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Investigation of secondary metabolism in the industrial butanol hyper-producer *Clostridium saccharoperbutylacetonicum* N1-4

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Abstract

Clostridium saccharoperbutylacetonicum N1–4 (*Csa*) is a historically significant anaerobic bacterium which can perform saccharolytic fermentations to produce acetone, butanol, and ethanol (ABE). Recent genomic analyses have highlighted this organism's potential to produce polyketide and nonribosomal peptide secondary metabolites, but little is known regarding the identity and function of these metabolites. This study provides a detailed bioinformatic analysis of seven biosynthetic gene clusters (BGCs) present in the *Csa* genome that are predicted to produce polyketides/nonribosomal peptides. An RNA-seq based untargeted transcriptomic approach revealed that five of seven BGCs were expressed during ABE fermentation. Additional characterization of a highly expressed nonribosomal peptide synthetase gene led to the discovery of its associated metabolite and its biosynthetic pathway. Transcriptomic analysis suggested an association of this nonribosomal peptide synthetase gene with butanol tolerance, which was supported by butanol challenge assays.

Keywords

Anaerobe; ABE fermentation; polyketide synthase; nonribosomal peptide synthetase; transcriptomics

Introduction

Clostridium saccharoperbutylacetonicum N1–4 (*Csa*) is a Gram-positive, spore-forming obligate anaerobe. After its isolation from soil in 1959, it was patented by the Sanraku Distillers Company in 1960 [32] for use in saccharolytic fermentations to produce organic solvents, including acetone, butanol, and ethanol (ABE). In subsequent decades, ABE

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fermentation largely fell out of favor due to competition from the petrochemical industry [21]. Today, interest in renewably produced butanol as a drop-in biofuel [15] has revived investigations into ABE fermentation as a sustainable source of chemical energy [12, 31, 44]. Despite the advantages of historical precedent [54], substrate flexibility [2, 5, 6, 8, 23, 24, 33, 35, 36, 42, 48, 51, 52, 58, 59], and butanol hyper-production for *Csa*, challenges remain to improve ABE productivity, titer, and yield of this organism. A deeper understanding of the biology of *Csa* could provide insights necessary to further improve its industrial fermentation traits [37].

Microbial secondary metabolites are known to possess diverse functions relating to the metabolism, physiology, differentiation, interspecies competition, etc [41]. For example, the plant pathogen, *C. puniceum*, produced an aromatic polyketide, clostrubin, which enabled *C. puniceum* to both survive in an oxygen-rich environment and inhibit other plant pathogenic bacteria [39, 46]. An ABE model organism, *C. acetobutylicum*, produced a glycosylated polyketide, clostrienose, which promoted sporulation and granulose accumulation [18]. A mutant of *C. acetobutylicum* lacking the capacity to produce clostrienose downregulated these differentiation processes, resulting in improved butanol titer and productivity. The secondary metabolism of clostridia could thus be manipulated to improve traits relevant for industrial applications. Among all secondary metabolites, polyketides (PKs) and non-ribosomal peptides (NRPs) are two major families noted for their chemical diversity, range of potent bioactivities, and well-studied mechanism of biosynthesis [20, 55]. PKs and NRPs are biosynthesized by polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), the thio-templated assembly-line enzymes, together with diverse tailoring enzymes. The biosynthetic genes for a particular metabolite often co-localize on the genome in a biosynthetic gene cluster (BGC), facilitating discovery *in silico* [29]. Notably, while *Csa* has a relatively large genome (6.6 Mb) [9, 40] among clostridia and putative PKS and NRPS encoding genes are widespread in its genome [26], little is known regarding the regulation and function of these genes and no PK and NRP metabolites have been reported.

We here study the PK/NRP-related secondary metabolism in *Csa* for the first time. First, an in-depth bioinformatic analysis is performed to profile BGCs encoding putative PKSs and NRPSs. Then, an untargeted transcriptomics approach is utilized to probe BGC expression in ABE fermentation culture conditions. Finally, one of the identified highly expressed BGCs is investigated to reveal its associated metabolite and possible role in ABE fermentation.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study are listed in Supplementary Table S1. *E. coli* strains were cultured in lysogeny broth (LB) containing the appropriate antibiotics at 37°C. Cloning was performed in an *E. coli* XL1-blue background. Protein work and *E. coli* in vivo assays were performed in *E. coli* BAP1 [38]. *Csa* was cultured at 37°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) containing an atmosphere of 97% nitrogen and 3% hydrogen. For routine culture and genetics work, *Csa* was incubated in 2x YTG (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, 10 g/liter glucose, 15 g/liter agar for solid

media) supplemented with 40 μg/ml erythromycin when necessary and adjusted to pH 6.5 with 1 N HCl.

Transcriptomic analysis

Fermentation samples were obtained 12 h after inoculation. 1 ml triplicate samples were pelleted and suspended in 1 ml TRIzol reagent (Ambion). These were stored at −80°C before processing based on a procedure modified from a previous method [56]. Samples were thawed and processed according to the manufacturer's instructions; samples were spun down and supernatants were extracted in chloroform. The aqueous phase of the three-phase mixture was pipetted into a new RNase-free centrifuge tube and mixed with 1 volume 70% ethanol, then applied to a RNeasy Mini Kit (Qiagen North America, Germantown, MD) for cleanup. The mixture was filtered by spinning through another Qiagen mini kit column, followed by the standard RW1 and RPE washes, and RNA was obtained in 60 μl DEPC-treated water. Residual genomic DNA was depleted using RQ1 DNase (Promega, Madison, WI). The samples were adjusted to 100 μl with DEPC-treated water, mixed with 350 μl RLT buffer and 250 μl ethanol, and returned to Qiagen spin columns for desalting according to the manufacturer's cleanup procedure. This yielded 60 μl samples containing 0.3–2.4 μg total RNA. RNA quality control, library construction, and library sequencing were performed by the University of California-Berkeley QB3 Functional Genomics Laboratory and Vincent J. Coates Genomic Sequencing Laboratory. RNA quality and concentration were assessed using a nanochip on an Agilent 2100 Bioanalyzer. Bacterial 16S and 23S rRNA was depleted using a RiboZero Kit (Illumina, San Diego, CA). The remaining RNA was converted to an RNA-seq library using an Illumina mRNA-Seq library construction kit. RNA library sequencing was performed on an Illumina HiSeq4000 instrument using 100 bp paired-end reads. After adapter trimming, reads were quality controlled using FastQC [4]. Base calls with a Phred score > 30 were kept for downstream analysis. Quality-controlled reads were mapped to the *Csa* chromosome (Refseq: NC_020291.1) and megaplasmid (Refseq: NC_020292.1) using Bowtie2 [25]. Read counts were extracted using HTSeq [3] and normalized to gene length. Differential expression analysis between wild-type and *Δnrps3* strains was carried out using DESeq2 [28].

Plasmid construction

Oligonucleotides were provided by IDT (Coralville, IA). Phusion polymerase (NEB, Ipswich, MA) was used for all PCR reactions. Detailed cloning procedures are provided under Supplementary Methods. All synthetic oligonucleotide sequences are described in Supplementary Table S2. After Gibson assembly [10], the reaction mix was transformed into chemically competent *E. coli* XL1-blue (Agilent Technologies, Santa Clara, CA). Clones were isolated and DNA was extracted using a plasmid Qiagen Miniprep kit. Constructs were validated by restriction digest patterning and Sanger sequencing.

Generation of *Csa nrps3* knockout mutant

Wild-type *Csa* was transformed with pNK52 using a previously reported electroporation method [18], with some adjustments. Briefly, competent cell stocks of *Csa* were prepared from overnight cultures (37°C, PL7 media) from glycerol stocks stored at −80°C. After reaching an OD_{600} of 0.6, overnight cultures were subcultured in 60 ml liquid 2x YTG (10%

inoculum) and incubated for 3 to 5 h until reaching an OD_{600} of 0.6. The subcultures were centrifuged (room temperature, 3,500×g, 15 min), decanted, and the pellet was resuspended in 6 ml room-temperature EPB (270 mM sucrose, 5 mM NaH_2PO_4 pH 7.4). We found that competent cell stock could be stored at −80°C in 20% glycerol, albeit with some loss of transformation efficiency. 500 μl electrocompetent cells were aliquoted into 4 mm Bio-Rad (Hercules, CA) cuvettes pre-chilled to 4°C, and 2 μg plasmid DNA was added. Electric pulses were delivered by a Bio-Rad Gene Pulser Xcell with parameters as follows: mode, exponential pulse; voltage, 2.0 kV; resistance, 200 Ω; capacitance, 25 μF. Following electroporation (yielding time constants of ~4 ms), cells were immediately resuspended in 10 ml 2x YTG and allowed to recover for 16 h at 37°C. Recovery cultures were centrifuged again and concentrated in 1 ml fresh media. 100 μl cells were plated on 2x YTG plates containing 40 μg/ml erythromycin. Colonies were picked and transferred into 10 ml liquid 2x YTG with antibiotic for 24–48 hours to allow Cas9-nickase mediated homologous recombination to delete 90% of the gene, using the recombination template present on pNK52 (Supplementary Fig. S1a). Dilutions were then plated on nonselective 2x YTG plates to allow curing of pNK52. After two days' incubation, colonies were replica plated on both nonselective and selective 2x YTG to detect successful loss of the plasmid. Colony PCR was used to screen for the genotype of the *Csa* Δ*nrps3* CRISPR mutants (Supplementary Fig. S1b).

Flask fermentations of *Csa*

Overnight cultures of *Csa* were prepared in a derivative of clostridial growth medium (CGM) [13], PL7 (30 g/liter glucose, 5 g/liter yeast extract, 2.67 g/liter ammonium sulfate, 1 g/liter NaCl, 0.75 g/liter monobasic sodium phosphate, 0.75 g/liter dibasic sodium phosphate, 0.5 g/liter cysteine-HCl monohydrate, 0.7 g/liter magnesium sulfate heptahydrate, 20 mg/liter manganese sulfate monohydrate, and 20 mg/liter iron sulfate heptahydrate, with the initial pH adjusted to 6.3 using 1 N HCl) from a single colony from a 2x YTG plate. At exponential phase, with OD_{600} of 0.4 to 0.6, a 4% inoculum was added to 25 ml of PL7G (PL7 with glucose increased to 35 g/liter, and 6 g/liter $CaCO_3$ for pH control) in loosely capped 50 ml centrifuge tubes to avoid pressurization. All fermentations were performed as biological triplicates in static batch culture. 1 ml samples were drawn at intervals of 12, 24, 48, and 72 h after inoculation. An additional 1 ml sample was drawn for metabolome analysis at the same time intervals.

Fermentation analytical procedures

Concentrations of acetone, butanol, ethanol, acetate, butyrate, lactate, and residual glucose were quantified using calibration curves generated on a Shimadzu Prominence UFLC system with refractive index and diode array detection (Shimadzu America, Inc., Columbia, MD). Prior to analysis, samples of culture supernatant were filtered using 0.22 μm polyvinylidene difluoride syringe (PVDF) filters (Restek, Bellefonte, PA). The resulting filtrate was resolved using a Bio-Rad Aminex HPX-87H column (300 mm by 7.8 mm) and detected by refractive index (for glucose, butanol, ethanol, acetate, and lactate), or by UV absorbance (for acetone, 265 nm; for butyrate, 208 nm). The method used a column temperature of 35°C, a 35 min run duration, and the manufacturer-recommended mobile phase (0.01 N H_2SO_4) at a flow rate of 0.7 ml/min.

Metabolomic analysis

For untargeted metabolomic analyses, samples were analyzed, in biological quadruplicate, using liquid chromatography-high resolution mass spectrometry (LC-HRMS). Samples containing 1 ml culture were extracted in 1 volume ethyl acetate. Mixtures were vortexed and spun down (6000×g, 1 min). The upper phase solvent layer was pipetted into a new centrifuge tube and dried under N_2, then resuspended in 100 μl methanol. 10 μl injections were analyzed on an Agilent Technologies 6545 Accurate-Mass QTOF LC-MS instrument fitted with an Agilent Eclipse Plus C18 column (4.6×100 mm). The run method used a linear gradient of 2–98% CH_3CN (v/v) over 57 min in H_2O with 0.1% formic acid (v/v) at a flow rate of 0.5 ml/min. Metabolomics data were analyzed using XCMS [11] to identify metabolites unique to either strain. The following parameters were used: p-value < 0.0005, fold change > 10, peak intensity > 10,000.

In vitro reconstitution of the CSPA_RS10760 NRPS

For in vitro assays, N-terminally his-tagged NRPS was overexpressed and purified from *E. coli* BAP1 pXZ247. Protein was purified as follows: A single colony was inoculated into 10 ml LB + 100 μg/ml carbenicillin for overnight growth at 37°C. About 5 ml was used to inoculate 700 ml LB + 100 μg/ml carbenicillin, and the culture was shaken at 240 rpm and 37°C until the OD_{600} reached 0.4. The culture was iced for ten minutes and isopropyl thio-β-D-1-galactopyranoside (IPTG) was added to a final concentration of 120 μM to induce protein expression. The culture was incubated at 16 °C for 16 h. Cells were harvested by centrifugation (5500×g, 4°C, 20 min), resuspended in 25 ml lysis buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, 5 mM imidazole), and lysed by homogenization over ice. Cell debris was removed by centrifugation (17,700×g, 4 °C, 60 min), and Ni-NTA agarose resin (Thermo Fisher Scientific, Waltham, MA) was added to the supernatant (2 ml/liter culture). The mixture was nutated at 4 °C for 1 h, loaded onto a gravity flow column, and the NRPS protein was eluted with increasing concentrations of imidazole in Buffer A (50 mM HEPES, pH 8.0, 1 mM DTT). Purified NRPS protein was concentrated and buffer exchanged into Buffer A + 10% glycerol using an Amicon Ultra-15 spin filter (MilliporeSigma, Burlington, MA) with nominal molecular weight cutoff of 100 kDa. Aliquots of purified NRPS protein were aliquoted and flash frozen in liquid nitrogen. The full in vitro reaction mixture contained 50 mM HEPES pH 8, 2 mM $MgCl_2$, 1 mM TCEP, 5 mM ATP, 4 mM NADPH, 5 mM L-valine, 5 mM L-leucine, 10 mM butyric acid, and 5 mM CoA, 0.01 mM Orf35 (a promiscuous CoA ligase) [60] and 0.01 mM NRPS in a 50 μl reaction volume. After 30 min, reactions were quenched with 2 volumes methanol and spun down (14000×g, 1 min) and the supernatant was collected for LC-MS analysis.

Substrate feeding in a heterologous host

E. coli BAP1 pXZ247 was cultured in 30 ml volumes of LB supplemented with 100 μg/ml carbenicillin at 37°C until the OD_{600} reached between 0.4 and 0.6. Cultures were cooled to 16°C and supplemented with 120 μM IPTG, 1 mM L-valine, 1 mM L-leucine, and 1 mM of various short-chain fatty acids (acetic acid, propionic acid, butyric acid, hexanoic acid, or octanoic acid). After 48 h incubation, cultures were spun down (4000×g, 5 min) and 10 ml

of supernatant was extracted twice using 5 ml ethyl acetate. The resulting products were detected by LC-HRMS under the conditions described above.

Butanol challenge assays

Butanol challenge assays were modified from a method used in *C. acetobutylicum* [53]. *Csa* wild-type and Δ*nrps3* cultures (in biological quadruplicate) were incubated overnight at 37°C in 50 ml centrifuge tubes containing 30 ml PL7G. At the onset of exponential growth at 8 h, cultures were challenged with the addition of water or butanol to a final concentration of 0, 2.5, or 5 g/liter butanol. Sampling was performed at 2–3 h time intervals by taking 200 μl volumes. OD_{600} was monitored in 96-well plates (Corning, NY) using a SpectraMax M2 instrument (Molecular Devices, San Jose, CA).

Results and discussion

Bioinformatic analysis of PK/NRP BGCs in *Csa*

As an initial step in exploring the secondary metabolism of *Csa*, we performed *in silico* analysis of the published genome of the type strain (NCBI accessions NC_020291.1 and NC_020292.1). The *Csa* genome consists of a 6.53 Mb circular chromosome and a 136 kb circular megaplasmid, giving this organism the largest reported genome of sequenced clostridia to date [9, 40]. The bioinformatic pipelines AntiSMASH [7] and PRISM [49] were used to identify putative BGC loci, and MultiGeneBLAST [30] and BiG-SCAPE [34] were used to query for homologous BGCs in other organisms. We identified seven BGCs which contain genes characteristic of PK or NRP biosynthesis. These can be categorized into four predicted NRPS gene clusters and three hybrid PKS-NRPS gene clusters (Fig. 1). We trimmed the BGCs conservatively to define putative boundaries by removing genes that were not in operon with a biosynthetic gene or that had predicted functions other than biosynthetic/transporter/regulatory/hypothetical. In the case of *hyb2* and *hyb3*, one locus was defined as two adjacent BGCs based on the fractured nature of biosynthetic genes in *hyb2*, the *cis*- vs *trans*-acyltransferase PKS modules, and the presence of distinct termination domains. More in-depth cluster-specific analyses are presented in Supplementary Table S3. The relative abundance of these secondary metabolism genes in *Csa* compared to other *Clostridium* species may suggest an important role of secondary metabolites in the evolutionary biology of *Csa*. Several of the BGCs appear to be conserved in other Firmicutes (Supplementary Fig. S2–S4). Notably, the megasynthetase gene of *nrps3* has homologs in two other ABE-producing Clostridia, although their respective neighborhoods are not conserved. This suggests the true boundaries of the BGC encompass the singular gene.

Transcriptional profiling of PK/NRP BGCs during ABE fermentation

A transcriptomic approach was used to study gene expression levels of the BGCs in an ABE fermentation context. High quality RNA (RIN ~8) was prepared from *Csa* batch cultures grown to log phase with four biological replicates. The resulting RNA-seq dataset (SRA: PRJNA551507) was quality controlled using FastQC. An average of 91.4% or 86.3 million reads per biological replicate were used as input for downstream analysis, well above the 5–10 million reads suggested to be sufficient for bacterial transcriptome analysis [16]. Next, input reads were mapped to either the *Csa* chromosome or the megaplasmid. Reads mapping

to multiple loci were counted once for each locus. FPKM values were calculated for each locus and compiled (Table S4) to assess relative gene expression.

Most of the genome is expressed, with 99.8% of genes having at least one mapped read and 97.8% of genes having at least 10 mapped reads. For each BGC, the core PKS/NRPS gene with the minimum average expression level was used to represent overall BGC expression. The *gyrB* (DNA gyrase subunit B) housekeeping gene was used as an expression benchmark [43]. The resulting profile of expression is shown in Fig. 2. Five of seven BGCs demonstrated some baseline expression (FPKM > 1), with *nrps2* and *hyb2* falling below the expression cutoff. Two BGCs, *nrps3* and *nrps4*, were expressed at a level comparable to that of *gyrB*. A detailed compilation of BGC gene expression levels is presented in Supplementary Table S4.

Characterization of *nrps3*-associated secondary metabolite

As *nrps*3 is both highly expressed and conserved in other solvent-producing *Clostridium* species, we selected this BGC for metabolite interrogation. In order to discover the secondary metabolite associated with *nrps3*, CSPA_RS10760 was disrupted using a CRISPR/Cas9-nickase targeted homologous recombination strategy [27, 57]. The resulting strain, Δ*nrps3*, was genotyped by PCR to confirm successful editing at the target locus (Supplementary Fig. S1). Next, chemical extracts of the wild-type and Δ*nrps3* cultures were analyzed using LC-HRMS. Untargeted metabolomic comparison of the strains was performed using XCMS, enabling identification of a new compound, **1**, with molecular formula $C_{15}H_{30}N_2O_3$ (calculated for $C_{15}H_{31}N_2O_3^+$: 287.2329; found: 287.2327) (Supplementary Fig. S5). A majority of **1** was found in spent culture medium rather than pelleted cell mass, suggesting that it is secreted upon biosynthesis.

To obtain enough material for structural characterization, three liters of *Csa* batch culture was extracted with ethyl acetate and purified using a size-exclusion column packed with Sephadex LH-20, followed by several rounds of high-performance liquid chromatography (HPLC) purification. A detailed method is available under Supplementary Methods. This yielded 2 mg of pure compound that was subjected to 1D and 2D NMR analysis for structural elucidation, including ^1H, ^{13}C, HSQC, COSY, and HMBC (Supplementary Fig. S6). Compound **1** was determined to be an *N*-acylated dipeptidyl alcohol derived from butyric acid, valine and leucine monomers. A chemical standard was synthesized (Supplementary Fig. S7) and the proposed structure was verified by HPLC retention time and tandem MS spectrum.

The domain organization of the di-modular NRPS offers insights into the biosynthesis of **1**. The proposed mechanism (Fig. 3a) channels substrates through successive modules to form two peptide bonds between the butyryl starting unit and subsequent L-valine and L-leucine monomers. Then, the reductase domain releases the alcohol product from the assembly line through a four-electron reduction. To confirm that the NRPS encoded by CSPA_RS10760 is sufficient to produce the identified compound, we overexpressed the gene in *E. coli* to probe for possible heterologous metabolite production (Supplementary Fig. S8). The recombinant *E. coli* strain produced the expected product **1**, consistent with the predicted function of the di-modular NRPS (Fig. 3b). We further probed the starter unit substrate promiscuity of the

assembly line by feeding fatty acids of varying chain length (Fig. 3c). New products corresponding to the two-, three-, six-, and eight-carbon head groups were identified upon feeding of the corresponding fatty acids, demonstrating that the C domain of the NRPS has a relaxed substrate specificity toward short- to medium-chain fatty acyl groups, albeit with C3–4 substrates preferred. Finally, to unequivocally confirm the function of the NRPS, the intact megasynthetase was purified from *E. coli* for in vitro activity reconstitution (Fig. 3d). The enzymatic reaction mixture containing butyryl-CoA, L-valine, L-leucine, ATP and NADPH successfully produced compound **1** as a product, confirming that the NRPS is sufficient to make this product.

The product of CSPA_RS10760 resembles a class of reported dipeptidyl aldehyde compounds associated with various human gut commensal microorganisms [14, 45]. This class of compounds represents potent inhibitors of a variety of human and bacterial proteases. Both these human-gut derived metabolites and **1** are biosynthesized by small NRPS genes terminating in R domains. However, several key differences can be observed between these secondary metabolites. The majority of NRPSs associated with protease inhibition lack the *N*-terminal C domain, with the resulting lack of *N*-acylation in the product leading to the formation of pyrazinone shunt metabolites. Moreover, two NRPSs with an identical domain organization to the NRPS encoded by CSPA_RS10760 have been expressed in heterologous *E. coli* hosts, and *N*-acylated dipeptidyl aldehydes, instead of alcohols, have been reported as products. In contrast, the major products of CSPA_RS10760 seemed to be alcohols from both anaerobic culture of *Csa* and aerobic heterologous expression in *E. coli*, and compound **1** did not have obvious protease inhibition activity toward cathepsin B. Thus, the R domain encoded by CSPA_RS10760 is a more efficient reductase which catalyzes the iterative reduction of the peptidyl carboxyl to a terminal alcohol, distinct from the homologous NRPSs from human gut microbes.

We then examined the possible role of *nrps3* in solvent production. Initial batch fermentations of the wild-type *Csa* and Δ*nrps3* showed that *nrps3* does not have a direct impact on batch ABE titers (Fig. 4a–b) or glucose consumption (Supplementary Fig. S9) after a 48 hour fermentation. In addition, the wild-type *Csa* and Δ*nrps3* strains exhibited similar colony morphologies and were indistinguishable in assays assessing for swimming-motility and granulose accumulation (data not shown). We next turned to an untargeted method to identify a possible impact of *nrps3* disruption; differential gene expression analysis was carried out using RNA-seq data representing cultures of *Csa* wild-type and Δ*nrps3*. Filtering the data for two-fold differential gene expression and p-value < 0.001 yielded CSPA_RS10760 (the NRPS) and six protein-coding genes which were revealed by STRING analysis [50] to comprise a putative glycerol metabolism operon (Fig. 4c). While *Csa* cannot use glycerol as a sole carbon source [22], glycerol metabolism genes have been reported as part of the solvent tolerance response in a wide variety of fermentation hosts, including *E. coli* (*glpBCFQ* in response to hexanes stress [17], *glpC* in response to xylene and cyclohexane [47], and *C. acetobutylicum* (up-regulation of *glpA* and *glpF* in response to 3.7 g/liter butanol) [1]. To probe whether *nrps3* mediates a solvent stress response in *Csa*, we collected growth data comparing wild-type and Δ*nrps3* after butanol challenge [1]. We found that Δ*nrps3* exhibited a growth defect during the exponential phase relative to the wild-type

(Fig. 4d), albeit the optical densities of Δ*nrps3* cultures converged with *Csa* wild-type cultures during the stationary phase. These results support an association between *nrps3* and *glp*-mediated solvent tolerance revealed through transcriptomic analysis, although further work will be required to determine the underlying molecular mechanism.

Conclusions

In summary, this study provides first insights into the secondary metabolism of *Csa*, an anaerobic bacterium historically valued for industrial solvent production. An in-depth bioinformatic analysis of *Csa* revealed seven uncharacterized PK/NRP BGCs in its genome, of which five were expressed under solvent-production conditions, as shown by transcriptomic analysis. Further investigation of one of the highly expressed BGCs, *nrps3*, led to the identification of its associated metabolite, an *N*-acylated dipeptidyl alcohol, and its biosynthetic mechanism. While phenotypic comparisons between the *Csa* wild-type and Δ*nrps3* showed no difference in the batch culture solvent production titers, a comparative transcriptomic analysis followed by butanol challenge assays suggested a possible role of *nrps3* in *glp*-mediated butanol tolerance. This work thus demonstrated another example of a small-molecule secondary metabolite affecting traits relevant for microbial industrial applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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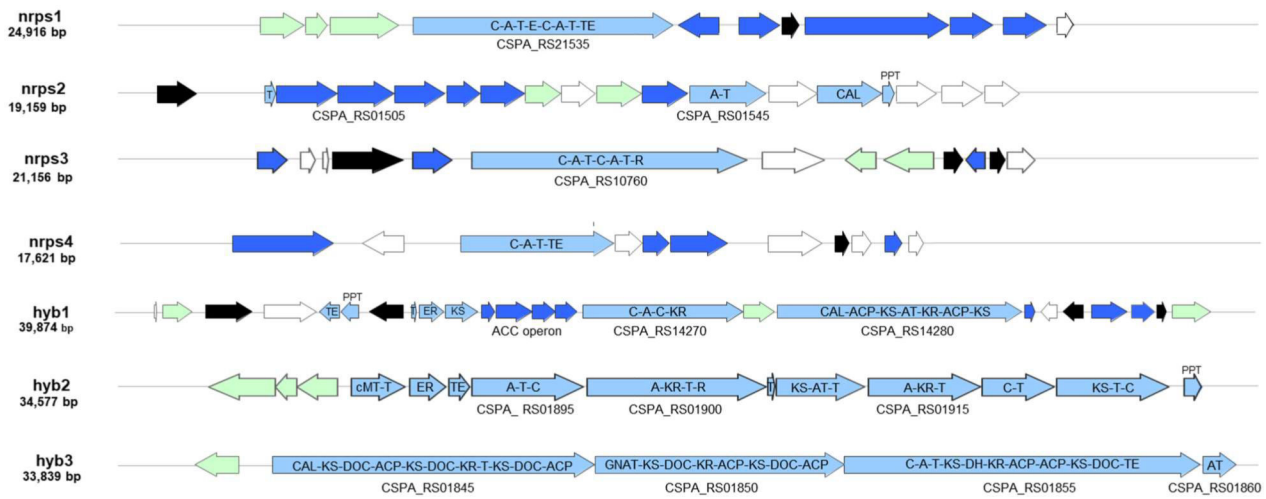


Fig. 1.

BGCs of *Csa* which contain PK or NRP biosynthetic genes. Gene color indicates putative function: light blue, core biosynthetic genes; dark blue, additional biosynthetic gene; black, transcriptional regulator; light green, transporter; white, other. Domain organization is indicated for PKS/NRPS genes. Abbreviations: C, condensation; A, adenylation; T, thiolation/peptide carrier; E, epimerization; TE, thioesterase; R, reductive release; KR, ketoreductase; CAL, Acyl-CoA ligase; KS, ketosynthase; cMT, C-methyltransferase; ER, enoyl-reductase; DOC, *trans*-AT docking; ACP, acyl-carrier protein; GNAT, Gcn5-related *N*-acetyltransferase; DH, dehydratase; PPT, 4-phosphopantetheinyl transferase

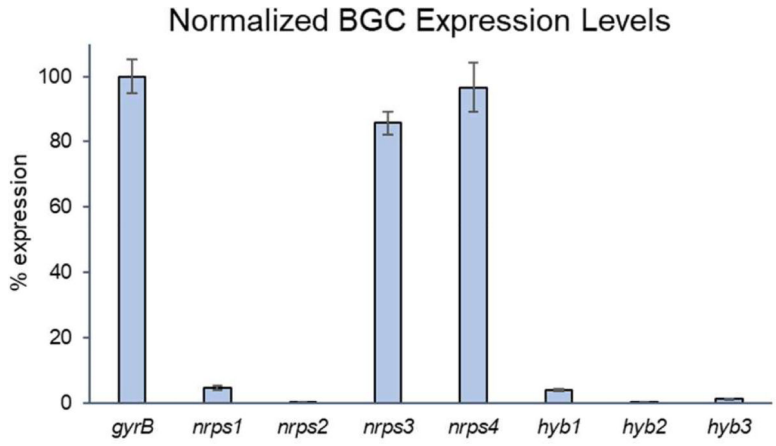


Fig. 2.

Expression level of the BGCs of *Csa*. Values are normalized to that of *gyrB*, a housekeeping benchmark.

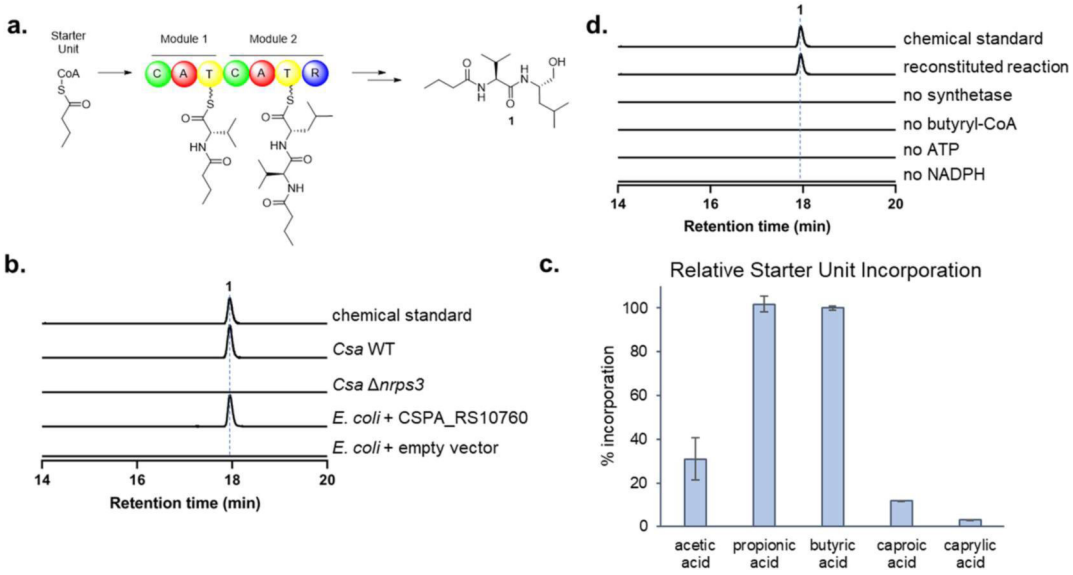
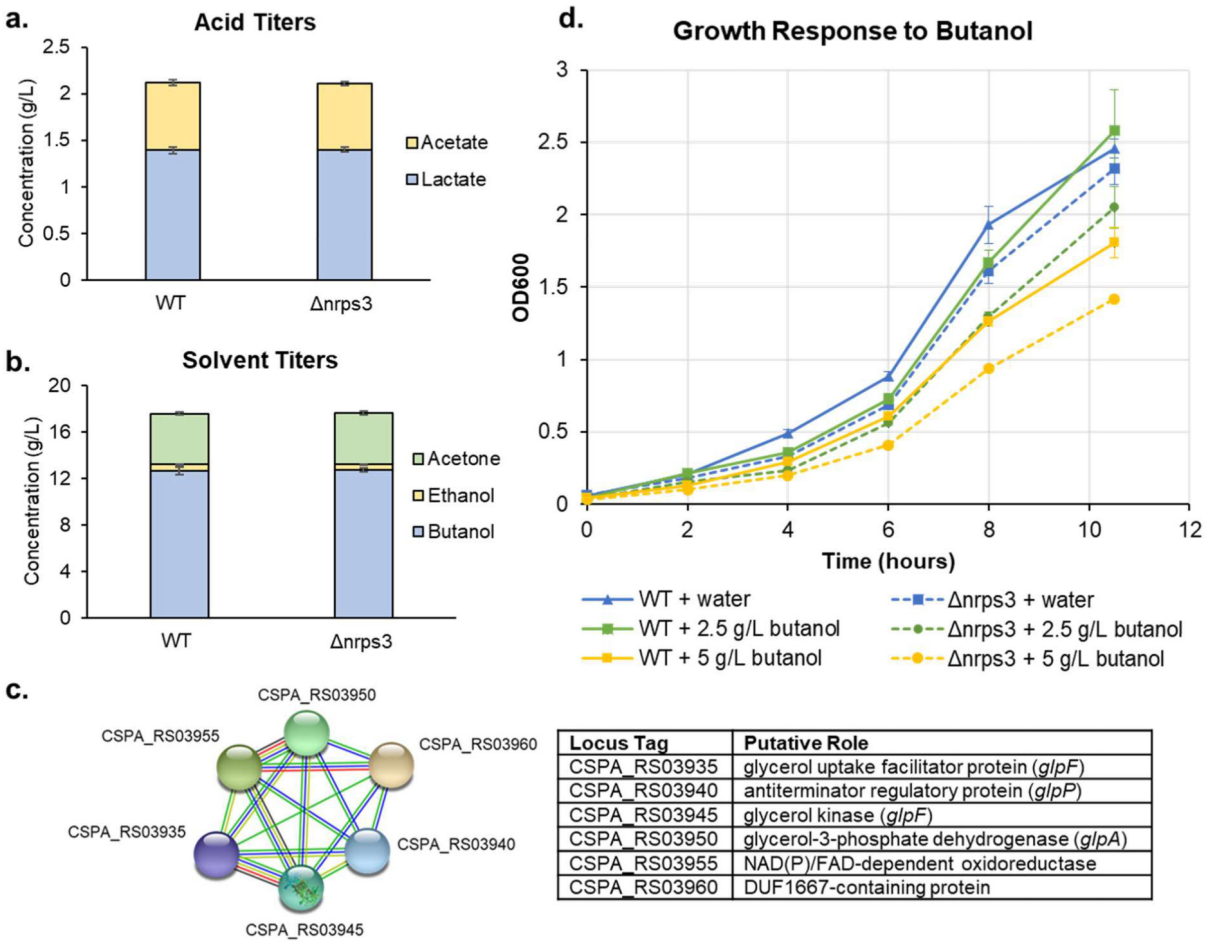


Fig. 3.

Characterization of *nrps3*-associated secondary metabolite, **1**. **a** Proposed biosynthesis of the secondary metabolite **1**, an *N*-acylated dipeptidyl alcohol. **b** HRMS extracted ion chromatograms demonstrating requirement of CSPA_RS10760 in *Csa* for compound biosynthesis, and heterologous production of **1** in a heterologous *E. coli* host expressing CSPA_RS10760. **c** Relative starter unit promiscuity of the NRPS demonstrated by substrate feeding of varying-length short-chain fatty acids in *E. coli* + CSPA_RS10760. **d** HRMS extracted ion chromatograms demonstrating biosynthesis of **1** in vitro. The calculated mass for **1** (287.2329) with 10 ppm mass error tolerance was used for each trace in b and d.



Locus Tag	Putative Role
CSPA_RS03935	glycerol uptake facilitator protein (*glpF*)
CSPA_RS03940	antiterminator regulatory protein (*glpP*)
CSPA_RS03945	glycerol kinase (*glpF*)
CSPA_RS03950	glycerol-3-phosphate dehydrogenase (*glpA*)
CSPA_RS03955	NAD(P)/FAD-dependent oxidoreductase
CSPA_RS03960	DUF1667-containing protein

Fig. 4.

Comparison of wild-type *Csa* and Δ*nrps3* in batch cultures under ABE fermentation conditions. **a** Production of organic acids. **b** Production of ABE. **c** STRING network analysis of RNA-seq data comparing wild-type and Δ*nrps3* fermentations identified a differentially regulated genetic pathway in Δ*nrps3*. The nodes represent the six down-regulated genes, CSPA_RS03935- CSPA_RS03960, summarized in the accompanying table. This glp operon has been associated with solvent stress response in another ABE producer, *C. acetobutylicum*. The edge colors reflect different lines of evidence for gene functional relationships: light green, gene neighborhood; red, gene fusions; blue, gene co-occurrence; olive green, textmining; black, co-expression. **d** Butanol challenge assay demonstrating a growth defect in Δ*nrps3* relative to wild-type Csa. Each assay was repeated at least three times independently.