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Analysis of a *bac* operon-silenced strain suggests pleiotropic effects of bacilysin in *Bacillus subtilis*[§]

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Bacilysin, as the simplest peptide antibiotic made up of only L-alanine and L-anticapsin, is produced and excreted by *Bacillus subtilis* under the control of quorum sensing. We analyzed bacilysin-nonproducing strain OGU1 which was obtained by bacA-targeted pMutin T3 insertion into the parental strain genome resulting in a genomic organization (*bacA′::lacZ::erm::bacABCDEF*) to form an IPTG-inducible *bac* operon. Although IPTG induction provided 3- to 5-fold increment in the transcription of *bac* operon genes, no bacilysin activity was detectable in bioassays and inability of the OGU1 to form bacilysin was confirmed by UPLC-mass spectrometry analysis. Phenotypic analyses revealed the deficiencies in OGU1 with respect to colony pigmentation, spore coat proteins, spore resistance and germination, which could be rescued by external addition of bacilysin concentrate into its cultures. 2DE MALDI-TOF/MS and nanoLC-MS/MS were used as complementary approaches to compare cytosolic proteomes of OGU1. 2-DE identified 159 differentially expressed proteins corresponding to 121 distinct ORFs. In nanoLC-MS/MS, 76 proteins were differentially expressed in OGU1. Quantitative transcript analyses of selected genes validated the proteomic findings. Overall, the results pointed to the impact of bacilysin on expression of certain proteins of sporulation and morphogenesis; the members of mother cell compartment-specific σ^E and σ^K regulons in particular, quorum sensing and two component-global regulatory systems, peptide transport, stress response as well as CodY- and ScoC-regulated proteins.

Keywords: *Bacillus subtilis*, bacilysin, *bac* operon, sporulation, chemical complementation, pleiotropy, comparative proteomics, RT-qPCR

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Introduction

As an enzymatically synthesized dipeptide first identified from *Bacillus subtilis,* bacilysin is made up of L-alanine and the non-proteinogenic amino acid, L-anticapsin (Walker and Abraham, 1970), the latter being responsible for its antibiotic action in susceptible bacterial and fungal cells (Kenig *et al*., 1976; Milewsky *et al*., 1986). *ywfBCDEFG* operon, and the monocistronic gene *ywfH* together encode the enzymes of pathway leading to anticapsin and bacilysin (Inaoka *et al*., 2003). These genes showing up to 88.6% sequence identity from different *Bacillus* species were renamed as *bacABCDEF* and *bacG*, for further use in genetic, biochemical and biophysical studies (Steinborn *et al*., 2005; Rajavel *et al*., 2009; Mahlstedt and Walsh, 2010; Parker and Walsh, 2012, 2013; Shomura *et al*., 2012). Of these genes, only *bacE* is not involved in bacilysin formation, but its product pumps out bacilysin to prevent suicide (Steinborn *et al*., 2005).

As recently reviewed (Özcengiz and Öğülür, 2015) (Fig. 1), bacilysin biosynthesis is one of the targets of quorum sensing control in its producer, requiring the functions of ComQ/ComX, Phr peptides C, F and K, ComP/ComA, Spo0K (Opp) and *srfA* operon (Yazgan *et al*., 2001; Karataş *et al*., 2003). As to the other elements operating in the regulatory circuit of bacilysin formation, Inaoka *et al*. (2003) reported the involvement of ppGpp and GTP in transcriptional regulation of *bac* operon in a pleiotropic CodY repressor-mediated manner, and then their finding on regulation of the operon by the transition state transcriptional repressor ScoC (Hpr) (Inaoka *et al*., 2009). While there is a competition between two key activators, ComA and Spo0A for *bac* operon promoter (P_{bac}), the negative transcriptional regulators AbrB and CodY simultaneously bind to P_{bac} (Köroğlu *et al*., 2011). The DegS/DegU two-component signal transduction system is also involved in positive regulation of *bac* operon and *bacG*, as shown in *B. amyloliquefaciens* (Mariappan *et al*., 2012).

Since the complete *B. subtilis* 168 genome sequence was first published (Kunst *et al*., 1997) and updated (Barbe *et al*., 2009), much effort has been devoted to deeply understand the physiology of this model organism by postgenomic studies. For instance, proteomics has been widely used in order to visualize the responses of *B. subtilis* cells to various types of stimuli like oxidative stress and nutrient starvation, elucidating the relevant gene expression networks (Hecker *et al*., 2009).

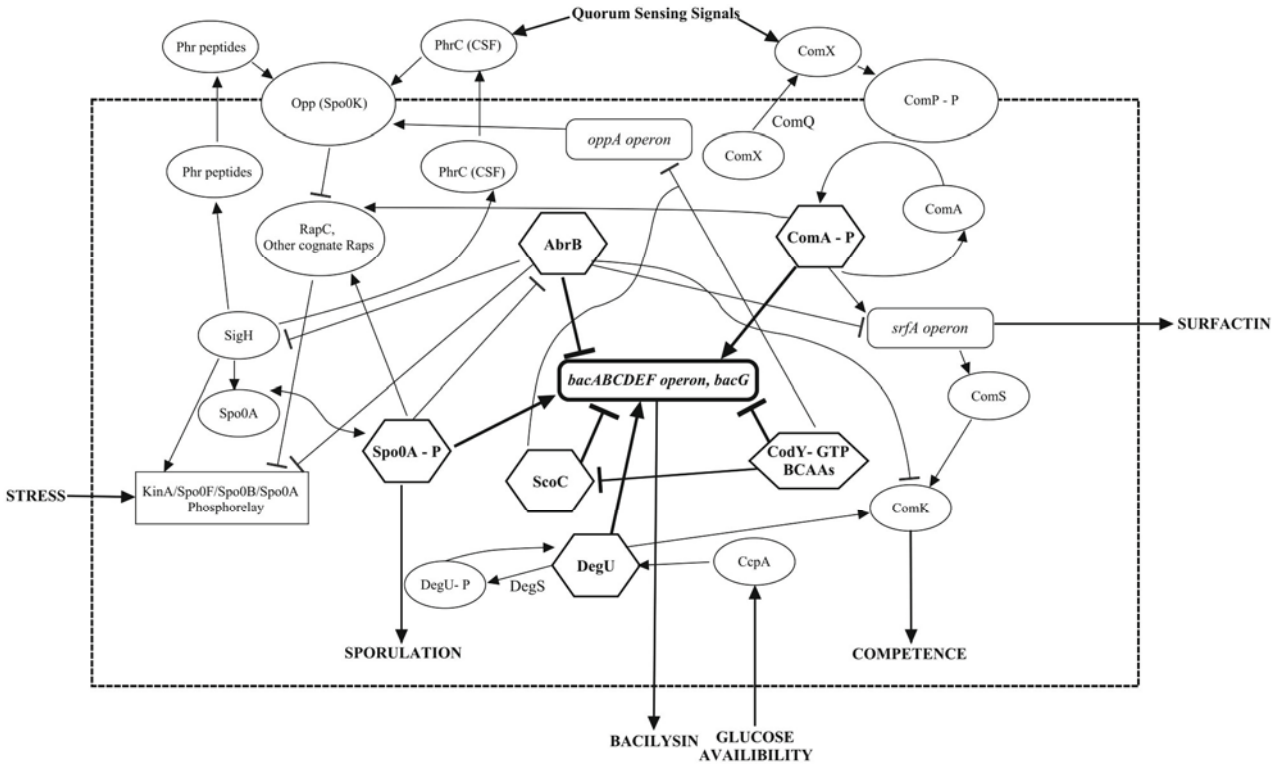


Fig. 1. Molecular regulation of bacilysin biosynthetic *bac* operon. Quorum sensing system and pleiotropic regulators that cross-link bacilysin biosynthesis with surfactin biosynthesis, competence development and sporulation in response to quorum sensing signals, stress and glucose availability. Arrows indicate activation; repression is denoted by T-bars. Bold lines refer to direct interaction with P_{bac} (adopted from Özcengiz and Öğülür, 2015).

The complexity of the regulation of bacilysin formation brings up the question of whether bacilysin itself has some role(s) in its producer and how *B. subtilis* cells physiologically respond to the loss of bacilysin biosynthetic function. In the present study, we showed that a bacilysin biosynthetic operon-silenced mutant, namely OGU1 (Köroğlu *et al.*, 2011), exhibited deficiencies in colony pigmentation, spore coat protein profile, spore resistance and germination. Externally added bacilysin concentrate provided chemical complementation and restored wild-type phenotype in this strain. A view of the alterations in cytosolic proteome was obtained by identifying differentially expressed proteins in this mutant by employing 2DE- and nanoLC proteomics as complementary approaches and validated by quantitative transcript analysis for the selected genes. The data presented herein document a variety of pleiotropic effects resulting from the loss of bacilysin biosynthesis.

Materials and Methods

Bacillus subtilis strains and the cultures

B. subtilis PY79 (Youngman *et al.*, 1984) and its bacilysin-non-producer (*bacA′::lacZ::erm::bacABCDEF*) derivative OGU1 (Köroğlu *et al.*, 2011) were the strains used throughout this study. PA medium (Perry and Abraham, 1979) without any antibiotics was the principal cultivation medium for suspended cultures. For OGU1, erythromycin and lincomycin were incorporated into LA (Luria Agar) cultures and their concentrations were as in Köroğlu *et al.* (2011). Fresh LA cultures of the strains were used to inoculate the cells into 10 ml of PA medium. The cultures grown overnight at 37°C by shaking were then inoculated into 100 ml of PA medium to an initial OD_{595} of 0.1 and subsequent cultivation was done by shaking at 37°C (200 rpm) for 16 h.

IPTG induction of OGU1 cultures

Three different experiments were conducted for IPTG induction of *bac* operon in OGU1. Hundred milliliter of PA medium without any antibiotics were inoculated with OGU1 overnight cultures to an initial OD_{595} of 0.1. In the first set of experiments, IPTG induction was made by adding 1 mM IPTG to the culture at 8^{th} h. The samples were collected at 12^{th}, 14^{th}, and 16^{th} h for detection of bacilysin activity by bioassays. In the second set of experiments, an 8^{th} h OGU1 culture received 1 mM IPTG to which 0.5 mM IPTG was re-added when it reached to 14^{th} h. The samplings for the determination of bacilysin activity were made at 16^{th}, 18^{th}, and 20^{th} h. In the third round of experiments, 1 mM IPTG was administered to 15^{th} h cultures from which the samples were withdrawn after 20, 30, 40, 60, and 80 min, respectively, for bioassays. In all of the experiments mentioned above, PY79 and uninduced OGU1 cultures were also run in parallel and the samples were collected at the same time intervals as the positive and negative controls, respectively.

Bacilysin bioassay

Bacilysin activity in culture fluids was determined by the paper disc-agar diffusion assay using *S. aureus* ATCC 9144 as the test organism and the antibiotic activity was estimated as in Özcengiz *et al.* (1990).

Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis

Two hundred microliter of culture supernatants were extracted with one-fourth volume of butanol and lyophilized to dryness. After dissolved in 10 µl of a solvent mixture (H_2O: 5% CH_3CN + 1% HCOOH), samples were analyzed by UPLC-MS (Waters Acquity UPLC H-Class and Water Synapt G2-Si HDMS) at the positive detection mode. 7.5 µl of sample was injected onto a Waters Acquity Peptide BEH C18 column (2.1 mm × 100 mm) equilibrated in solution mixture (1% CH_3CN + 0.1% HCOOH + H_2O) with a flow rate 0.2 ml/min, and column temperature was kept at 65°C. Bound compounds were eluted with a gradient prepared with Solution A (0.1% HCOOH:H_2O) and Solution B (CH_3CN) as follows: 1% Solution B for 1 min, 1–40% of Solution B over 10 min, 40–80% Solution B over 1 min and kept for 2 min, 80–1% of Solution B over 50 sec, and 1% Solution B reequilibration for 10 min before loading the next sample.

Whole genome resequencing (WGRS)

WGRS of *B. subtilis* OGU1 and its parental strain PY79 were performed on individual colonies on Illumina Truseq in 2 × 150 bp paired end configuration (Macrogen, Inc.). Average coverage was 1–2 Gb/sample. The raw data were processed and aligned to reference genome of Bacillus Genetic Stock Center (BGSC)-deposited *B. subtilis* PY79 (DDBJ/EMBL/GenBank databases with GenBank accession no. CP006881.1) with Burrows-Wheeler Aligner for SNPs and small indels analysis (Schroeder and Simmons, 2013). The data from OGU1 and its parental PY79 were deposited at DDBJ/ENA/GenBank databases under the accession no. QLNM00000000.1 and WOWX00000000, respectively. SAMTools and Picard were used to remove duplicated reads and find variant information for further confirmation.

Preparation of bacilysin concentrates and chemical complementation

Bacilysin concentrates were prepared from 16 h PA cultures of PY79 as in Roscoe and Abraham (1966), sterilized by filtration, and kept in -20°C until use. For chemical complementation, the concentrate was added to a 100 ml of growing cultures of OGU1 at 7^{th} h of incubation to introduce ca. 150 Units/ml and the incubation was continued. As another negative control, OGU1 broth concentrates were also prepared in the same way and added in the same volume to a parallel OGU1 culture.

Protein extraction

Bacilysin levels were routinely monitored in cultures used for protein extraction. To isolate cytosolic proteins, 15 ml aliquots of 16 h cultures were centrifuged at 6,000 rpm for 5 min, after which the pellet was washed twice with cold TE buffer. After resuspending in lysozyme (40 mg/ml in 0.04 M TE buffer) and incubating for 1 h at 37°C, the samples were centrifuged at 13,000 rpm for 10 min at RT. After adding 8 M urea onto the pellet and vortexing at 4°C, another centrifugation was made at 13,000 rpm for 15 min to collect the supernatant. Protein concentration determinations were made as in Ramagli and Rodrigez (1985), the concentrations were equalized for each sample and the resulting protein samples were stored at -20°C.

1DE

For each strain, two independent biological samples were run, each with two technical replicates. A 50 µg protein aliquot was mixed with SDS-PAGE sample buffer and protein separation was done in 12% acrylamide/bis-acrylamide gels (Bio-Rad Cell System) by applying 16 mA/gel, after which the proteins were stained with CCB (Neuhoff *et al.*, 1988). Ten equidistant pieces were cut from each lane of 1D gel for the tryptic in-gel digestion and peptide elution for liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS).

2-DE and image analysis

For each strain, two independent biological samples were run, each with two technical replicates. Isoelectric focusing (IEF) was performed as described in Görg *et al.* (2004) using the linear IPG-strips: 17 cm, pH 4–7, Bio-Rad; 18 cm, GE Healthcare Immobiline DryStrip pH 4.5–5.5, and 18 cm, GE Healthcare Immobiline DryStrip pH 5.5–6.7, after rehydration and IEF, 12% acrylamide/bis-acrylamide gels were run for the second dimension on a Biorad Protean Xii electrophoresis system (Bio-Rad), applying approximately 25 mA/gel. After CCB staining and digitizing the gels using an HP scanner, the 2D image analysis software Delta2D version 3.3 (Decodon) was employed for the processing of gel images and gel-based relative quantitation of protein spots. Gels in each group (*B. subtilis* OGU1 and *B. subtilis* PY79) were used to generate a fused master gel image which contained all the spots coming from each replicate. By using the volume percentage ratios for each spot, a spot intensity showing at least a 2.5-fold difference between *B. subtilis* OGU1 and PY79 was selected for identification by MALDI-TOF/MS analysis. Standard deviations of the spot intensities from the replicates remained in the range of 20%.

MALDI-TOF/MS and NanoLC-MS/MS

The MS analyses of the spots excised from 2DE gels and equidistant gel pieces from 1D gels were performed at the Institute of Microbiology, Ernst-Moritz-Arndt-University, as in Eymann *et al.* (2004). After destaining of spots, tryptic digestions and peptide elutions, MALDI-TOF/TOF Proteome-Analyzer 4,700 (Applied Biosystems) was used for the peptide mass determination. Three most abundant peptides in each MS spectrum were chosen and the resulting sequence data were included in the database search. A linear trap quadrupole (LTQ) Oribtrap (Thermo Fisher Scientific) equipped with a nanoACQUITY UPLC (Waters) was employed for nanoLC–MS/MS analysis of the peptides eluted from 1D gels. Trap and elute mode was used to separate peptide

mixture by LC system as described by Eymann *et al.* (2004). The mass spectrometric analysis started with a full survey scan in the Orbitrap (m/z 300–2000, resolution of 60,000) followed by collision-induced dissociation and acquisition of MS/MS spectra of the five most abundant precursor ions in the LTQ. Precursors were dynamically excluded for 30 sec, and unassigned charge states as well as singly charged ions were rejected. For quantification of abundance differences, the relative spectral counts (R_{sc}) were calculated for quantitation of the abundance differences of the proteins identified from the strains by using the formula provided by Old *et al.* (2005) : $[R_{sc}=\log2[(n_2+f)/n_1+f)] +\log2[(t_1-n_1+f)/(t_2-n_2+f)]$ R_{SC} is the log2 ratio of abundance between Samples 1 and 2; n_1 and n_2 are spectral counts for the protein in Samples 1 and 2, respectively; t_1 and t_2 are total numbers of spectra over all proteins in the two samples and f is the correction factor. In the present study, the correction factor f was set to 1.25 in calculations and the proteins with R_{sc} values higher than 2.0 were selected as differentially expressed.

Database search and bioinformatic analyses

B. subtilis genome project web address (http://genolist.pasteur.fr/ SubtiList/) was used to obtain the amino acid sequences of the proteins. Briefly, the database search was performed by using the SEQUEST software (Bioworks v.3.2, Thermo Electron) and the results were imported to Scaffold 2.02.01 (Proteome Software) for validation of MS/MS-based peptide and protein identifications. The data obtained from MALDI-TOF/MS measurement were analyzed by using "Peptide Mass Fingerprint (PMF)" and "MS/MS Ion Search" engines of MASCOT software (http://www.matrixscience.com/) against the *B. subtilis* PMF database. For protein identifications, the protein score values higher than 98 were accepted. Protein accession numbers of the identified spots which were included in the MASCOT output were searched in the SubtiList Web Server and Protein Knowledgebase (UniProtKB) (http://www.uniprot.org/) for finding out the putative functions of

Table 1. Primer sets used for real-time quantitative PCR

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)
rpoB	TGAACATCGGGCAGGTATTG	GTTTCCCAGACATCCTCTTCTC
dpaA	GGAACGATCATGCTGGCTAT	AACTTCTTGCCCCCACTTTT
dpaB	TTTTATTCCATTCGGGCAAG	GTGCATGAGTGCCTTTTCAA
cotA	GCCGCTTCTTATCACAGACC	CCGGAACGATTGAAGGATTA
ytfJ	CGAAAAGAAATCGGAAGACG	ATTCTGATGCCTGTCGATCC
yhcQ	TCAAAACAGGAAGCGAACCT	ATTTGTCGGCTGATGATGCT
yxbC	GAACTCGCTGTAAATCATCAATCC	GGTCAATGTCTCTGCGTTCT
cheV	AGTTTGGCGTGGGTGAAA	CTTCTACATGCTGATGGGAGTG
resD	TGAAGCGGGAACAGATGATT	GAGGTTTGGGATGCTCTTCT
phoP	TTTGGAACGGTCAGGCTATG	GCATCACATCAAGCACAATCAA
degU	GCGGTTAAAGTAGTGGCTGA	TGTTGAGGGTGTGCAGAAA
degS	CAGAGGACGGATTCCAAGAAA	CAAGGGCCATCGGTCTTAAA
gerE	CTCGTTCAAGATAAGACAACAAAGG	CACACCCAATTTCTGCATGG
codY	TGAGGAGCTTGACGGAAATG	GAGTGCGTTCACAATAACAGAAC
abrB	TGCCAAGTAACTGGTGAAGTT	GGATTTCGCTGATGATTTGCTC
ccpA	TGGACGGCATCGTGTTTAT	GTGTTTCCTCCTGCTCTTCTAC
abbA	ACGATATAGAGACAGGGACCA	CAAATCGAAATCGGTCGTATAGC
spo0A	AGTGATCGGCGTTGCTTAT	CCGTCTAGATGCGGCATAAT
yugI	GCTGCTCAAGTAAGCGAAGA	TAAGGTCTTTGCGGTTGGAC
dnaK	ATTCAGGGCGGCGTTATC	TGTTGCGGTCGATCAGTTT
yceE	GGCATCACAGTGACCATTCA	CTCCCAAATCGAAGCGAAGA
ytkL	ACCTTGCTTTCCTTCCGATT	TTCGATGACCGGGAATGTAT
luxS	GAAGCCTGACACCATTCACA	GGCAGCCCATTGGAGAAATA
albE	TCACTGATCCCTTCTCCTACTC	GAATCGCCTTTATCCTGTCTCTC
ispA	AGCTTCCTGAGGGCATTAAA	GTCGCAGCCTGTGTCTAATA
oppD	CTGCTGGAATTAGTCGGTATCC	GCAATGACAACCCTCTGTCT
dnaA	CGTCAAATCGCCATGTACTTATC	ATCACGTCCTCCAAACTCTTC
glmS	GCTCGTGAATACCTGACTGTATC	CCTTCTGCCTGGATGTAAGAAA
glnA	GACCTCGGCTTCAGTGATTT	TCCGCCTTTGTCGTTTAGTT
malS	GTTTTAGATGCGGGGACAAA	GCGCAACGTAGTCGTCAATA
bacA	CTTCTACAAGGGCTGAACAGTAA	GCATGTGGGACGATGGTATATT
bacB	CTGATGATGACTGTCGGAGATG	TCTTGGTCTGTGTCGTTTCTT
bacC	ATGTGGTTGTGGCGGATATT	TCGTCTGTGATGTCCGTTTG
bacD	CATAACAAGCCTGAGGAAGAGG	CGCCATCGGAGCAATGAATA
bacE	GTCATGTCGTCCTTGGAATCA	CAGATCCGCCTAACAGAAAGAG
bacF	GGGAATACGGAGTGACAATCAA	GATTCGGGACTAAAGCGATGT
bacG	TGTCGGAAATCTGTGGAAAGAA	GGAGCAAGCTGGATGGAAATA

proteins. The functional classification of the proteins was according to the categories in the *Subti*Wiki database (Michna *et al*., 2016; Zhu and Stülke, 2018). PSORT web server (http://wolfpsort.hgc.jp) and the SignalP web server (http://www.cbs.dtu.dk/services/SignalP/) were used for protein subcellular localization and signal peptide predictions, respectively.

RNA extraction and RT-qPCR

For total RNA extraction, PY79 and OGU1 overnight cultures were used to inoculate 100 ml of fresh PA media without any antibiotics to an initial OD_{595} of 0.1. IPTG induction was carried out by adding 1 mM IPTG to OGU1 culture at 15th h. All cultures including the IPTG induced one were harvested at 16th h and checked for bacilysin levels prior to RNA extraction. Total RNA isolation from *B. subtilis* PY79, OGU1, and IPTG induced OGU1 cells was performed with Nucleospin® RNA Kit (Macherey-Nagel GmbH & Co) according to the manufacturer's instructions with minor modifications. These modifications were namely usage of 450 µl of lysis buffer RA1 and 70% ethanol instead of 350 µl. The quality and the concentration of total RNA samples were measured with Nanodrop (BioDrop) spectrophotometrically at both 260 and 280 nm. For the integrity of total RNA samples, two important criteria – RIN (RNA integrity number) and 23S/16S rRNA ratio – were checked with Bioanalyzer 2100 (Agilent). Total RNA samples were kept at -80°C until further usage.

cDNA synthesis from total RNA samples was achieved with usage of iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions. In this reaction, 1 µg of total RNA was converted into total volume of 20 µl cDNA with mixture of oligo(dT) and random hexamer primers. Synthesized cDNAs were used as the template in RT-qPCR reactions which were performed in 0.2 ml 8-Tube PCR Strips with caps (Bio-Rad Laboratories). RT-qPCR reactions were carried out with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) following the manufacturer's instructions and the fluorescent signal detection was done with CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). Primer sets are listed in Table 1.

For the analysis of every gene, two independent qPCR runs were performed and each run contained three biological and two technical replicates of both PY79 and OGU1. Each run also included a no template control sample having nuclease free water instead of cDNA. To observe possible contaminant PCR products, melting curve analyses were performed for each run covering the temperature range of 40–95°C. Fold changes were calculated with $2^{-\Delta\Delta CT}$ method improved with efficiency correction (Rao *et al*., 2013) and *rpoB* gene that encodes for RNA polymerase beta subunit in *B. subtilis* was selected as the internal control gene. For statistical analysis, Student's t-test was carried out with GraphPad Prism 7 (Graphpad Software) and statistical significance reported as ns (no significance, $p > 0.05$), *($p < 0.05$), **($p < 0.01$), and *** ($p < 0.001$).

Characterization of colony morphologies

Colony morphologies were visualized on 1 µl of initial culture spotted DSM plates incubated for 18 days at 30°C. Epson Perfection V750 Pro scanner was used to obtain the images.

Mature spore purification

The procedure used was adopted from Nicholson and Setlow (1990). DSM-grown *B. subtilis* cells were harvested after 48 h incubation by centrifugation at 10,000 × *g*, for 10 min. After purifying spores by washing twice with ice-cold dH_2O and treatment with lysozyme (0.1 mg/ml) for 10 min at 37°C, spores were collected after re-washing several times and re-suspending in ice-cold dH_2O and kept in dark at -20°C.

Determination of sporulation efficiency and spore resistance

After culturing in DSM at 37°C for 48 h, the aliquots taken from the cultures were serially diluted in saline solution and plated on LA before and after heat treatment at 80°C for 30 min. Sporulation efficiency was expressed as the number of sporulated cells over total CFU/ml.

In order to determine heat resistance profiles, viable counts were made for the purified spores heated at 80°C for 30 min. Chloroform and lysozyme resistance of purified spores were determined as in Nicholson and Setlow (1990). Survival ratios were expressed as the ratio of CFU/ml before and after treatment.

Dipicolinic acid (DPA) content of spores

DPA content of purified spores was analyzed as described by Nicholson and Setlow (1990) after diluting, pelleting and resuspending them in ice-cold dH_2O.

Spore germination

Germination assay with purified spores was as in Nicholson and Setlow (1990). The OD_{585} readings were normalized according to the first reading (t_0) and expressed as averages with ± standard deviations. As proposed by Atluri *et al.* (2006), a 60% decrease in relative OD_{585} was accepted to correspond to germination of the whole spore population.

Extraction of coat proteins and small acid soluble proteins (SASPs) from spores

The procedure originally described by Takamatsu *et al.* (2000) was used for solubilization of the majority of spore coat proteins. Briefly, harvested mature spores were washed, suspended in lysozyme buffer, re-washed and solubilized in loading buffer. The resulting samples were subsequently analyzed by SDS-PAGE (15% acrylamide/bis-acrylamide; Bio-Rad Cell System) by applying 20 mA/gel, after which the proteins were stained with CCB (Neuhoff *et al*., 1988). After excision and destaining of selected bands, tryptic digestions and peptide elutions, the MS analyses were performed at the HUNITECH Center of Hacettepe University, Ankara, using an Ultraflextreme MALDI-TOF (Bruker Daltonics). MS/MS-based peptide and protein identifications were made as described above. SASPs were extracted from the purified spores by using a dental amalgamator for dry rupture of spores followed by < 5,000 Da cut off dialysis (Nicholson and Setlow, 1990). The lyophilized material was dissolved in 8 M urea before PAGE at low pH.

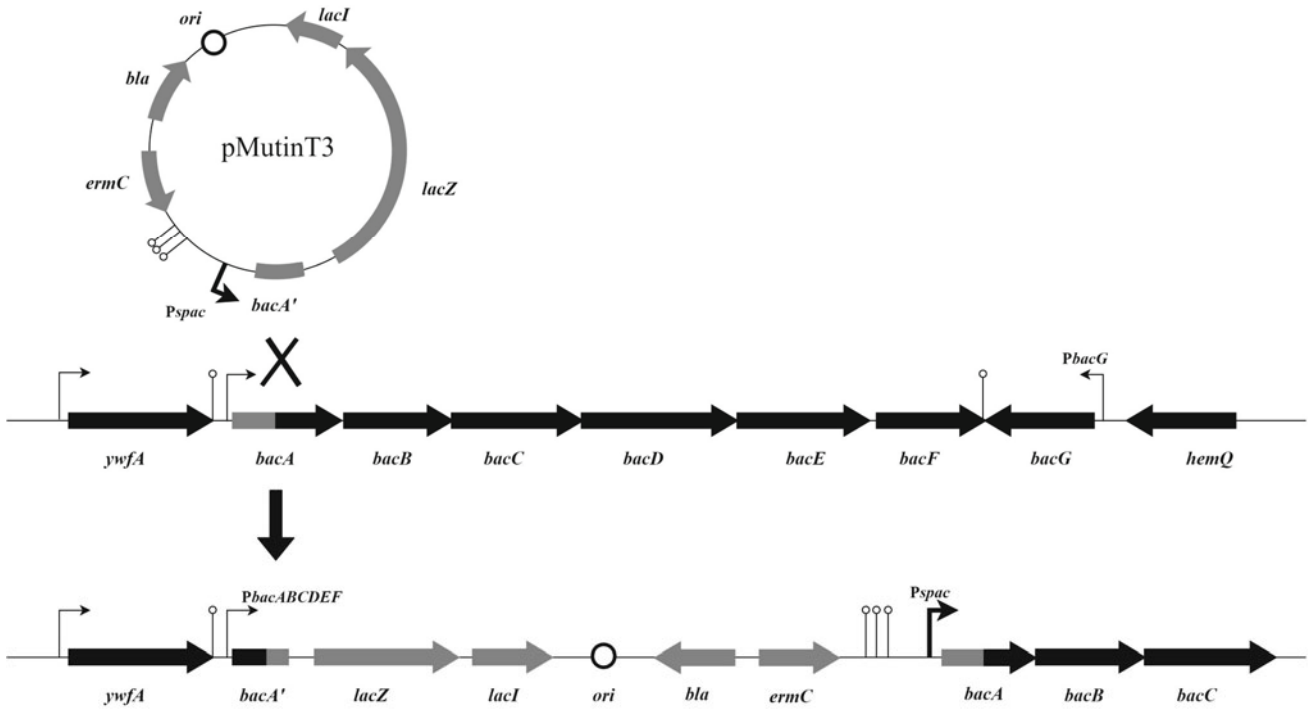


Fig. 2. The diagram of *bacA*-targeted pMutin T3 insertion into OGU1 genome. The resulting genomic organization (*bacA′::lacZ::erm::bacABCDEF* and the flanking regions) contains P_{bac}, 5′-region of *bacA* (*bacA′*), pMutin T3 followed by the whole *bac* operon under P_{spac}, ending up with an IPTG inducible *bac* operon.

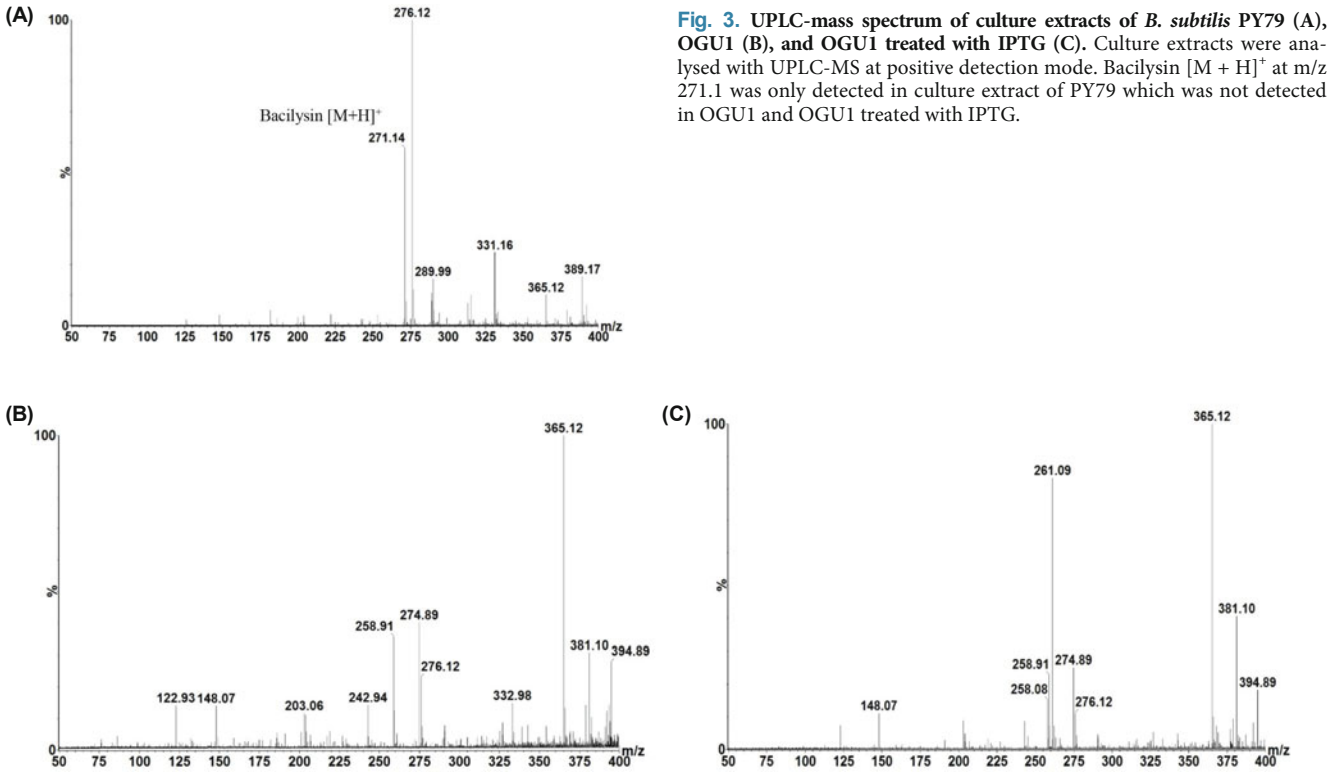


Fig. 3. UPLC-mass spectrum of culture extracts of *B. subtilis* PY79 (A), OGU1 (B), and OGU1 treated with IPTG (C). Culture extracts were analysed with UPLC-MS at positive detection mode. Bacilysin $[M + H]^+$ at m/z 271.1 was only detected in culture extract of PY79 which was not detected in OGU1 and OGU1 treated with IPTG.

Biofilm formation

Microtitre plate assay of biofilm formation was performed according to the protocol described by Hamon and Lazazzera (2001).

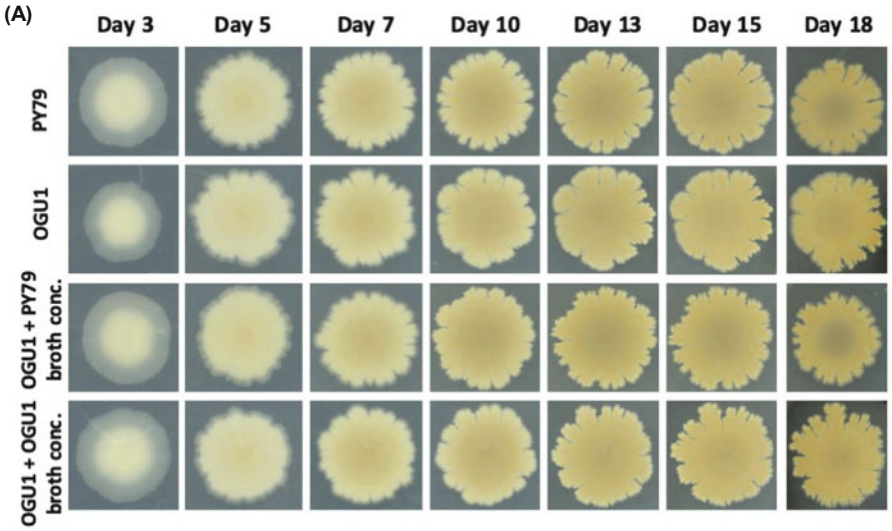
Results and Discussion

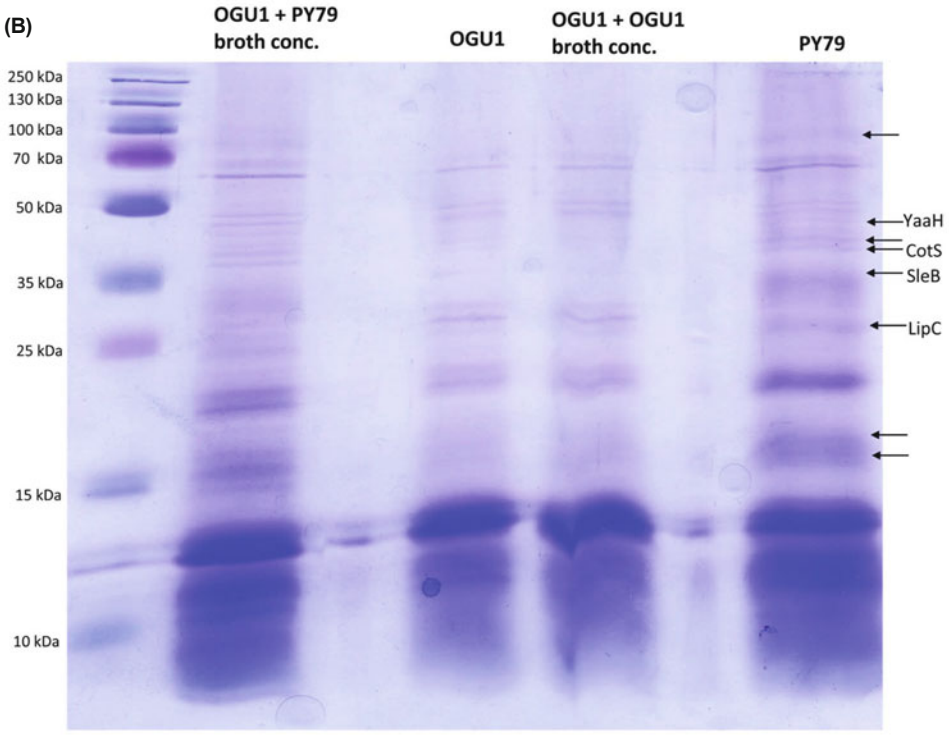
Supplementary data Table S1 shows whole genome resequencing (WGRS) of OGU1 (GenBank accession no. QLNM-00000000.1) and its parental strain PY79 of our laboratory (GenBank accession no. WOWX00000000) in reference to BGSC-deposited PY79 (GenBank accession no. CP006881.1). Accordingly, OGU1 has a total of 5 SNPs and 1 deletion and its parental PY79 has 3 SNPs and 5 indels. Three SNPs (in two putative deacetylase genes, *ylxY* and *yxkH*, and the non-coding region between *ywcC* gene and 5'UTR of *slrA*) and one deletion (at K^6 of *swrAA/1* [swarming motility protein; control of DegU activity]) are common to both our PY79 and OGU1 (Supplementary data Table S1). To be more specific, the SNP at the non-coding region is located at a site to which no transcription factor is theoretically bound. The other SNPs result in missense mutations in *ylxY* and *yxkH* at P^{264} to K^{264} and V^{168} to M^{168}, respectively while the common deletion results in a frameshift mutation. In addition, OGU1 and its parental PY79 have some unique alterations as well. The parental strain has four indels, two of them being present in *fruA* (fructose-specific PTS), one in *bmrU* (multidrug resistance protein), and another one in 16S RNA coding *rrnB*, the former two causing alterations in protein structure. On the other hand, OGU1 has only two distinct SNPs, which are located in the coding region of *prkA* (putative serine protein kinase; control of SigK-dependent gene expression) and 16S RNA coding *rrnE*. The SNP in *prkA* results in a silent mutation ($R^{525}R$).

As deduced from the analysis of the whole sequence of *bac* operon and its flanking regions in OGU1, pMutin T3 containing 5′-region of *bacA* (designated as *bacA*′) has been inserted in between P_{bac} and the remaining 3′-region of *bacA*. Thus, an intact *bacA* gene and the rest of *bac* operon under P_{spac} were obtained in OGU1. Accordingly, the resulting genomic organization (*bacA*′::*lacZ*::*erm*::*bacABCDEF*) has constituted an IPTG-inducible operon (Fig. 2). The sequence of *bacABC* – pMutin T3 – *bacA*′ – *ywfA* genomic region in OGU1 is presented as Supplementary Data 1. The OGU1 cultures were induced with IPTG at different stages of growth and for different time durations. The samples were then collected at time intervals for determination of bacilysin activity by paper disc-agar diffusion assay. Culture fluids of IPTG-induced and uninduced OGU1 cultures gave no zone of inhibition in bioassays while the expected bioactivities were obtained from PY79 culture fluids in all trials (data not shown). UPLC-MS analysis was performed to confirm the lack of bacilysin production upon IPTG induction of OGU1. The peak with an *m/z* of 271.1 corresponding to the mass of bacilysin $(M + H)^+$ (Walker and Abraham, 1970; Yazgan *et al*., 2001) was absent from the culture fluid extracts of IPTG-induced and uninduced OGU1, but present in that of PY79 as the positive control (Fig. 3).

OGU1 cells had a sporulation efficiency of 90%, which was almost equal to that of PY79. Purified mature spores of this strain were as heat-resistant as those of PY79, but more sensitive to lysozyme and chloroform (Table 3). When the yellowish-brown pigmentation of spores was assessed, the less pigmented phenotype of OGU1 colonies was evident starting from the day 10 (Fig. 4A). SDS-PAGE of spore coat proteins revealed at least 8 underrepresented and/or missing bands in OGU1 as compared to PY79 (Fig. 4B). As to the germination response, OGU1 spores displayed a germination delay of 25 min in rich (2xYT) medium and 30 min in minimal (SMM) medium with respect to PY79 spores (Fig. 4C). There were no differences in DPA level (16.139 ± 1.438 and 15.828 ± 1.758 μg per OD600, for OGU1 and PY79, respectively) and alpha/beta- and gamma-type SASPs of spores (data not shown) between two strains. On the other hand, the strains formed no biofilms, and the swarming behavior of the strains was almost the same (data not shown). When the parallel growing cultures of OGU1 were supplemented with broth concentrates of PY79 and OGU1, all of the above-mentioned deficiencies were almost completely cured in cultures that received the former concentrate, but not the latter (Table 3 and Fig. 4). This finding proves the ability of bacilysin to reverse *bac* operon silencing-induced phenotypic changes in *B. subtilis*. Bacilysin concentrate addition did not harm or alter the growth of OGU1 (Fig. 5A) and the activity of externally introduced bacilysin declined rapidly as it could not be compensated by newly synthesized bacilysin (Fig. 5B). Among the spore coat proteins recovered upon bacilysin addition to OGU1 culture, the identity of 4 could be determined as YaaH, SleB, LipC, and CotS (Fig. 4B). Except for CotS, all these proteins were also identified to be downregulated in OGU1 by our proteomic analyses (Table 2). CotS, on the other hand, is formed simultaneously with CotSA during T5 to T7 of sporulation and responsible for assembly of CotSA (Takamatsu *et al*., 1999), the latter being another underrepresented spore coat proteins we detected.

In PA cultures of PY79, bacilysin activity peaks at the 16^{th} h of incubation, upon entry into stationary phase and decreases abruptly during late stationary phase (Özcengiz *et al*., 1990; Köroğlu *et al*., 2011). For this reason, the cells were collected at 16^{th} h of incubation to check for bacilysin, and to prepare samples for proteomic analyses and RT-qPCR experiments. Since *B. subtilis* cytosolic proteome analysis in one single 2-DE gel with a pH range of 4–7 is known to cover two third of all proteins (Eymann *et al*., 2002), we also employed two narrow pH ranges of 5.5–6.7 and 4.5–5.5 in order to resolve the regions of spot crowding in this range. A total of 1986 spots were detected by dual channel 2-DE imaging in three different pI ranges of 4.0–7.0, 4.5–5.5, and 5.5–6.7 with the separation of 1100, 243, and 643 protein spots, respectively (Supplementary data Fig. S1A, B, and C). Over 250 protein spots were differentially expressed between PY79 and OGU1. One hundred and fifty-nine differentially expressed proteins could be identified which corresponded to 121 distinct ORFs (Supplementary data Table S2). Of these, the levels of 63 were found to be decreased, 33 were expressed more abundantly, 20 were found to be absent in bacilysin-nonproducer OGU1 and 1 protein spot was detected only in this strain. Fifteen proteins appeared in multiple spots, suggesting post-translational charge and/or mass modifications although ar-





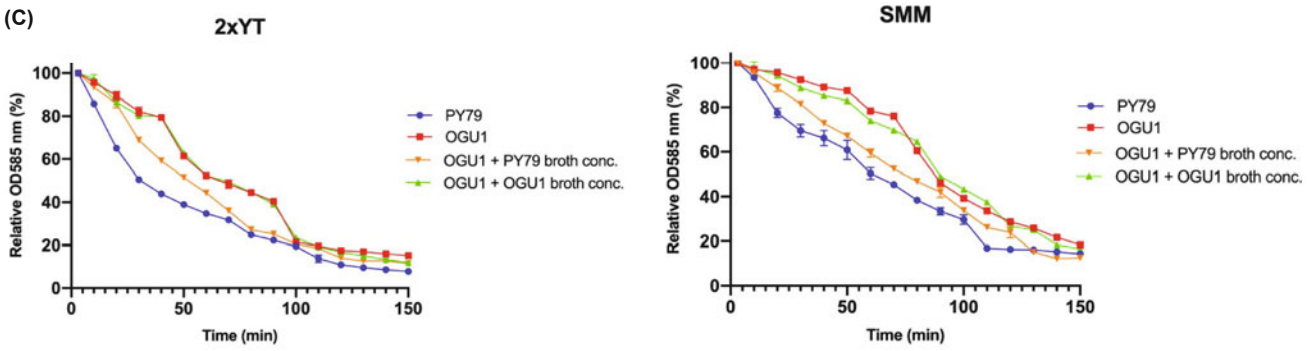


Fig. 4. Morphological effects of bacilysin silencing and its chemical complementation in OGU1. All tests included the cultures of OGU1 supplemented (OGU1 + PY79) and non-supplemented with bacilysin broth concentrate (OGU1 only; negative control) as well as PY79 itself (positive control) and OGU1 supplemented with its own broth concentrate (OGU1 + OGU1; further negative control). The cultures were grown for 16 h in PA medium, after which the morphology of the diluted spots was visualized on DSM plates at intervals for 18 days (A). Germination responses of spores purified from 48 h DSM broth cultures in rich (2xYT) and minimal (SMM) medium (B). SDS-PAGE analysis of coat proteins isolated from spores harvested and purified after 48 h incubation. Arrows point to the protein bands missing or underrepresented in OGU1 and restored upon chemical complementation (C).

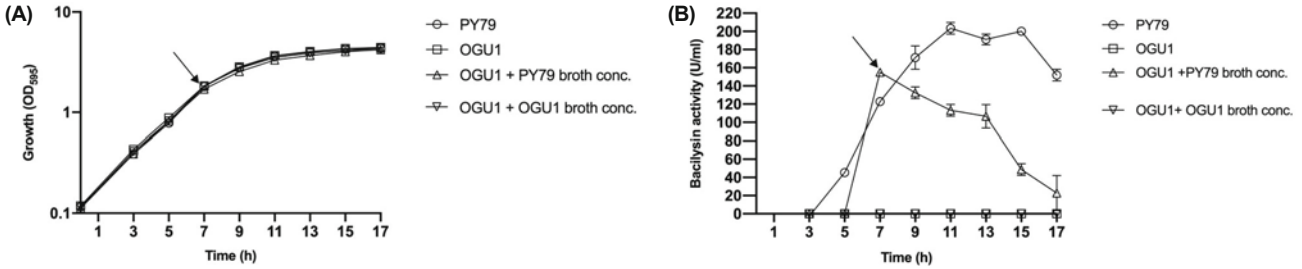


Fig. 5. Growth and bacilysin titers in OGU1 cultures supplemented with broth concentrates from PY79 and OGU1. Broth concentrates of PY79 and OGU1 obtained from 16 h PA cultures were added to parallel PA cultures of OGU1 at the time indicated by arrow. Growth (A) and bacilysin titers (B) of these cultures were next monitored. Parallel cultures of PY79 and OGU1 were also included as positive and negative controls.

tificial chemical modifications/unintended protein degradation occurring during protein preparation cannot be ruled out. In addition, 4 distinct proteins (#31, #33, #41, and #74) displayed opposing ratios either in the same or a different pI range. This might be due to protein comigration and partial comigration that can occur in 2D gels, imposing a challenge in quantification. NanoLC-MS/MS led to the identification of 1282 proteins from cytosolic proteome of PY79

Table 2. Significantly underrepresented proteins in OGU1 as the members of sporulation σ regulons in *B. subtilis*

Regulon*	Protein	Product/Function	Approach**
	AsnO	Asparagine synthase (glutamine-hydrolysing)/ biosynthesis of asparagine	NanoLC
	GerQ (YwdL)	Spore coat protein necessary for the proper localization of CwlJ/spore germination	2DE
	SodF	Superoxide dismutase/ detoxification of oxygen radicals	NanoLC
	SpoVR	Unknown/ involved in spore cortex synthesis	NanoLC
	SpsB[c,g,m]	Unknown/ spore coat polysaccharide synthesis	NanoLC
SigE	SpsJ[c,g,m]	dTDP -glucose-4,6-dehydratase/ rhamnose biosynthesis, spore coat polysaccharide synthesis	NanoLC
	SpsK[c,g,m]	dTDP -4-dehydrorhamnose reductase/rhamnose biosynthesis, spore coat polysaccharide synthesis	NanoLC
	YheC[c,j]	Unknown/ ATP-binding spore coat protein	NanoLC
	YodQ	Putative deacylase/ unknown	NanoLC
	CtpB[e]	Carboxy-terminal processing serine protease, cleaves SpoIVFA/ processing of pro-SigK; control of SigK activation	NanoLC
	YaaH[b,j]	N-acetylglucosaminidase/ survival of ethanol stress, protection of the spore	2DE
	Gpr[d,e,k]	Germination protease/ degradation of SASPs	NanoLC
SigF	YtfJ	Unknown; germination	2DE
	KatA[r]	Major catalase in spores; detoxification of hydrogen peroxide	NanoLC
	SleB	Spore cortex lytic enzyme/ degradation of spore cortex, germination	NanoLC
	TepA[k]	Orphan ClpP-like germination protease/degradation of SASP	NanoLC
SigG	YhcQ	Unknown	2DE
	YpeB	Germination protein/ essential for SleB assembly in spores	NanoLC
	YvaB[p]	Azoreductase/ sporulation protein; resistance to 2-methylhydroquinone	2DE
	GalM (YoxA)[b]	Aldolase-1-epimerase (mutarotase)/ unknown	2DE
SigH	MinC[a,f,h,i,l]	Cell-division inhibitor (septum placement)/ destabilizes Z ring placement	2DE
	MinD[a,f,i,l]	Septum placement/ cell division inhibitor	2DE
	CgeA[m,n]	Spore crust protein/maturation of the outermost layer of the spore	NanoLC
	CotA[m]	Laccase, bilirubin oxidase, spore coat protein (outer)/ resistance of the spore	2DE, NanoLC
	CotE[c,m]	Outer spore coat morphogenetic protein/ controls the assembly of the outer spore coat	NanoLC
	CotI	Spore coat protein/ spore envelope	NanoLC
	CotSA[m]	Spore coat protein/ resistance of the spore	NanoLC
	CotU[m,n]	Spore coat protein/ resistance of the spore	NanoLC
SigK	CwlC	N-acetylmuramoyl-L-alanine amidase/ mother cell lysis	NanoLC
	LipC[g,m,n,o,s]	Spore coat phospholipase B/ spore germination	NanoLC
	OxdD[m]	Oxalate decarboxylase/ inner spore coat protein; protection of the spore	NanoLC
	DpaA (SpoVFA)	Dipicolinate synthase (subunit A)/ dipicolic acid production	2DE
	DpaB (SpoVFB)	Dipicolinate synthase (subunit B)/ dipicolic acid production	NanoLC
	YfnG[c,m]	Similar to CDP-glucose 4, 6-dehydratase/ unknown	NanoLC
	YitD[c]	Unknown	NanoLC

* A member of other regulon(s) as well: [a] SigA, [b] SigB, [c] SigE, [d] SigF, [e] SigG, [f] SigH, [g] SigK, [h] SigM, [i] SigV, [j] SpoIIID, [k] SpoVT, [l] ComK; [m] GerE, [n] GerR, [o] KipR, [p] MhqR, [r] PerR, [s] TnrA.
** Identified by 2DE, 2-DE MALDI-TOF/MS; NanoLC, NanoLC-MS/MS

Table 3. Heat, chloroform, and lysozyme resistance of spores purified from DSM cultures of PY79 and OGU1 after 48 h incubation

Strain	Heat resistance	Chloroform resistance	Lysozyme resistance
PY79	0.88 ± 0.03	0.47 ± 0.03	0.83 ± 0.08
OGU1	0.91 ± 0.04	0.25 ± 0.03	0.49 ± 0.11
OGU1 + OGU1 broth conc.	0.89 ± 0.05	0.26 ± 0.02	0.53 ± 0.08
OGU1 + PY79 broth conc.	0.92 ± 0.06	0.44 ± 0.03	0.78 ± 0.15

and OGU1. Seventy six of these proteins were differentially expressed in OGU1 by comparing the spectral counts of the samples, with 50 proteins absent, 7 underrepresented and 19 found only in OGU1 (Supplementary data Table S3). Indeed, there were only a few differentially expressed proteins common to both proteomic approaches, including the enzymes of *bac* operon, CotA, DegU, GlmS, MalS, SpoVF, YhcQ, YtfJ, and YtkL. NanoLC-MS/MS could actually detect many of the proteins identified by 2DE approach, but the high criteria employed to quantitate the relative spectral counts resulted in a large subtraction from the final list.

According to the relative functional distributions of differentially expressed proteins identified by both 2-DE and nanoLC approaches (Fig. 6A and B), "Metabolism" and "Lifestyles" were the two top-level functional categories according to the categories in *Subti*Wiki database. The subcategory "Sporulation and Germination" under the category "Lifestyles" constituted as high as 58% of all missing and downregulated proteins of OGU1 strain identified by nanoLC-MS/MS. The present study identified 35 underrepresented sporulation and germination proteins in OGU1, which belong to 5 sporulation sigma regulons (Table 2). Of these regulons, the mother cell compartment-specific σE and σK harbored the majority of these proteins. It is noteworthy that the total percentage of the subcategory "Unknown functions" was higher among the upregulated proteins than the downregulated ones, in both proteomic approaches.

In order to confirm the alterations in abundance (over- or

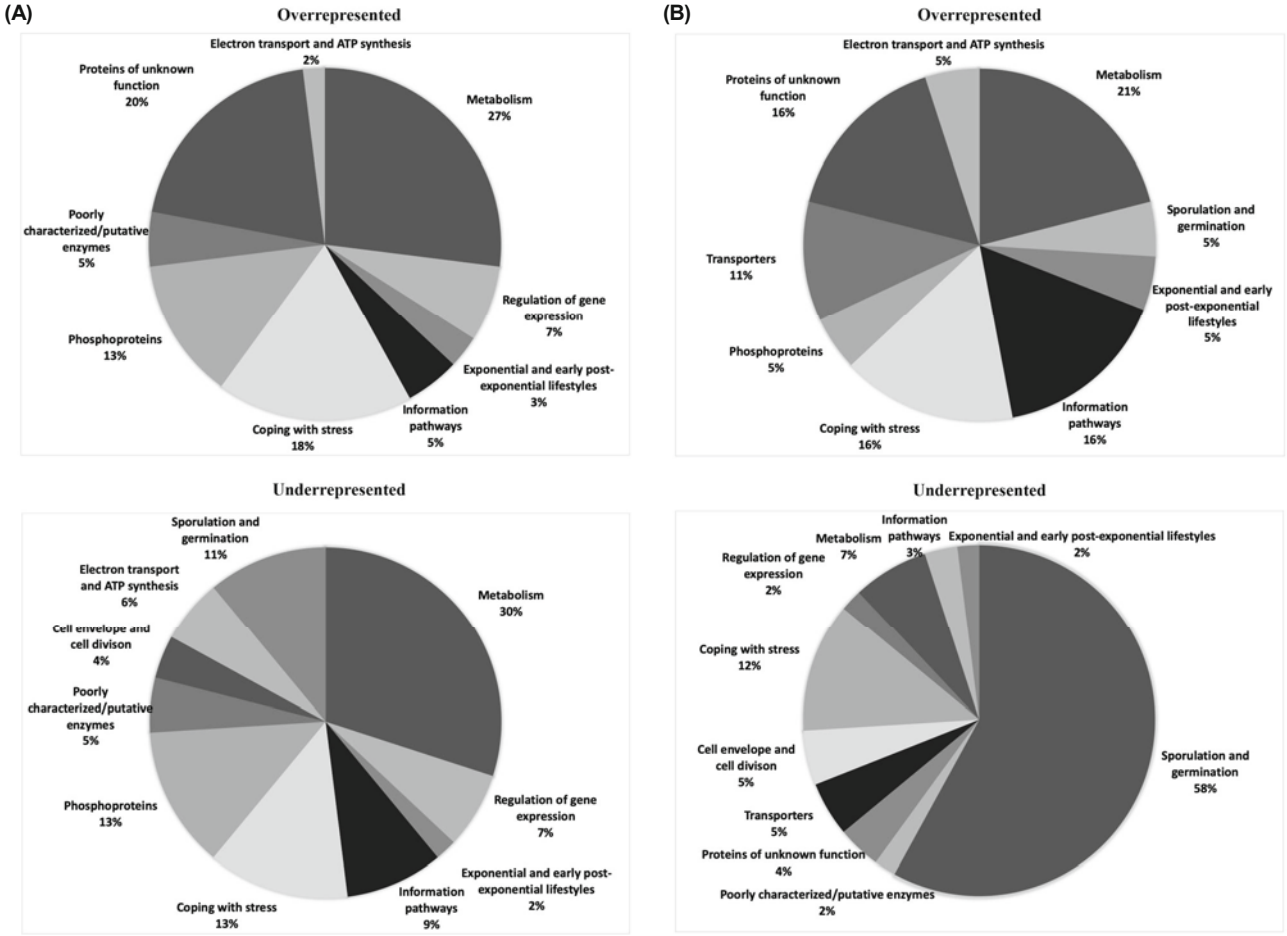


Fig. 6. Differentially expressed proteins in OGU1 with respect to their functional classes. Distribution of overrepresented (above charts) and underrepresented (below charts) proteins to major functional classes of cellular physiology in bacilysin non-producer OGU1 as determined by 2-DE MALDI-TOF/MS (A) and nanoLC-MS/MS (B) approaches.

under-representation) of each protein, the genes coding for selected proteins (a total of 36) were next subjected to quantitative transcript analysis. Majority of these genes were selected as indicated by our proteomic data, and 9 of them (*phoP*, *degS*, *gerE*, *codY*, *abrB*, *ccpA*, *abbA*, *spo0A*, and *dnaA*) that could not be identified by the present proteome work were also included because of their significant global regulatory roles in cellular physiology, sporulation in particular, and/or their interaction with master regulators. The results expressed in fold change were tabulated separately with respect to the functions of the genes; "sporulation and germination" (*dpaA*, *dpaB*, *cotA*, *ytfJ*, *yhcQ*, and *yxbC*), "two-component systems" (*cheV*, *resD*, *phoP*, *degU*, and *degS*), "global regulators" (*gerE*, *codY*, *abrB*, *ccpA*, *abbA*, and *spo0A*), "stress response" (*yugI*, *dnaK*, *yceE*, and *ytkL*), "bacilysin biosynthesis" (*bacA*, *bacB*, *bacC*, *bacD*, *bacE*, *bacF*, and the monocistronic gene *bacG*), and "others" (*luxS*, *albE*, *ispA*, *oppD*, *dnaA*, *glmS*, *glnA*, and *malS*), as shown in Fig. 7A–F. No contaminant PCR products were observed according to melting curve analyses. Comparative RT-qPCR analysis between OGU1 and PY79 for these genes generally indicated a good correlation between transcript levels and proteomic data. Particularly, the transcripts of *bac* operon genes of uninduced OGU1 were 2- to 4-fold less abundant with respect to PY79 (Fig. 7E), consistent with our nanoLC-MS/MS data wherein the levels of corresponding proteins displayed 3- to 5.5-fold decrease (Supplementary data Table S3). Except for monocistronic *bacG*, IPTG induction provided 3- to 7-fold increment in *bac* transcription. Thus, the lack of bacilysin in IPTG-induced OGU1 cultures is quite intriguing and deserves further investigation.

Combining the data obtained from the whole proteomics survey, the differentially expressed proteins are discussed with respect to their cellular functions:

Proteins of sporulation and germination

In all spore-forming bacteria, the network of cellular development and differentiation shows a well-conserved and strictly regulated hierarchical organization. In this, the mother cell and forespore compartment- and sporulation stage-dependent sigma factors and the master regulator Spo0A are sequentially activated (de Hoon *et al*., 2010). In the present study, the majority of underrepresented sporulation and germination proteins in OGU1 belonged to σE and σK regulons. While the former plays a decisive role in spore morphogenesis, the latter is activated after the completion of engulfment and directs the expression of genes required for synthesis of the spore cortex and coat proteins as well as those required for spore germination and mother-cell lysis (Steil *et al*., 2005). GerE, as a component of σK regulon and the final regulator turning on the expression of last genes in mother cell compartment according to the hierarchical programme of sporulation, harboured 9 of the differentially expressed protein genes of σE and σK regulons in its own regulon. As a small regulatory protein (8.43 kDa) with a high pI value (9.64), GerE was not detected in our proteomics work. Nonetheless, RT-qPCR analysis revealed 3.2-fold down-regulation of its gene in OGU1 compared to PY79 (Fig. 7C).

Of about 70 proteins identified in the spore coat, the coat morphogenetic proteins that organize the assembly of different subsets of coat proteins include CotE, CotH, CotO,

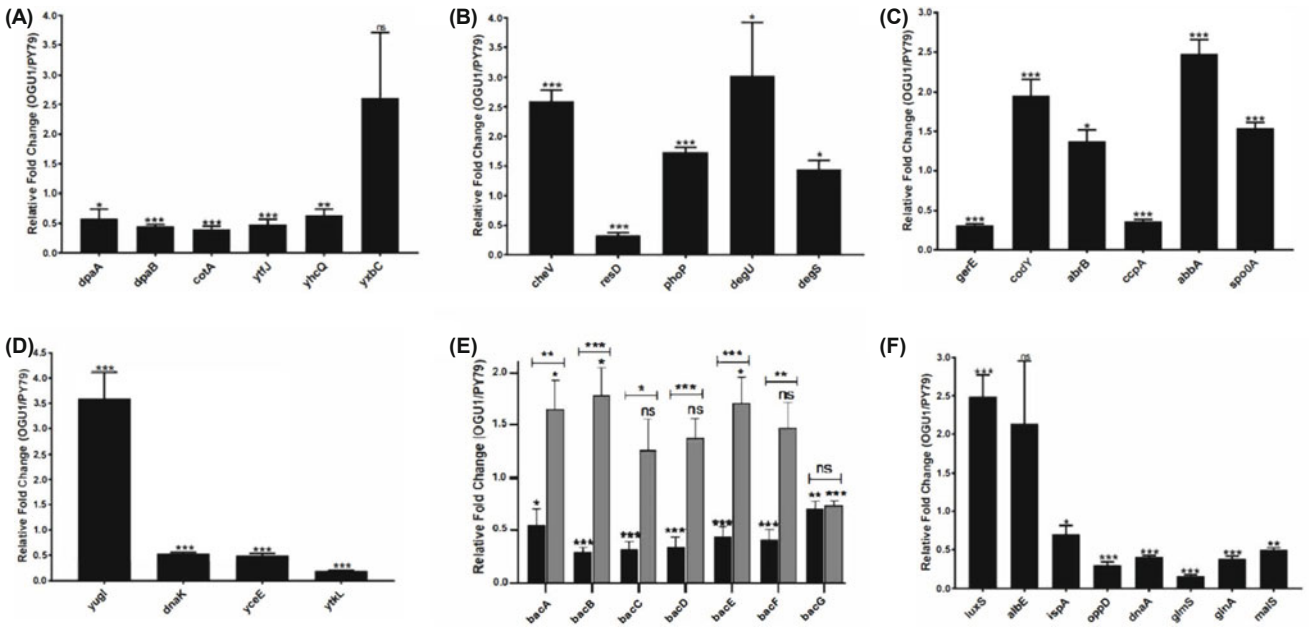


Fig. 7. RT qPCR analysis for the genes corresponding to selected differentially expressed proteins. Analysis by RT qPCR of the transcripts of the genes encoding selected proteins, which are differentially expressed in bacilysin-negative OGU1 and grouped with respect to their functions: "sporulation and germination" (A), "two-component systems" (B), "global regulators" (C), "stress response" (D), "bacilysin biosynthesis" (E) and "others" (F). In (E), bacilysin biosynthetic genes included those of *bac* operon (*bacABCDEF*) as well as the monocistronic gene *bacG*. The gray bars show the relative transcript levels upon 1 h IPTG induction at 15th h, and the dark bars with no IPTG. The significance levels are represented as ns (no significance, $p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Error bars represent standard error of the mean.

SafA, and SpoVID since the loss of any of them would drastically interfere with overall coat architecture (Zheng *et al.*, 1988; Plomp *et al.*, 2014). Our study revealed the absence of many coat proteins, namely CgeA, CotA, CotB, CotI, CotSA, CotU, and OxdD and significant downregulation of CotE in OGU1 accounting for altered coat protein profile and increased sensitivity of OGU1 spores to lysozyme and chloroform. A very significant downregulation of ca. 65-kDa CotA, as validated by 2.5 fold decreased level of its transcript must have accounted for the loss of brownish pigmentation in OGU1 (Jicinska, 1960) (Fig. 4A). Like spore coat proteins, spore coat polysaccharide synthesis proteins SpsB, SpsJ, SpsK, and cortex synthesis protein SpoVR were lost from OGU1.

Alanine utilization enzyme L-alanine dehydrogenase Ald (SpoVN), as a sporulation and germination protein, was absent in OGU1. Other proteins of this category, which were strongly underexpressed in this strain were 3-hydroxyacyl-CoA dehydrogenase FadN, MRP family transition state regulator SalA, spore cortex-lytic enzyme SleB, unknown germination protein YtfJ. SalA is a phosphorylation-dependent repressor of ScoC (Derouiche *et al.*, 2015) which is a well-known transition state negative regulator of sporulation, exoprotease production and *bac* operon (Inaoka *et al.*, 2009). DPA is essential for development of spore heat resistance and disruption of the *spoVF* or *spoVA* operons impairs sporulation (Daniel and Errington, 1993). The levels of dipicolinate synthase subunits A and B (DpaA and DpaB) as well as their transcripts were decreased 1.7 and 2.2 fold in OGU1, respectively, but with no alteration in DPA content of spores. Oligopeptide ABC transporter OppD (Spo0KD) as a component of CSF-mediated quorum sensing for initiation of sporulation and competence development was also underrepresented in OGU1 with a concomitant 3.3 times downregulation of its transcript. *opp* operon is among the targets of ScoC repression (Koide *et al.*, 1999). To note, the insertional inactivation of *spo0K* operon leads to complete loss of bacilysin biosynthetic function (Yazgan *et al.*, 2001). The germination protein YtfJ (GerW) and the unknown spore wall protein YhcQ gave an OGU1 to PY79 ratio of 0.1 and 0.3, respectively, in 2DE-based proteomics. Quantitative RT-PCR results generally well accorded with these protein representation ratios, demonstrating 2.1 and 1.6-fold decrease, respectively, in their mRNA levels.

Collectively, above-mentioned findings might indicate that bacilysin displays complex, but yet undefined functions in cellular differentiation, which phenotypically affect spore resistance and germination.

Global regulatory proteins

Some essential two-component regulator proteins that play the critical role in many cellular functions, like the adaptation to nutrient shortage, chemotaxic motility, competence development, sporulation and existence in biofilms (Hoch, 2000; Mascher *et al.*, 2006; Gao *et al.*, 2007) were differentially expressed between OGU1 and PY79.

In *B. subtilis*, the two-component system ResDE constitutes a key switch to ensure adaptation to anaerobiosis. ResD, which is activated upon phosphorylation by ResE, turns on the genes that function in nitrite and nitrate respiration as well as the gene coding for the anaerobic gene regulator Fnr (Nakano *et al.*, 2000b). We found that ResD level in OGU1 is much lower than that in the parental strain which was transcriptionally validated (3-fold decrease) by RT-qPCR assay.

LuxS/AI-2 (autoinducer-2 synthase/autoinducer-2) couple form a cell-to-cell language in which AI-2 coordinates morphogenesis and intra- and interspecific social behavior of *B. subtilis*. The master regulators Spo0A and SinR of pluricellular behavior have negative effect on *luxS* expression although LuxS has no effect on spore development (Lombardia *et al.*, 2006). Biofilm formation and swarming behavior in undomesticated strains of *B. subtilis* rely on LuxS-based signalling. Intriguingly, LuxS was found 2.7 fold overexpressed and there was a 2.5-fold increase in its transcript abundance in OGU1 compared to PY79, however neither of the strains displayed biofilm formation or a remarkable swarming motility, as expected from surfactin-negative, domesticated strains (Kearns and Losick, 2003; McLoon *et al.*, 2011).

In *B. subtilis*, CodY is a global pleiotropic regulator that represses during growth over 100 genes and operons, which play functions for adaptation to nutrient inavailability, such as peptide and amino acid transport systems, degradative enzymes, competence, antibiotic production, flagellin and early sporulation genes. For stimulation of its DNA-binding activity, it has to first bind to the branched-chain amino acids (BCAAs) and GTP, which may be regarded as the signals of the metabolic and energetic status of the cell (Molle *et al.*, 2003; Brinsmade *et al.*, 2014). Thus, limitation of nutrients at the early stationary phase is associated with a transient drop in intracellular GTP and BCAA pools, thus derepression of CodY targets occurs when cells encounter with starvation. The negative transcriptional regulator ScoC is directly repressed by CodY, therefore the potential repressive effect of ScoC can be underestimated as soon as CodY is active (Belitsky *et al.*, 2015). However, CodY uses ScoC in its regulatory cascade to repress certain genes when it loses its own activity and thus it initiates multiple forms of cascade regulation. For instance, the *opp* operon which is crucial for onset of sporulation and competence is repressed by both ScoC and CodY, but *codY* or *scoC* single mutants are not much affected. Our group showed earlier that CodY represses *bac* operon by co-binding with AbrB repressor to P_{bac} (Köroğlu *et al.*, 2011). The present proteomic analysis demonstrated that many proteins encoded by the members of CodY regulon such as CodY-activated acetate kinase (*ackA*) and inosine-monophosphate dehydrogenase (*guaB*) and CodY-repressed D-alanyl-aminopeptidase (*dppA*), L-alanine-D/L-glutamate epimerase (*ykfB*), aconitase (*citB*), alpha subunit of urease (*ureC*), an amidohydrolase of unknown function (*amhX*), the oligopeptide ABC transporter (*oppD*; *spo0KD*), intracellular serine protease (*ispA*), an unknown protein (*yxbD*) and some proteins involved in branched-chain amino acid biosynthesis, ketol-acid reductoisomerase (*ilvC*), dihydroxy-acid dehydratase (*ilvD*), 3-isopropylmalate dehydratase subunit (*leuC*) as well as 2-oxoisovalerate dehydrogenase involved in utilization of branched-chain keto acids (*lpdV*) were all significantly underrepresented or absent from OGU1 when compared to PY79. Our current findings on general downregulation of the proteins of CodY and ScoC regulons with a concomitant increase of ca. 2 fold in *codY* transcript level in bacilysin-negative mutant led us to hypothesize that

the absence of bacilysin during transition phase might somehow potentiates the transcription of CodY pleiotropic repressor which awaits several experimental tests.

Considering the possible impacts of *bac* operon silencing on early sporulation genes, the transcript abundances of the crucial proteins like phosphorelay response regulator (master regulator) Spo0A and the transition state global regulator AbrB were also determined in OGU1. In parallel to an increment in the transcript level of *Spo0A*, *DnaA* (a transcription factor repressed by Spo0A) transcript abundance was decreased by 2.4 fold. AbbA is activated by Spo0A and acts as an anti-repressor protein by inhibiting AbrB activity (Banse *et al*., 2008). Carbon catabolite control protein CcpA is known to repress *abbA*. Interestingly, *ccpA* transcription was found to be 2.7 fold decreased in OGU1 which might account for 2.5 fold elevated transcript abundance of *abbA* in this strain (Fig. 7C).

As an important protein functioning in sensory adaptation in response to attractants (Karatan *et al*., 2001), the response regulator CheV was found to be 4.3-fold overrepresented in OGU1 with a 2.6 times elevated transcript levels. Likewise, DegU protein of DegS-DegU two-component system that regulates transition state specific events, degradative enzyme production, competence and motility (Mader *et al*., 2002) was 2.7-fold overrepresented in OGU1. *degU* is known to be positively regulated by CcpA (Ishii *et al*., 2013). The transcript abundance of *degU* exhibited 3-fold increase, and its partner *degS* displayed 1.4-fold increase in OGU1, while *ccpA* is strongly downregulated. These increments upon the loss of bacilysin biosynthetic function may be particularly important knowing that DegU, in a nonphosphorylated form, positively regulates *bac* operon as well as the monocistronic *ywfH* (*bacG*) in *B. amyloliquefaciens* (Mariappan *et al*., 2012). PhoPR is another two-component response regulator in *B. subtilis*, regulating phosphate metabolism. PhoPR is subjected to cumulative repression by ScoC and CcpA (Kaushal *et al*., 2010). In our work, *phoP* transcript abundance was found to be 1.7 times higher in OGU1.

Stress response proteins

Alternative sigma factor σ^B is activated in response to chemical stress and starvation in *B. subtilis* (Hecker *et al*., 2007). SigB regulon proteins underrepresented in OGU1 included NadE (NH_3-dependent NAD^+ synthetase, so-called sporulation protein OutB), YaaH (N-acetylglucosaminidase/ survival of ethanol stress, protection of the spore), YceE (similar to tellurium resistance protein; required for survival of ethanol stress and low temperature), YceD (similar to tellurium resistance protein; required for survival of ethanol stress), YceH (similar to toxic anion resistance protein), and KatX (major catalase in spores for detoxification of hydrogen peroxide). On the other hand, general stress protein YtkL induced by ethanol shock and by treatment of antibiotic enduracidin (Rukmana *et al*., 2009) and multidrug-efflux transporter BmrA were 3.4 and 2.1 times overrepresented in OGU1, respectively. Analysis for transcript abundance validated a downregulation at the level of gene expression for YceE.

Other stress proteins which are not the members of SigB regulon and identified as significantly less abundant in OGU1 were MsrA (peptide methionine sulfoxide reductase), expression of which is elevated under disulfide stress (Leichert *et al*. 2003), YvaB (resistance to 2-methylhydroquinone), YgaF (resistance against oxidative stress) (Horsburgh *et al*., 2001), YjlD (NADH dehydrogenase, negatively regulated in stringent response), and YfkO (protection against NaOCl stress) while DnaK (class I heat shock protein) was absent. The stress proteins strongly overexpressed in OGU1 were YugI (a member of stringent response regulon) and YppQ (regeneration of methionine and restoration of protein function after oxidative damage), with 8.5 and 7.6 fold increments. AhpC (resistance against peroxide stress) and YaaN (cell envelope stress induced by salt shock) (Petersohn *et al*., 2001) were also increased by 2.5 and 4.4 times, respectively. Of abovementioned proteins, DnaK and YugI were selected for RT-qPCR experiments and a 1.8 times decrease in *dnaK* and a 3.6 times increase in *yugI* transcripts confirmed the corresponding alterations in protein levels.

Secondary metabolite production and resistance

In *B. subtilis*, *sbo-alb* operon is responsible for the production and secretion of antilisterial bacteriocin subtilosin. AlbE encoded by this operon is a signal peptide protease that acts for subtilosin export. Induction of the *sboA-alb* operon under anaerobic conditions occurs in response to oxygen limitation and nutritional stress, which absolutely requires ResDE signal transduction complex. The operon is subjected to an independent repression by AbrB (Nakano *et al*., 2000a). AlbE was found as a 3-fold upregulated protein in OGU1 while ResD was simultaneously downregulated. The transcript levels of *albE* and *resD* fit well to the proteomic data in that while *albE* transcription showed a 2.1-fold increase (ns, $p > 0.05$), *resD* transcription showed an opposite behavior with a strong decrease of 3 fold. Therefore, AlbE overrepresentation did not seem to be a manifestation of ResDE signal transduction complex, but rather due to an elevated level of AbrB in OGU1. On the other hand, an increase in AlbE expression upon the loss of bacilysin biosynthetic function might confer a competitive adaptation since the secretion of a new antimicrobial into the environment would compensate the loss of another.

bceA and *bceB* genes encode the nucleotide-binding domain and the membrane-spanning domain subunits of an ABC transporter for bacitracin, plectasin, mersacidin and actagardine export in *B. subtilis* (Ohki *et al*., 2003). The *bceRS* genes that code for a two-component system reside just upstream of *bceAB* and when induced by bacitracin, the activated response regulator *bceR* upregulates *bceAB* genes. In the present study, BceB protein was overrepresented in OGU1. Another antibiotic transporter protein is the multidrug ABC transporter Bmr3 that confers multiple antibiotic resistance to *B. subtilis* (Steinfels *et al*., 2004). Interestingly, overexpression of both BceB and Bmr3 proteins in OGU1 was recorded.

Other proteins

As in several other Gram-positive bacteria, the *dlt* operon is responsible in *B. subtilis* for D-alanylation of lipoteichoic acids, which are the major cell wall components (Cao and

Helmann, 2004). By generating a net positive charge on cell walls and repulsing positively charged molecules, this confers resistance to cationic antimicrobial peptides and lysozyme. DltA (D-alanine-D-alanyl carrier protein ligase) inactivation results in a higher susceptibility to such agents in *B. subtilis* (May *et al*., 2005). This protein of cell wall biosynthesis was less abundantly expressed in OGU1.

The level of MutT repair protein of mismatch recognition was shown to confer protection against oxidative stress (Castellanos-Juárez *et al*., 2006). MutS, another protein with the same function, was among the proteins found in elevated levels in OGU1. Three other proteins, all unknown, but increased 8.3, 7.6, and 3 times in OGU1, were YwhD, YwlG, and YciC (putative metallochaperone), respectively.

glmS Codes for glutamine-fructose-6-phosphate transaminase for cell wall synthesis. It is a glucosamine 6-phosphate (GlcN6P)-responsive small ribozyme, self-cleavage activity of which is triggered by binding of GlcN6P as a cofactor so that the gene is only expressed in the absence of GlcN6P (Collins *et al*., 2007). This protein was strongly downregulated in OGU1 in our proteome work, with a 6.25-fold decrease in its transcript abundance. The antimicrobial action of bacilysin is due to the inhibition of GlcN6P synthase activity by its C-terminal anticapsin moiety, following the entry of bacilysin into susceptible target cells and its cleavage by intracellular proteases into the constituent amino acids. The striking downregulation of *glmS* ribozyme upon the loss of bacilysin biosynthetic activity is indicative of elevated levels of GlcN6P, which does not seem to be the case in the bacilysin producer strain. It might then be speculated that, like other peptide pheromones, a fraction of externalized bacilysin is transported back into the producer cells to play some functions, meanwhile inhibiting GlmS, lowering intracellular GlcN6P pool, and creating conditions for GlmS expression. In the opposite situation that bacilysin is not produced by the cells, as in OGU1, *glmS* riboswitch is expected to gain self-cleavage activity which may explain a sharp decrease in *glmS* transcript levels as recorded in our work.

Antibiotic-mediated heterologous signalling involves the mechanisms by which subinhibitory concentrations of certain antibiotics trigger specific transcriptional responses in target pathogens influencing (i) their virulence, (ii) SOS-response and other responses to stress, (iii) stabilization of mRNA and ribosome stalling, (iv) two-component systems and (v) quorum-sensing (Fajardo and Martinez, 2008). For instance, aminoglycosides and tetracycline, at subinhibitory concentrations, increase biofilm formation by *Pseudomonas aeruginosa* and tetracycline increases cytotoxicity of this pathogen, both being beneficial in natural environments (Linares *et al*., 2006). Autologous signaling, on the other hand, generally occur among members of the same species although some antibiotics may act as both autologous signals and heterologous inhibitors. Of a number of 2-alkyl-4(1H)-quinolones produced by *P. aeruginosa*, the quinolone signal and 2-heptyl-4-hydroxyquinoline act as quorum-sensing signal molecules controlling the expression of many virulence genes as a function of cell population density (Heeb *et al*., 2011). The polyketide germicidin A and chalcone which are produced during germination of the *Streptomyces coelicolor* had an inhibitory effect on the germination process, by acting as quorum sensing molecules coordinating the development of their producer while repressing competitive microflora in natural environments (Čihák *et al*., 2017). The antitumor agent prodiginine produced by *S. coelicolor* and aromatic polyketide chromomycin produced by *S. flavisclerotic*us constitute two other examples of functions in the producer genus, as the former triggers the programmed massive death round of the vegetative mycelium during morphogenesis (Tenconi *et al*., 2018), and the latter acts as an antioxidant (Prajapati *et al*., 2019), explaining the pleiotropic phenotypes of the deletion mutants for these antibiotics. When it comes to lantibiotics and other antimicrobial peptides, the presence of the classical quorum sensing autoinducers having antimicrobial activity gives more power to the conception that antimicrobials play a dual role as signals and weapons. Especially, quorum-sensing mechanisms are widespread in lantibiotic biosynthesis, including nisin (Kuipers *et al*., 1995; Kleerebezem *et al*., 2004), subtilin (Kleerebezem *et al*., 2004), salivaricin A (Upton *et al*., 2001), streptin (Wescombe and Tagg, 2003) and mersacidin (Schmitz *et al*., 2006) that autoregulate their own biosyntheses. Paracrine signaling was extensively studied in recent years to understand the interaction between different co-existing subpopulations (surfactin-producing and matrix-producing, hence biofilm forming cells; sporulating cells; flagellated and motile ones; cells that secrete peptide toxins to cannibalize their siblings; and cells that become competent), all making up different cell types of *B. subtilis* multicellularity. The basic signals acting in response are ComX pheromone and the cyclic lipopeptide antibiotic surfactin, the former triggering surfactin production, and the latter triggering matrix-production and the ultimate aim looks like the maintenance of all cell types over generations (Shank and Kolter, 2011). *B. subtilis* 916 is capable of co-producing four different nonribosomally synthesized lipopeptide (LP) antibiotics, namely surfactin, bacillomycin (iturin family), fengycin, and a new family called locillomycin, and Luo *et al.* (2015), attempted to uncover the phenotypic features associated with each via the deletion of respective biosynthetic operons. Regarding the features like antifungal activity, colony morphology, hemolytic activity, swarming motility, and biofilm formation of the producer, each LP contributed in an intricate way by affecting the production of the others. In view of afore-mentioned studies and demonstrated functions, it might be plausible to attribute the proteome-wide alterations to the loss/gain-of-function in our bacilysin-negative mutant of *B. subtilis*, hence to the possibility that bacilysin itself, either directly or indirectly, acts as an autoregulator to modulate the level and timing of the distinct biological responses for adaptation to changing conditions. A small, heat-stable and proteinase K susceptible extracellular factor named FacX, which accumulates to high levels only during post-exponential growth, was reported by Ababneh *et al.* (2015). It was found to differ from known quorum-sensing peptides in that its activity neither requires the oligopeptide transporter systems, nor depends on SigH, Spo0A, or ComX. This study indicated the presence of yet unknown quorum sensing signals other than well-known peptide pheromones.

Remarkably, many critical two-component systems as well as global response regulators that play the critical roles in

diverse physiological functions were differentially expressed in bacilysin-negative OGU1 cells. Our IPTG-inducible *bac* operon itself was theoretically suitable for complementation, however no detectable bacilysin was produced in spite of enhanced transcription of *bac* operon genes upon IPTG induction. The finding that OGU1 is phenotypically rescued by adding bacilysin concentrate into its cultures provided a strong clue for a connection between bacilysin and the altered functions reported herein. Although our data pointed to pleiotropic effects of bacilysin on cellular physiology in *B. subtilis*, it will be highly interesting to know how it takes part or cooperates in highly complex, integrated regulatory network(s).

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Conflict of Interest

The authors declare no conflict of interest.

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