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Whole-Genome Shotgun Sequencing of Two β-Proteobacterial Species in Search of the Bulgecin Biosynthetic Cluster

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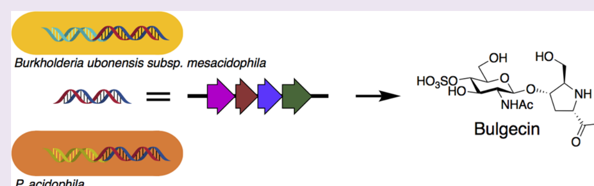
Supporting Information

ABSTRACT: We have produced draft whole-genome sequences for two bacterial strains reported to produce the bulgecins as well as NRPS-derived monobactam β-lactam antibiotics. We propose classification of ATCC 31363 as *Paraburkholderia acidophila.* We further reaffirm that ATCC 31433 (*Burkholderia ubonensis subsp. mesacidophila*) is a taxonomically distinct producer of bulgecins with notable gene regions shared with *Paraburkholderia acidophila.* We use RAST multiple-gene comparison and MASH distancing with published genomes to order the draft contigs and identify unique gene regions for characterization. Forty-eight natural-product gene clusters are presented from PATRIC (RASTtk) and antiSMASH annotations. We present evidence that the 10 genes that follow the sulfazecin and isosulfazecin pathways in both species are likely involved in bulgecin A biosynthesis.



Resistance to antibiotics in Gram-negative bacteria has created a state of crisis, as many classes of antibiotics have become obsolete.[1−3] Morbidity and mortality from infections by these bacteria have reached high levels for the past 50 years, necessitating novel strategies for clinical intervention.[4] Efforts in discovery of new classes of antibiotics for Gram-negative bacteria have been largely fruitless,[1] which have prompted a re-evaluation of old known compounds that were not developed previously. The polymyxin colistin, a decades-old antibiotic, is an example.[1] This antibiotic was abandoned because of its nephrotoxicity, but it has found clinical applications of late in light of the dearth of options.

In this vein, we have been interested in the bulgecins, natural products that were discovered in the 1980s.[5−7] These compounds potentiate β-lactam antibiotics in killing Gram-negative bacteria.[5,8] Bulgecins—of which three are known (bulgecins A–C, Figure 1A)—are inhibitors of bacterial lytic transglycosylases.[5,8] As the β-lactam antibiotic inhibits cross-linking of the cell-wall peptidoglycan (the transpeptidase reaction), linear chains of peptidoglycan are accumulated.[9] Lytic transglycosylases turn over these aberrant peptidoglycan structures. In the presence of a bulgecin, initiation of the repair processes is abrogated, which leads to cidal activity on Gram-negative bacteria. The reason why bulgecins were abandoned has not been reported. However, it is possible that since there was clinical recourse in treatment of Gram-negative bacteria at the time of their discovery, the commercial field might have been too crowded for a successful development. Unfortunately, bulgecins are no longer available to reassess their activities, which prompted the present study. In an effort to identify the gene cluster responsible for the biosynthesis of bulgecins, we undertook the present sequencing of two strains: *Pseudomonas acidophila* (ATCC 31363) and [*Pseudomonas*] *mesoacidophila*[10] (ATCC 31433, Taxonomy ID: 265293). We report herein that both strains possess 10 genes downstream of a cluster for the biosynthesis of a monobactam (sulfazecin or isosulfazecin, Figure 1B) that we attribute to the bulgecin cluster. Both strains have been characterized phenotypically as unique pseudomonads in the literature.[11] Recently, Loveridge and co-workers proposed classification of ATCC 31433 as a member of the *Burkholderia cepacia* complex.[10] We report concurrent sequencing efforts on ATCC 31433 as well as ATCC 31363. Additionally, we describe a conserved biosynthetic cluster whose disruption generates a mutant strain of ATCC 31363 deficient in bulgecin A production. Our phylogenetic analysis supports designation of ATCC 31433 as *Burkholderia ubonensis subsp. mesacidophila* and argues a classification of ATCC 31363 as *Paraburkholderia acidophila.* For the sake of clarity in the report, we will refer to the organisms hereafter exclusively by their ATCC designation.

We attempted purification of bulgecins from microbial culture, according to the reported methodology.[7,12] We were able to produce an extract from the growth that shows the potentiation activity by microbiological assays (Figure 1C). Cell-free supernatant from the culture of ATCC 31433 was treated with a base to inactivate the coproduced monobactam. Additionally, we were able to observe bulgecin A in the extract by liquid chromatography−mass spectrometry (LC-MS; Figure

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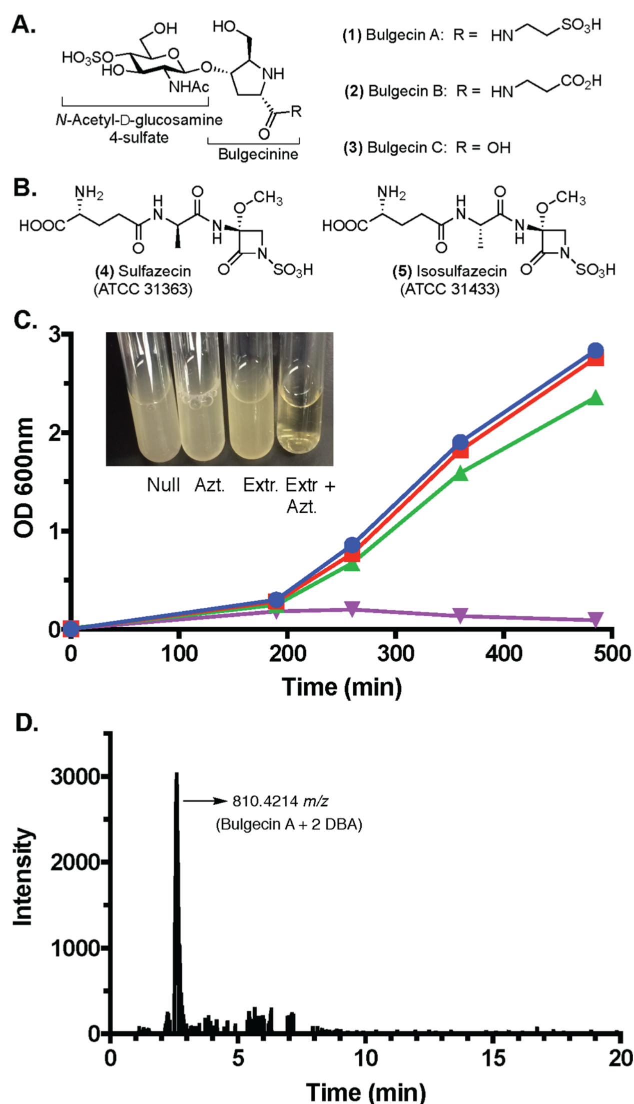

Figure 1. Chemical structures of bulgecins (A) and monobactams (B) produced by ATCC 31363 and ATCC 31433. (C) Bulgecin extract and aztreonam display potentiation against *E. coli* MC1061 in liquid culture and (D) detection of bulgecin A in culture extract. (C) *E. coli* MC1061 grown without aztreonam or bulgecin extract (blue), with aztreonam (0.05 mg L^{-1}, red), bulgecin extract (10%, green), or both bulgecin and aztreonam (purple). Inset shows the cultures at 500 min of incubation. (D) An extracted-ion chromatogram (XIC, 810.42 ± 0.01 *m*/*z*) from LC-MS analysis of culture extract showing the detection of bulgecin A as the bis-dibutylammonium (DBA) salt (observed *m*/*z*, 810.4214; calculated *m*/*z*, 810.4199).

1D). Previously, we had found the ion-pairing reagent di-*n*-butylamine acetate to be useful for the retention of sulfates on LC; this reagent proved to be helpful for the detection of bulgecin A, which was observed as a salt with two di-*n*-butylamine (DBA) molecules (observed *m*/*z*, 810.4214; calculated *m*/*z*, 810.4199).

Draft genomes for ATCC 31433 and ATCC 31363 were obtained through Illumina Miseq sequencing and SPAdes assembly. The ATCC 31433 assembly was 154 contigs, 7.7 Mb large, and 67.1% GC, while that of ATCC 31363 had a higher degree of assembly (11 contigs), smaller content (7.2 Mb), and notably lower GC% content (62.1%). These features are consistent with genome-wide sequence comparisons (MASH distances, RAST nearest neighbors, as well as 16S rRNA analysis, see accompanying Supporting Information, SI) that show ATCC 31433 as a member of the *Burkholderia* subclade[13] (average GC content of 67.2%, similar to *Burkholderia cepacia* complex member *B. ubonensis*, previously proposed as a member of *Burkholderia*[10]) and ATCC 31363 as a member of the *Paraburkholderia* subclade[14] (average GC content of 62.9%, similar to sp. 9120 (NCBI accession PRJNA247916), proposed here as *Paraburkholderia acidophila*). Our results with ATCC 31433 sequencing agree with those of Loveridge *et al.*, which was carried out recently independent of our work.[10] The distribution of genes dedicated to the different metabolic functions is largely conserved between these two species (SI), but only 56.7% (3704) of the ATCC 31363 genes have bidirectional matches in the ATCC 31433 genome, and the latter contains more secondary metabolite genes overall (see accompanying SI).[15]

Genome comparisons identified 70 genes uniquely shared among ATCC 31363, ATCC 31433, and suspected bulgecin producer *B. gladioli*[16] (previously sequenced[17]), yet not found in closely ATCC 31433-related strains that lack the sulfazecin cluster (SI). These 70 genes are likely candidates for production of unique metabolites, such as bulgecinine, a core constituent of the bulgecin structures (Figure 1A), as well as for the other transformations in the assembly of the larger bulgecin structure(s). There are two large syntenic clusters of these identified genes, one of which includes the recently identified sulfazecin cluster,[18] along with several adjoining genes. ATCC 31433 and ATCC 31363 share the monobactam (sulfazecin/isosulfazecin) pathway, as well as 10 genes downstream of *sulP*, which was defined as the last essential gene in the core sulfazecin cluster (Figure 2).[18]

These 10 genes, contiguous with the monobactam cluster, may constitute the cluster for the biosynthesis of bulgecin. The cluster is seen in both producers studied herein, as well as in *B. gladioli* 10248; each exhibits phenotypic microbiological activities equivalent to bulgecin-like natural products. In essence, every known producer has this cluster, which is not seen in other related strains. Furthermore, as we will outline below, disruption of this cluster leads to the reversal of the phenotype. Potential functions for the gene products of bulA−H and sat1−2 are outlined in Table 1.

The proximity of the genes from Table 1 to the sulfazecin cluster potentially allows for coregulation of monobactam and bulgecin production. Three regions surrounding the sulfazecin cluster (Figure 2) are notable for having several potential promoter sites (SI).[19] These include the sequence upstream of *sulM* (suggesting *sulM*−*sat1* as a theoretical transcriptional unit) and two regions in between *sulH* and *akn* (suggesting *akn*−*sulL* and *sulH*−*sulD* as possible transcriptional units). This configuration may restrict bulgecin production to situations in which the monobactam pathway is expressed.

The putative bulgecin cluster (Figure 2) codes for a sulfotransferase and a glycosyltransferase, as expected, as well as core genes (*sat2*, *sat1*) for the assembly of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), a metabolic sulfate donor. Additionally, BulC and BulD likely form a transketolase, which others have suggested is another requirement of the bulgecin pathway.[10] Interestingly, genes needed for taurine, the side chain found in bulgecin A, are not found within the pathway itself.

Remarkably, the *sulI* to *sat2* region is observed in at least 384 publicly deposited genomic sequences of bacterial isolates (SI), though bulgecin production in these strains has not been

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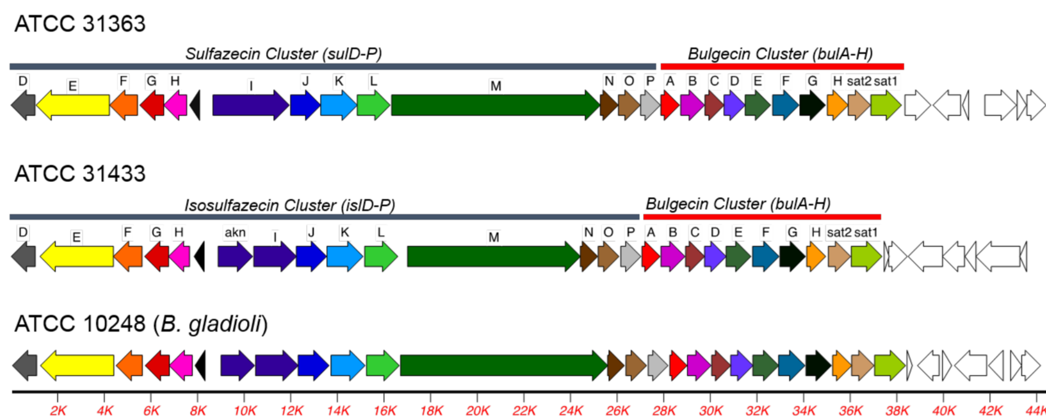

Figure 2. Gene cluster analysis in ATCC 31363, ATCC 31433, and *B. gladioli*. ORFs bulA−H, sat1, and sat2 represent genes common to producers of bulgecins and include predicted genes for a sulfate adenylyltransferase, a glycosyl transferase, and a sulfotransferase. The PATRIC-assembled contig containing the cluster in ATCC 31433 ends in the middle of sat2; additional scaffolding[10] supports contig 034 as the extension of the cluster. The completed cluster is shown. The ORF bulA was originally designated as sulQ but was found to be not essential for sulfazecin production. The monobactam cluster of gladioli was annotated *via* antiSMASH[15] using the complete genome.

Table 1. Attribution of Potential Functions for BulA−H and Sat1−2 Based on Sequence Similarity to Known Genes[a]

ORF	similar function	length (aa)	ATCC 31363 identity/similarity	*B. gladioli* ATCC 10248 locus tag	*B. gladioli* ATCC 10248 identity/similarity
bulA	ATP-grasp family	259	85/90	BM43_2804	84/88
bulB	sulfotransferase	341	77/85	BM43_2805	67/76
bulC	ThDP-dependent transketolase, N-terminal thiamine diphosphate binding domain	272	85/89	BM43_2806	81/86
bulD	transketolase, C-terminal pyrimidine binding domain	308	85/88	BM43_2807	83/88
bulE	dehydrogenase (see SI)	355	68/77	BM43_2808	66/73
bulF	acyltransferase 3	375	76/86	BM43_2809	80/88
bulG	phosphoserine transaminase	364	78/87	BM43_2810	82/89
bulH	glycosyl transferase group 2 family	270	84/91	BM43_2811	80/88
sat2	sulfate adenylyltransferase small subunit	320	91/93	BM43_2812	94/95
sat1	sulfate adenylyltransferase large subunit	438	85/90	BM43_2813	88/92

[a]Protein lengths in amino acids of ATCC 31433 are listed and % identity/similarity based on protein-level matches to ATCC 31433. Additional data are provided in Table S8.

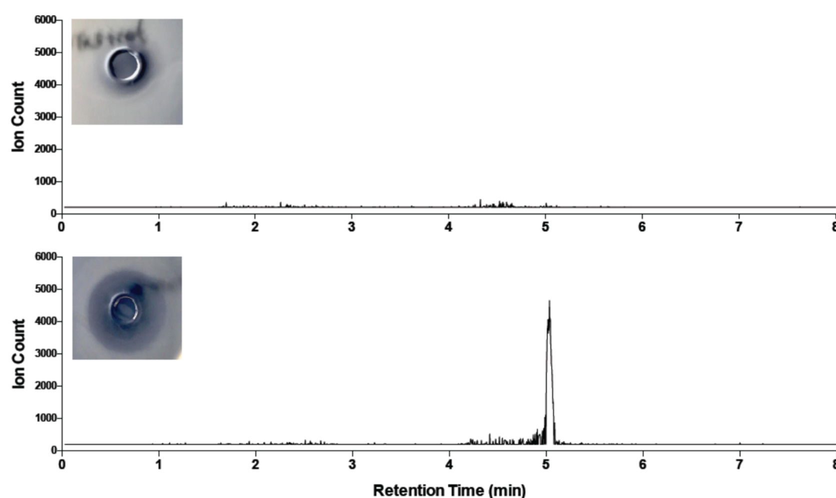

Figure 3. Screening of bulgecin nonproducer from transposon mutagenesis. Bioassay and UPLC-HRMS analyses of bulE::Tn5/sulG::Gm double mutant of ATCC 31363(top) and sulG::Gm single mutant of ATCC 31363, a bulgecin producer (bottom). Supernatants of fermentation broth were used directly for bioassay and for UPLC-HRMS after being concentrated 12.5× by partial lyophilization. Shown is the extracted ion chromatogram (XIC, 552.1164 ± 0.01) for the parent ion of bulgecin A (observed *m/z*, 552.1169; calculated *m/z*, 552.1164).

verified. Most commonly, *sulG* and *sulH* are omitted upstream and the ORF following *sat2* codes for a putative L-threonine kinase. In 21 draft genomes, the contig assembly ends at the start of the *sat2* ORF, whereas 15 genomes show a complete *sat2* followed by a partial or full stand-alone *sat1*, depending on the assembly. The majority (>80%) of *sulI* to *sat2* clusters are

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found in strains of *B. pseudomallei*, with lower proportions also found in examples of *B. mallei*, *B. thalliadensis*, *B. oklahomensis*, and *B. gladioli*; however, excluding ATCC 31363 and ATCC 31433, only four detected isolates (all *B. gladioli*) contain equivalents for *sulG* and *sulH* upstream of *sulI*. Enigmatically, eight *Chromobacterium* isolates possess a similar *sulG*, but in these cases, the remaining genes in the clusters are distributed throughout the genome.

In 1988, Gwynn *et al.* described a strain of *Burkholderia gladioli* (326–32B, genome sequence unknown) that they remarked "[in addition to producing a sulfazecin-like molecule (MM 42842)] members of the bulgecin family of antibiotics were detected in the same culture (S. J. Box and S. R. Spear; unpublished data)."[20] Additionally, Cooper *et al.* described *Chromobacterium violaceum* (ATCC 31532) as a producer of two bulgecin A analogues (SQ 28504 and SQ 28546), where taurine is replaced with peptides.[21,22] Subsequently, a draft genome was generated.[23,24] This genome draft (*Chromobacterium violaceum* strain CV017) was one of the eight *Chromobacterium violaceum* strains identified in the genome search.

Confirmation of the bulgecin biosynthetic gene cluster (BGC) in ATCC 31363 was carried out by a blind screen to obtain a bulgecin nonproducer using transposon mutagenesis.[18] The recipient strain was deficient in sulfazecin production by inactivation of *sulG*.[18] Approximately 2000 transconjugates were screened to identify the desired phenotype by bioassay. One transconjugant, named the *sulG::Gm/bulE::Tn5* double mutant of ATCC 31363, was identified. This strain was then fermented in sulfazecin production medium, partially purified, and concentrated. Direct UPLC-HRMS analysis confirmed that bulgecin production was completely eliminated in the *sulG::Gm/bulE::Tn5* double mutant (Figure 3). DNA sequencing analysis of the Tn5 insertional region in the *sulG::Gm/bulE::Tn5* double mutant revealed that *bulE* was disrupted by Tn5 transposon insertion. This finding clearly demonstrated that the bulgecin BGC is located downstream of the sulfazecin cluster.

BulE is recognized as a conserved hypothetical protein observed in similar *Burkholderia* strains; however, no member of this group has been assigned a function. Notwithstanding, in order to predict a role for BulE, the amino-acid sequence was aligned to well-characterized proteins with associated crystal structures (using I-TASSER,[25] see SI, Methods). BulE is predicted to adopt a Rossman fold similar to an NADH-dependent malate dehydrogenase and, thus, might function as a dehydrogenase in the assembly of the bulgecinine core.

Observed in both ATCC 31363 and ATCC 31433, the presence of genes involved in the production of PAPS and subsequent sulfation, a putative glycosyl transferase, a two-component transketolase, and a putative dehydrogenase, whose disruption eliminates the production of bulgecin A and modulates the synergistic properties of an extract from ATCC 31363, support this shared genomic region's involvement in bulgecin biosynthesis. Future work will focus on determination of the roles of these genes and the sequence of reactions leading to bulgecin biosynthesis.

■ METHODS

Bacterial Strains. *E. coli* MC1061 and ATCC 31433 (isolate originating at ATCC) were generously provided by Professor Marion Skalweit. ATCC 31363 was purchased from the American Type Culture Collection.

Growth and Potentiation Assays. For liquid culture potentiation assays, 40 mL of modified nutrient broth[26] (3 g/L meat extract powder, Himedia, and 5 g/L tryptone, VWR) in a 125 mL Erlenmyer flask was inoculated with 1 mL of overnight outgrowth of ATCC 31433 and 1 mL of dirt extract (6 g of moist dirt up to 20 mL with distilled water, vortexed, centrifuged 6000*g* for 5 min, cotton filtered, centrifuged at 21 100*g* for 3 min, and sterile-filtered). It was anticipated that the dirt extract would potentially up-regulate secondary metabolite pathways. Cultures were grown at 29 °C for 20 h with shaking at 200 rpm. The cells were pelleted by centrifugation (15 000*g*, 7 min). The supernatant was then base-treated with 2 M sodium hydroxide to pH 10 for 2 h to hydrolyze coproduced monobactams. The supernatant was concentrated 10-fold by rotary evaporation under reduced pressure to give the bulgecin extract. To assess potentiation, overnight outgrowths of *E. coli* MC1061[8] were diluted 1:1000 in LB medium, and 10% (v/v) of either nutrient broth (control) or bulgecin extract was added (culture volumes: 4 mL). Culture growth was assessed by measuring optical density (600 nm). To observe potentiation, 0.05 mg L^{-1} (final concentration) aztreonam was added to the culture. The potentiation assay is based on ref 27.

Tn5 Transconjugants Screen. The transposon mutagenesis library was constructed in the sulfazecin nonproducer *sulG::Gm* single mutant of ATCC 31363.[18] A total of 2000 Kan^R/Car^R transconjugants were analyzed for a loss of bulgecin production as previously reported[18] with the added supplementation of cefmenoxime (30 ng mL^{-1}) and *E. coli* DH5α to test potentiation after mating with the Tn5 delivery vehicle pGS9 in *E. coli* PR47.[18,28]

LC-MS Detection of Bulgecin A. The bulgecin extract from ATCC 31433 was prepared as described above, but the initial culture volume was scaled to 800 mL. Half of the extract was then concentrated to dryness using rotary evaporation under reduced pressure. Methanol (2 × 20 mL) was then added to the resulting residue, which was mixed and centrifuged (15 000*g*, 8 min) to remove insoluble materials. The solvent was removed *in vacuo*. The residue was dissolved in water (4 mL), which was further diluted 1–2 orders of magnitude for MS detection. LC-MS analysis was conducted on a Dionex UltiMate 3000 HPLC using an Acclaim RSLC 120 C18 column (120 Å, 2.2 μm, 2.1 × 100 mm). Initial conditions included 90% (v/v) solvent A (10 mM aqueous di-*n*-butylamine acetate) and 10% solvent B (10 mM di-*n*-butylamine acetate in acetonitrile), 0–2 min 10% B, 2–18 min up to 100% B, and 18–20 min 10% B. The flow rate was 0.4 mL min^{-1}. MS analysis of the bis-dibutylammonium salt was done using a coupled Bruker microTOF-Q II ESI Quadropole TOF mass spectrometer.

sulG::Gm/bulE::Tn5 and *sulG::Gm* double and single mutants of ATCC 31363 were fermented in sulfazecin-production medium for 60 h.[18] The supernatants (200 mL) were adjusted to pH 4.5 and applied to active charcoal columns (~100 mL). The columns were washed with 200 mL of ddH_2O and eluted with 125 mL of 50% acetone in five fractions. The 25 mL active fraction was confirmed by bioassay and further concentrated approximately 12.5-fold by lyophilization and filtered to remove any residual proteins (Millipore 3k filter). Ultraperformance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) experiments to directly detect bulgecin A (calculated m/z: 552.1164; observed: 552.1169) were carried out on a Waters Acquity H-class UPLC system in tandem with a Xevo-G2 high mass resolution Q-TOF MS/MS ESI system at the Johns Hopkins Mass Spectrometry Facility using the following UPLC-HRMS method, ESI+ [ternary gradient: water (solvent A), water (+1% (v/v) formic acid (solvent B), acetonitrile (solvent C)], 0.3 mL min^{-1}]: 0–1 min isocratic 10% (v/v) A, 10% B, 80% C; 1–7.5 min gradient 10% to 90% A, isocratic 10% B; 7.5–8.4 min isocratic 90% A, 10% B; 8.4–8.5 min gradient 90% to 10% A, isocratic 10% B; 8.5–10 min isocratic 10% A, 10% B, 80% C; Waters ACQUITY UPLC BEH Amide Column, 130 Å, 1.7 μm, 2.1 mm × 100 mm.

Illumina Paired-End MiSeq Sequencing. Genomic DNA was extracted from overnight cultures of ATCC 31363 and ATCC 31433 (ATCC) using a Wizard Genomic DNA purification kit (Promega). Each genomic DNA sample was incorporated into a sequencing library using an Illumina TruSeq Nano DNA Library Prep Kit. Each library

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was spiked with a quality control of PhiX174 genomic DNA (1:100 PhiX174 to library DNA), and the libraries were sequenced with paired-end reads using an Illumina MiSeq sequencer with a MiSeq Reagent Kit (v2, 500 cycles-PE) at the Genomics and Bioinformatics Core Facility at the University of Notre Dame.

Sequencing yielded 6 935 938 and 6 582 869 reads for ATCC 31363 and ATCC 31433, respectively. Assembly by SPAdes (v3.1.1)[29] on PATRIC produced 11 contigs for ATCC 31363 with a total size of 7 170 935 bases. The final average coverage was 178 fold with an N_{50} of 1 743 501. Running a SPAdes assembly on the ATCC 31433 reads produced 154 contigs with a total size of 7 750 452 bases. On average, the final coverage was 155 fold with an N_{50} of 100 069.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00687.

Additional methods (PDF)

Accession Codes

These Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accessions MTZU00000000 (ATCC 31433) and MTZV00000000 (ATCC 31363). The versions described in this paper are MTZU01000000 and MTZV01000000. The raw read data can be found at NCBI SRA accessions SRR5380803 (ATCC 31363) and SRR5380804 (ATCC 31433).

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Notes

The authors declare no competing financial interest.

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