₁₈H₂₁N₂O₂ 297.1603; Phe-Phe-DHPZN (imine): HRMS [M + H]⁺ *m/z* found 279.1498, calcd for C₁₈H₁₉N₂O 279.1497; Phe-Phe-PZN (compound **5**): HRMS [M + H]⁺ *m/z* found 277.1348, calcd for C₁₈H₁₇N₂O 277.1341.

The structures of peptide aldehydes after deprotection were verified by HRMS and HRMS-MS analyses. These compounds will fragment in a characterized manner as shown in Figure S5.

4. Estimation of the stability (half-life) of deprotected peptide aldehyde compounds

50% aqueous DMSO (for dissolving pyrazinone compounds with poor water solubility) in 50 mM potassium phosphate buffer (pH 7.2) was prepared. 1% of the reaction solution (in TFA) was added to the prepared buffer to yield a 1% TFA solution (pH 7.0). At different time points, 20 μ L of the mixed solution was injected into LCMS using the method as aforementioned. The amount of pyrazinone type compounds was measured by EIC as shown in [Figure S5](#).

Protease Inhibition Assays

For protease inhibition assays, all fluorescence measurements were made on a Biotek H4 instrument. Buffering reagents were purchased from Sigma-Aldrich and sterile-filtered prior to use. Compounds and substrates were diluted from DMSO stocks into water/DMSO such that final DMSO plate concentrations were below 5% during the reaction. All reactions were started by the addition of substrate to the enzyme/compound solutions.

Enzymes

Cathepsin L, cathepsin B, cathepsin C and cathepsin S were purchased from R&D Systems. trypsin, chymotrypsin, and calpain I were purchased from Sigma-Aldrich. Calpain I was also purchased from Abcam. Human 20S proteasome was purchased from Boston Biochem.

Substrates

z-FR-AMC, Boc-IEGR-AMC, Suc-ALPF-AMC and GR-AMC were purchased from Bachem. Suc-LLVY-AMC was purchased from Anaspec. Substrates were used without further purification.

Inhibitors

Carfilzomib was a generous gift from Onyx Pharmaceuticals and was used as a positive control in the proteasome inhibition assay. Pefabloc was purchased from Sigma-Aldrich and used as a positive control in the inhibition assays of trypsin and chymotrypsin. Leupeptin and Chymostatin were purchased from Research Products International. Leupeptin was used as a positive control in the inhibition assays of cathepsin B, cathepsin L, cathepsin S, and human calpain I. Chymostatin, as a positive control, was used in the inhibition assay of cathepsin C.

Enzyme assays

Cathepsin L (0.02 μ g/ml) and cathepsin B (0.2 μ g/ml) were assayed in 50 mM MES buffer (pH 5.5) containing 5 mM DTT, using z-FR-AMC at 20 μ M.

Trypsin (3 μ g/ml) was assayed in 40 mM Tris (pH 7.8), 0.01 M CaCl_2 using Boc-IEGR-AMC (10 μ M). Chymotrypsin (30 ng/ μ l) was assayed in 40 mM Tris (pH 7.8), 0.1 M CaCl_2 with Suc-ALPF-AMC (10 μ M). Human 20S proteasome was pre-activated for 1 hr in 20 mM Tris (pH 8.0), 0.5 mM EDTA, and 0.03% SDS at 10 nM prior to dilution to 1 nM for assays with Suc-LLVY-AMC (10 μ M).

Calpain I (10 μ g/ml) was assayed in 20 mM Imidazole (pH 7.5), 5 mM DTT, 5 mM CaCl_2 with Suc-LLVY-AMC (10 μ M). Cathepsin S was pre-activated for two hours at 10 μ g/ml in 50 mM MES buffer (pH 5.5), 5 mM DTT, and then diluted to 100 ng/ml for assays with z-FR-AMC (10 μ M). Cathepsin C/DPPI (200 μ g/ml) was incubated with cathepsin L (20 μ g/ml) in 25 mM MES (pH 5.5) 5 mM DTT for 1 hr prior to dilution to 0.25 ng/ μ l in 25 mM MES (pH 5.5), 5 mM DTT, 50 mM NaCl for assays with GR-AMC (10 μ M). Normalized enzyme activity data were fit using GraphPad Prism version 5.0 for Windows.

Target Identification by Chemical Proteomics

THP-1 cells (TIB-202), Ramos cells (CRL1596) and H1975 cells (CRL5908), obtained from ATCC, were grown at 37°C with 5% CO_2 and maintained at a low passage number (< 10 passages). THP1 and Ramos cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. THP-1 cells were further supplemented with 50 μ M β ME. H1975 cells were cultured in DMEM medium supplemented with 20% fetal bovine serum, penicillin, streptomycin, and glutamine. To induce differentiation of THP-1 cells, cells were treated with phorbol 12-myristate 13-acetate (PMA, final concentration = 200 nM) for 18 hr at which point the media was replaced and cells were allowed to proliferate for 3 additional days. Cells were harvested by centrifugation (1,400 g, 3 min, 4°C), pellets washed with cold PBS, lysed by sonication and fractionated (100,000 g, 45 min) to yield soluble and membrane fractions, which were then adjusted to a final protein concentration of 1.5 mg/mL for proteomics experiments. pH 5 and pH 5.5 samples were lysed into sodium acetate buffer adjusted to the indicated pH. Protein concentration was determined using the Bio-Rad DC protein assay kit. 500 μ L of the indicated proteome was treated with *bgc35* Phe-Phe-H (5 μ L of 2.5 mM aqueous solution containing 1% TFA, final concentration = 25 μ M) or with vehicle (5 μ L aqueous containing 1% TFA). For 2.5 μ M and 250 nM concentration treatments, samples were treated with 5 μ L of 100 \times compound stock solutions diluted from the parent stock solution into water. Samples were incubated for 30 min following which treated and control samples were further labeled for an additional 30 min with iodoacetamide-alkyne (IA-alkyne, 5 μ L of 10 mM stock in DMSO, final concentration = 100 μ M). All labeling steps were conducted at ambient temperature. Control and treated samples were then subjected to copper-mediated azide-alkyne cycloaddition (CuAAC) conjugation to isotopically labeled, TEV-cleavable biotinylated peptide tags, control, and treated samples combined, enriched on streptavidin resin (Pierce 20349) and subjected to sequential trypsin and TEV digests as has been reported previously ([Backus et al., 2016](#)). TEV digests were analyzed by multidimensional liquid chromatography tandem mass spectrometry (MudPIT), using an LTQ-Velos Orbitrap mass spectrometer (Thermo Scientific) coupled to an Agilent 1200- series quaternary pump and searched and analyzed as has been reported previously ([Backus et al., 2016](#)).

Detailed Structural Characterization of Purified Compounds Isolated in This Study

Compounds identified in this study can be grouped into three classes: dihydropyrazinones (compounds 1 and 2, for example), pyrazinones (compounds 3-15), and N-acyl peptide aldehydes (compound 16) (Figure 2). Their biosynthetic origin, from a group of NRPSs which take amino acids as substrates, facilitates the structural elucidation process.

For dihydropyrazinones, compound 1 was purified as an amorphous yellowish solid and its molecular formula was determined to be $C_{20}H_{19}N_3O$ by its HRMS spectral data, suggesting thirteen indices of hydrogen deficiency (IHD). This compound and compound 2 are slowly degrading upon isolation. The 1H , ^{13}C , gHMQC, and gHMBC NMR spectroscopic data of compound 1 (Data S1, Table a) including the six phenyl carbons [δC 138.3 (C-4), δC 128.6 (C-5 and C-9), δC 129.4 (C-6 and C-8), the five aromatic protons [H-5 and H-9, δH 7.38 (2H, d, $J = 12.0$ Hz); H-6 and H-8, δH 7.29 (2H, m); H-7, δH 7.28 (1H, m)], the CH_2 -3 methylene group [δH 3.16 (1H, d, $J = 12.0$ Hz), δH 3.03 (1H, d, $J = 12.0$ Hz), and δC 40.6] exhibit a typical phenylalanine side chain. A tryptophan side chain was also established from the 1H , ^{13}C , gHMQC, and gHMBC NMR spectroscopic data. The ^{13}C spectrum exhibits eight aromatic carbons (C-4' to C-11'). The 1H spectrum shows four aromatic protons exhibiting a typical coupling pattern of an indole ring (H-7' to H-10'). The gHMBC correlations between the indole 5'-NH [δH 10.73 (1H, s)] and four aromatic carbons (C-4', 5', 6', and 11') and the gHMBC correlations between the CH_2 -3' methylene group [δH 2.88 (1H, dd, $J = 12.0$, 6.0 Hz), δH 2.77 (1H, d, $J = 12.0$ Hz), and δC 26.4] and C-4' and 11' further suggests that compound 1 contains a tryptophan side chain moiety. The phenylalanine and tryptophan side chains, in combined, contributed 10 IHDs. The dihydropyrazinone core (and how these two side chains are connected to the core) was set up based on the following evidence: 1. The gHMBC correlations between the H_2 -3' and C-2' (δC 53.1) and one amide carbon C-1' (δC 171.5); 2. The gHMBC correlations between H_2 -3 and C-2 (δC 58.6); 3. The gCOSY correlations between one imine proton H-1 (δH 7.44, m) and H-2 (δH 3.53, m). Thus, the structure of compound 1 was assigned as shown in Figure 2 and we named it DHPZN1.

For compounds within the pyrazinone class, the verification of amino acid side chains are comparable to that of compound 1. Taking compound 7 (Data S1, Table g) as an example, its molecular formula was determined to be $C_{15}H_{18}N_2O_2$ by its HRMS spectral data, suggesting eight indices of hydrogen deficiency (IHD). The tyrosine side chain takes up four IHDs. The pyrazinone core (and how tyrosine and leucine side chains are connected to the core) was set up based on the following evidence: 1. Comparison to the published literature (MacDonald et al., 1976; Zimmermann and Fischbach, 2010); 2. The gHMBC correlations between the H_2 -3' [δH 2.60 (2H, d, $J = 10.0$ Hz)] and C-2' (δC 156.6) and one amide carbon C-1' (δC 157.3); 3. The gHMBC correlations between H_2 -3 [δH 3.76 (2H, s)] and C-2 (δC 139.3) and C-1 (δC 121.6). Thus, the structure of compound 7 was assigned as shown in Figure 2 and we named it PZN5.

Both Adenylation Domains Participate in Generating Side-Chain Chemical Diversity

Given that pyrazinones appear to be the native products of *bgc35* and *bgc52*, we next turned to the question of how a three-module NRPS gives rise to a diverse family of dimeric nonribosomal peptides. To address this question, we individually mutagenized the first and second adenylation domains (A_1 and A_2) from the NRPS in *bgc35*, expressed the mutagenized protein in *E. coli*, and profiled its culture extract by LC-MS (Figure S7). To our surprise, both individual mutants retained the production of a subset of the *bgc35* products. Reasoning that the residual activity could be due to A domain mutations that did not eliminate amino acid substrate binding, we constructed and profiled an A_1 - A_2 double mutant. LC-MS analysis of its culture extract revealed that activity had been completely abolished, indicating that each single domain mutant had effectively eliminated substrate binding. Collectively, these data suggest that each module in the NRPS is capable of acting iteratively. Consistent with this view, a truncated form of the *bgc35* NRPS consisting of only the second module (C_2 - A_2 - T_2 -R) is capable of synthesizing a subset of the pyrazinone and pyrazine products observed from *bgc35* (Figure S7; Table S4). These data suggest that both modules of the *bgc35* NRPS contribute to the diversity in product structure. Moreover, the observation that the product spectrum of the single A domain mutants is skewed toward pyrazines is consistent with the possibility that in iterative format, the terminal reductase favors the release of individual α -aminoaldehyde monomers rather than a dipeptide aldehyde.

DATA AND SOFTWARE AVAILABILITY

A supplemental dataset including all the NMR information about the compounds characterized in this study (Data S1) and a supplemental data table including all the cysteine targets identified in target identification experiments (Data S2) and can be found with this article online.

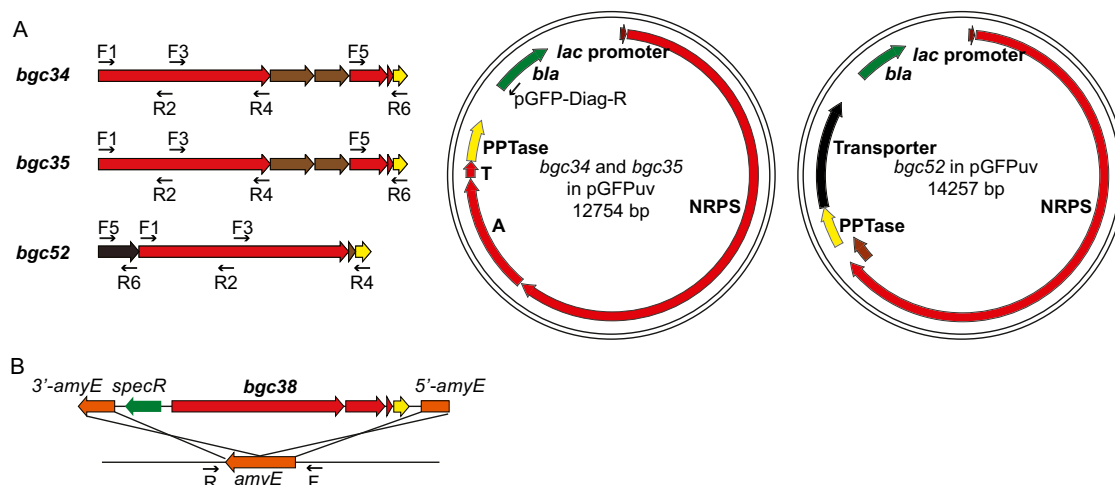


Figure S1. The Schemes for Molecular Genetic Cloning and Diagnostic PCR, Related to Figures 1 and 2

(A) *bgc34*, *bgc35*, and *bgc52* were synthesized via assembling of three fragments (F1 + R2, F3 + R4, F5 + R6) and pGFPuv vector. Transformants were selected on LB + Carb plates and were initially screened by diagnostic PCR using primer set F5 + pGFP-Diag-R (Table S3).

(B) An example of how those BGCs optimized for *B. subtilis* 168 are transformed into the host strain. pMSD is constructed in a manner in which the targeted BGC is inserted into 5'-*amyE* and 3'-*amyE*. Upon transformation, the targeted BGC, for example *bgc38*, and the spectinomycin resistant gene *specR* are inserted at the *amyE* locus of *B. subtilis* via homologous recombination. Diagnostic PCRs were performed using primer set BS_amyE_F + BS_amyE_R, which anneal just outside the *amyE* locus. If a single copy of BGC is inserted, the PCR fragment amplified from a correct transformant will be different in size from the fragment amplified if *amyE* is intact.

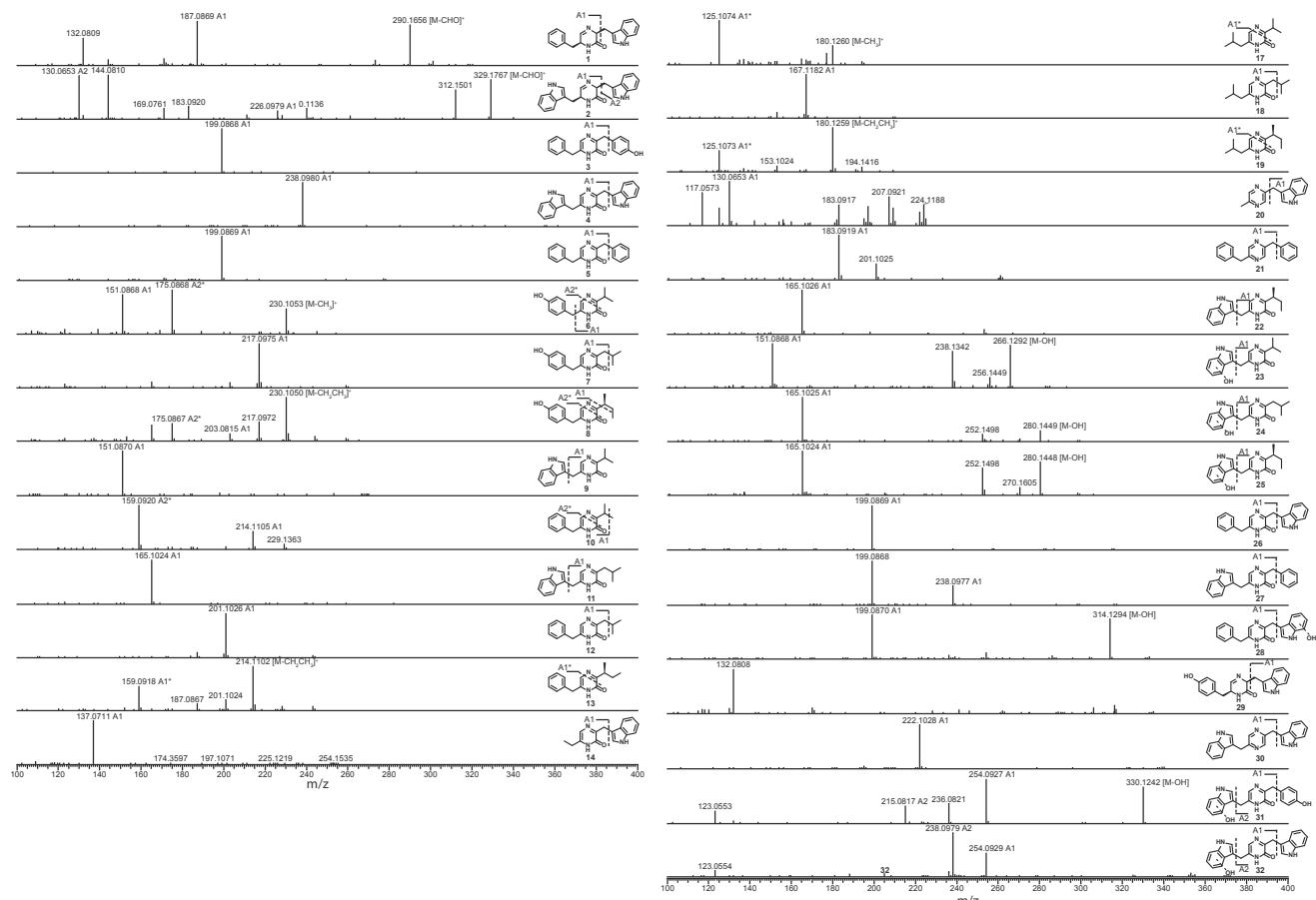


Figure S2. HRMS-MS Fragmentation Analyses of Pathway-Dependent Molecules, Related to Figure 2 and Table S4

The asterisk denotes a pattern reported by Wyatt et al. (2012).

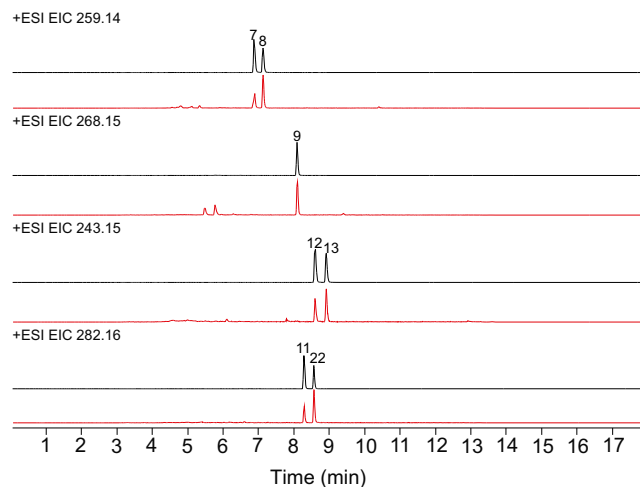


Figure S3. The Same Set of Pyrazinones Are Produced by *Ruminococcus sp.* 5_1_39BFAA, the Native Host of *bgc52*, Related to Figure 2 and Table S4

The compounds produced by *E. coli* + *bgc52* can also be found in the culture extract of *R. sp.* 5_1_39BFAA. Black: Extracted ion chromatogram of *E. coli* DH10β_ *bgc52* culture; Red: Extracted ion chromatogram of *R. sp.* 5_1_39BFAA culture. The numbering of the peaks in the figure corresponds to the natural products shown in Table S4.

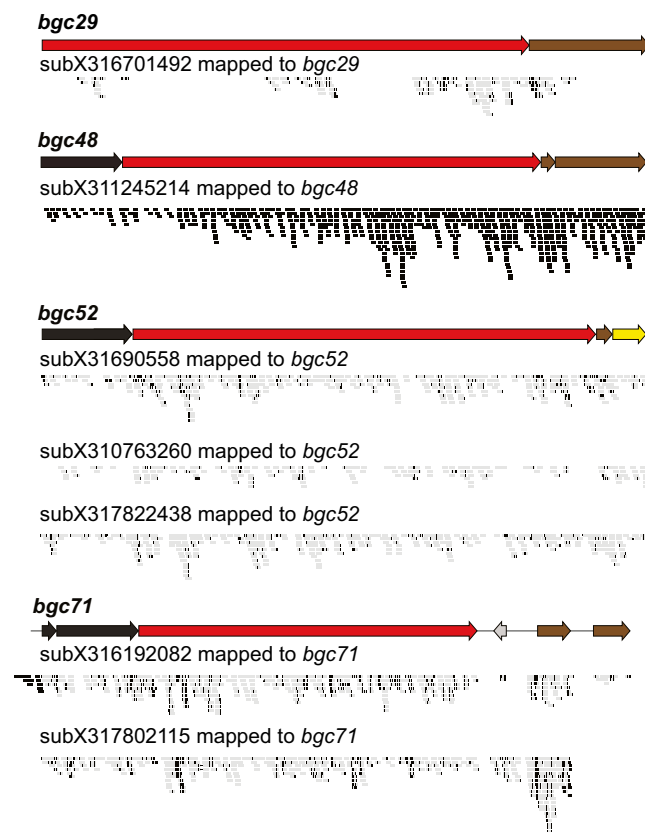


Figure S4. Metatranscriptomic Analyses of BGCs in Figure 1, Related to Figures 1 and 3

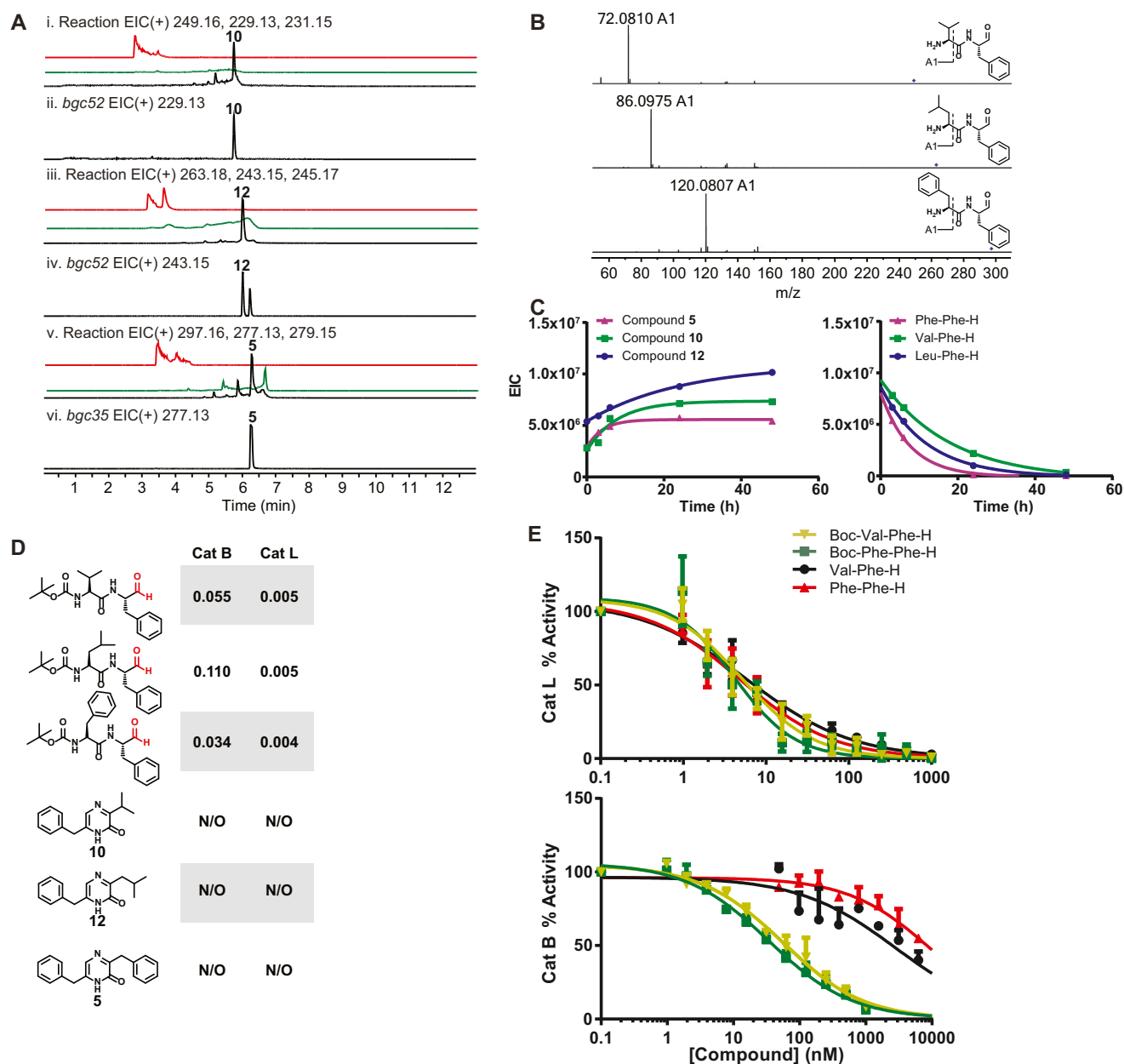


Figure S5. Analytical Chemistry and Biological Activity Analysis of Synthetic Dipeptide Aldehydes, Related to Figure 3

(A) HRMS analysis of TFA deprotection reactions. i. EIC (+) of 249.16 (Val-Phe-H, red), 229.13 (compound **10**, black), and 231.15 (corresponding imine form, green); ii. EIC (+) of 229.13 (**10**) from EA extracts of bacterial culture; iii. EIC (+) of 263.18 (Leu-Phe-H, red), 243.15 (**12**, black), and 245.17 (imine form, green); iv. EIC (+) of 243.15 (**12**) from EA extracts of bacterial culture; v. EIC (+) of 297.16 (Phe-Phe-H, red), 277.13 (**5**, black), and 279.15 (imine form, green); vi. EIC (+) of 277.13 (**5**) from EA extracts of bacterial culture.

(B) HRMS-MS fragmentation pattern of Val-Phe-H, Leu-Phe-H, and Phe-Phe-H.

(C) Stability measurement of dipeptide aldehydes in the experiment. The stability of dipeptide aldehydes is measured by (1) the rate of pyrazinone (**5**, **10**, and **12**) accumulation examined by the area under curve (AUC) of EIC (+) and (2) the rate of the dipeptide aldehyde disappearance as determined by the AUC of EIC (+).

(D) IC₅₀ values obtained in in vitro cathepsin B and cathepsin L inhibition assays using Boc-protected peptide aldehydes and pyrazinones. IC₅₀ values are shown in μ M. N/O = no inhibition observed.

(E) Inhibition curves of Val-Phe-H, Phe-Phe-H, and their Boc-protected peptide aldehydes against cathepsins B and L. The Boc-protected molecules could efficiently inhibit both cathepsin B and cathepsin L with IC₅₀ values at nM range. The deprotected molecules could efficiently inhibit cathepsin L (IC₅₀ at nM range) but not cathepsin B (IC₅₀ at μ M range).

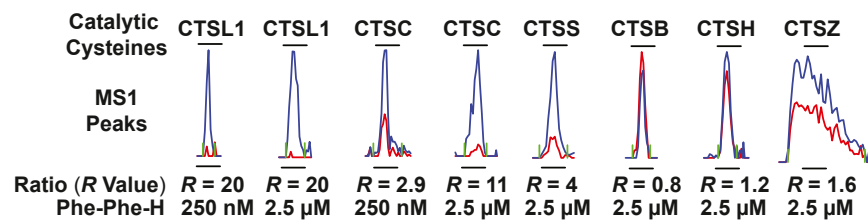


Figure S6. MS1 Chromatographic Peaks for Peptides in Target Identification Analyses, Related to Figure 3

This figure shows representative MS1 chromatographic peaks for peptides containing the catalytic cysteines from CTSL1, CTSC, CTSS, CTSB, CTSH and CTSZ. The membrane fraction of THP1 cells was treated with Phe-Phe-H at the indicated concentrations and evaluated by isoTOP-ABPP.

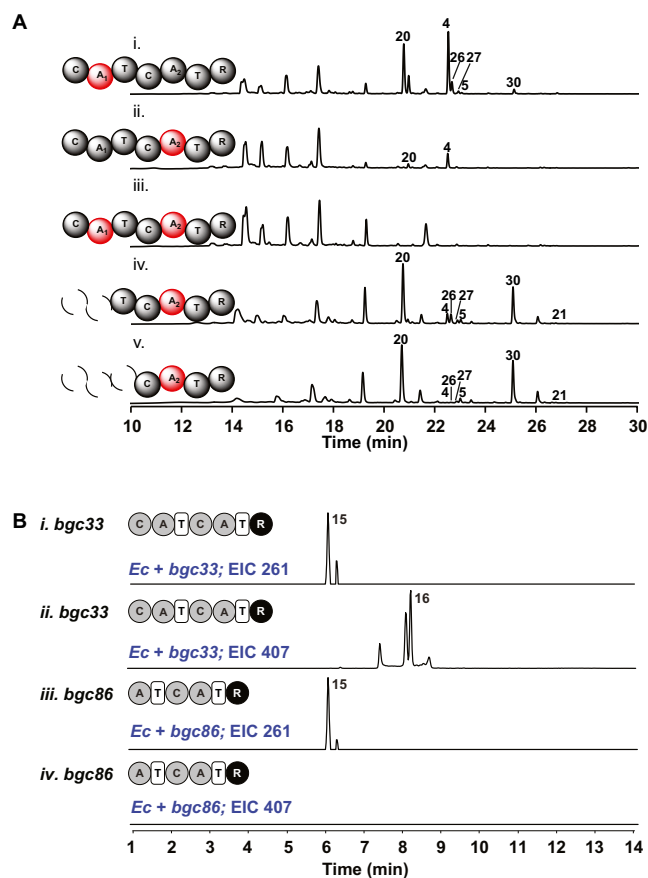


Figure S7. Metabolite Profiling of Strains Harboring *bgc35* Mutants, *bgc33*, and *bgc86*, Related to Figures 1 and 2 and Tables S4 and S5

(A) Point mutation and protein truncation analysis of the NRPS in *bgc35*. HPLC profiles of the point mutants heterologously expressing *bgc35* (i) D686A, (ii) D1713A, (iii) D686A and D1713A, (iv) *bgc35* NRPS from which C and A1 have been excised, (v) *bgc35* NRPS from which C, A1, and T1 have been excised, as detected by UV at 300 nm.

(B) The first condensation domain of *bgc33* participates in N-acylation of compound 16. The gene clusters *bgc33* and *bgc86* are closely related and differ only by the absence of the first condensation domain (Figure 1). Under the same cloning, fermentation, and extraction conditions as *bgc33*, *bgc86*-harboring *E. coli* BAP1 produced the pyrazinone 15 (i and iii), but not the acylated compound 16 (ii and iv).