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Characterization of sporulation dynamics of *Pseudoclostridium thermosuccinogenes* using flow cytometry

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ABSTRACT

Single-cell analysis of microbial population heterogeneity is a fast growing research area in microbiology due to its potential to identify and quantify the impact of subpopulations on microbial performance in, for example, industrial biotechnology, environmental biology, and pathogenesis. Although several tools have been developed, determination of population heterogenity in anaerobic bacteria, especially spore-forming clostridia species has been amply studied. In this study we applied single cell analysis techniques such as flow cytometry (FCM) and fluorescence-assisted cell sorting (FACS) on the spore-forming succinate producer *Pseudoclostridium thermosuccinogenes*. By combining FCM and FACS with fluorescent staining, we differentiated and enriched all sporulation-related morphologies of *P. thermosuccinogenes*. To evaluate the presence of metabolically active vegetative cells, a blend of the dyes propidium iodide (PI) and carboxy fluorescein diacetate (cFDA) tested best. Side scatter (SSC-H) in combination with metabolic indicator cFDA dye provided the best separation of sporulation populations. Based on this protocol, we successfully determined culture heterogeneity of *P. thermosuccinogenes* by discriminating between mature spores, forespores, dark and bright phase endospores, and vegetative cells populations. Henceforth, this methodology can be applied to further study sporulation dynamics and its impact on fermentation performance and product formation by *P. thermosuccinogenes*.

1. Introduction

Microbial population heterogeneity is an outcome from genetic diversity of the population and/or phenotypic variance among isogenic cells. Population diversity results from the response of cells to their environment, microbial interactions, or processes such as cell division or gene regulation. Recent developments showed the existence and relevance of cellular heterogeneity leading to research on single microbial cell analysis [1–4]. Therefore, single-cell analysis techniques focused on subpopulations will advance the understanding of the bacterial population dynamics.

The genus clostridia are obligate anaerobe, gram-positive, sporulating firmicutes that are phylogenetically diverse. These species are important to human or animal health as well as related to the industrial production of bio-based products [5]. Pathogenic species such as *Clostridium difficile*, *Clostridium tetani*, *Clostridium perfringens*, and *Clostridium botulinum* produces bacterial toxins [6]. The cellulolytic and solventogenic species such as *Clostridium saccharobutylicum*, *Clostridium cellulolyticum*, *Hungateiclostridium thermocellum*, *Clostridium acetobutylicum, Clostridium beijerinckii and Clostridium pasteurianum* have the potential for sustainable biofuel or biochemical production [7–10]. *Pseudoclostridium thermosuccinogenes* is the only known thermophilic spore-forming bacteria that ferments sugar to succinate. Anaerobic thermophilic production of succinic acid may become an attractive green alternative to the existing chemical processes [11,12]. Furthermore, other nontoxigenic, proteolytic species such as *Clostridium sporogenes* and *Clostridium novyi*, serve as chemotherapeutic vehicles for intravenous delivery of spores, to treat solid tumors [13].

Sporulation is common to all clostridia, but knowledge on its mode of action and regulation at molecular and cellular level is still limited. The sporulation process of clostridia is studied extensively for several industrial applications using their enzymes and metabolites impacting product formation and their use in the medical field for drug delivery [14,15]. Sporulation can be linked to relevant

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characteristics such as enterotoxin production in *C. perfringens* and *C. botulinum* [16,17] or solvent production in *C. acetobutylicum* [18] and *C. beijereinkii* [19], respectively. *C. sporogenes* and *C. novyi* spores are administered as therapeutics [13].

Sporulation in clostridial species can be studied with single-cell analysis techniques including Fourier transform infrared spectroscopy, Raman spectroscopy coupled with microscopy, and electro-optical measurement techniques [13,20–22]. However, these techniques need custom-built equipment, lack the option for high-throughput analysis and are unable to quantify bulk population heterogeneity with different phenotypes. Flow cytometry (FCM), potentially combined with fluorescence activated cell sorting (FACS), is ideal to characterize microbes on single cell level. It is a quick method to obtain multi-parametric data of individual cells from thousands of them within a sample [23]. Light scattering (LS) measurements such as Forward-scatter (FSC) offer data on the cell size, whereas side-scatter (SSC) is linked to diffraction of light based on cellular content and granularity [1,24]. Moreover, fluorescent dyes can provide insights into other cell properties such as viability or physiological state of cells [25–27]. For example, *Bacillus licheniformis* spores were differentiated from viable cells based on size and fluorescence using the SYBR green I dye [28]. Recently, automation of FCM method in combination with SYBR dye has enabled fast quantification and separation of *B. subtilis* sub-populations [29]. Several other applications of FCM include discrimination of microbial species in co-cultures, viability assays of bacteria and yeasts, studies on sporulation cycles, metabolic activity and membrane integrity [2,3,23,30–32].

Here we report the development of FCM methods for investigation of sporulation aspects of anaerobic thermophilic clostridia exemplified by *P. thermosuccinogenes*. Knowledge on the initiation and dynamics of the sporulation process may provide clues to further optimize succinate production. LS parameter (SSC-H) and cFDA/PI dyes were evaluated to discriminate cellular morphologies and viability. Microscopy and heat inactivation verified the identities of the sorted populations of mature spores, dark and bright phase endospores, forespores and vegetative cells of *P. thermosuccinogenes*. This paves the way for possible use of the described approach in monitoring industrial fermentations and to link sporulation dynamics to metabolite production.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. thermosuccinogenes DSM 5809 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *P. thermosuccinogenes* was grown anaerobically in CP medium adapted from Plugge, 2005. The final volume of 50 mL medium was dispensed in serum bottles under 80:20 N_2/CO_2 atmosphere with ~70 kPa overpressure and then autoclaved. Glucose as a carbon source was autoclaved separately and added later to a final concentration of 5.0 g/L [33].

2.2. Phase contrast and fluorescence microscopy

Phase contrast and fluorescence microscopy (Olympus BX51) were used to determine the morphological status of cells and their staining patterns. Bacterial cells were observed by phase contrast microscopy at 400x and 1000× magnifications. Counting of spores was performed with 400× magnification with oil immersion lens.

2.3. Transmission Electron Microscopy (TEM)

A suspension of spores of each strain was pelleted for 2 min at 10,000 rpm and the spores were suspended and fixed in 4% (v/v) gluteraldehyde (EMS, Washington D.C., USA) buffered at pH 7.2 with 0.1 M phosphate buffer for 18 h at 4 °C. After three times washing with buffer (20 min), the samples were post-fixed for 64 h at 4 °C with 1% (w/v) OsO_4 (EMS, Washington D.C., USA) in 0.1 M phosphate buffer pH 7.2. Next, the samples were washed 5 times (10 min) with dH_2O and dehydrated in a graded ethanol series (10, 30, 50, 70, 90 and 100% ethanol) 10 min per step. Between each step the spore samples were pelleted for 2 min at 10,000 rpm and vortexed again. Subsequently, the samples were infiltrated with quetol resin (EMS, Washington D.C., USA) by adding small amounts of resin during 6 h after which the quetol concentration was raised till 65%. Finally, for three days and by three changes of 100% quetol the samples were fully infiltrated with quetol resin. Before each refreshment the samples were centrifuged for 3 min at 14,000 rpm and the pelleted spores were resuspended in fresh quetol mixture. Hereafter a few droplets of quetol containing spores were put in conical tip BEEMTM capsules (EMS, Washington D.C., USA), covered with fresh quetol resin, centrifuged for 3 min at 14,000 rpm and polymerized in an embedding oven (Agar scientific, B702, England) for 48 h at 60 °C. Ultra-thin sections (80 nm) were made with an ultra-microtome (Reichert Ultra Cut S using a diamond knife. During sectioning, 1 μm distance was left between the sectioned areas. The sections were examined with a TEM (JEM 1011 Jeol, Tokyo, Japan).

2.4. Spore suspension preparation

P. thermosuccinogenes DSM 5809 was grown anaerobically at 60 °C overnight in CP medium. In order to trigger sporulation, 1 mL of pre-culture was added to sporulation medium adapted from Yang et al., 2009 and B Mearls et al., 2012 [34,35], cultured at 55 °C for 2 days. The Modified AEA Sporulation Medium Base (catalogue number 17170, Sigma Aldrich) was prepared and 30 mL dispensed into anaerobic bottles under 80:20 N_2/CO_2 with ~70 kPa overpressure and then autoclaved. After autoclavation the medium was supplemented with 4% (v/v) of glucose solution, freshly prepared and filter sterilized 1.33% (v/v) of sodium carbonate (Na_2CO_3) and 1.33% (v/v) cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), respectively. Then, 1.33% v/v of freshly prepared sodium ascorbate solution ($C_6H_7O_6Na$) was added. 1 mL of the pre-culture was added to the sporulation medium and incubated at 55 °C. Once spores were formed 1 mL of pre-culture from sporulation medium was inoculated into 50 mL CP medium and grown for 2 days at 60 °C. When mature spores were highly predominant, spore suspension was harvested aerobically by centrifugation at 4700 rpm for 15 min. Cell pellets were resuspended, gently layered on 50% Histodenz solution and centrifuged at 3000 g, 20 °C for 60 min. Spore pellet was collected and resuspended in 15 mL Milli-Q water. Centrifuged at 1500 rpm, 20 °C for 30 min and supernatant was discarded. Mature spores pellets were resuspended in 10 mL Milli-Q water. 1 mL aliquots of mature spores were stored at −80 °C.

2.5. Flow cytometry and FACS analysis

P. thermosuccinogenes was grown anaerobically in CP medium and 1 mL culture were collected at different time points (0, 5, 20 h). Cells were collected and immediately washed twice for 1 min, 1000 rpm with sterile, microfiltered (0.22 μm) 0.9% NaCl solution. Aliquots of 200 μL cells were stained with either cFDA (22 μM)/PI (15 μM) or Syto-9/PI (100 μM) combination of dyes. Samples were kept at room temperature in the dark and incubated for 10 min 20 μL of sample were diluted in 2 mL of ultrapure H_2O and immediately analysed in FCM. This protocol was adapted from Kolek et al., 2016 [36]. For the FCM, 10,000 events were analysed. FSC, SSC,

green (FL1; 515–565 nm) and red (FL3; >605 nm) fluorescence were measured using an Accuri C6 cytometer (BD Accuri Cytometer Inc., USA). It is equipped with 20 mW, 488 nm, Solid State Blue Laser. FSC and SSC characteristics used as trigger signal. Samples are stained at close range to the flow cytometer and analysed immediately following incubaton. For the FACS analysis the same sample preparation was followed. We used a FACSAria III (BD Biosciences, USA) with lasers 488 nm for FSC/SSC and cFDA/PI double staining (502 LP with 530/30 filter; 488 nm and 600 LP with 610/20 filter; 561 nm). Pre-selection of data occurred using FSC and SSC signals and removed background noise by gating of bacterial populations. For FSC/SSC signals both Height and Area parameters can be used for scatter properties, but we observed that the Height provided superior separation over the Area for *P. thermosuccinogenes*. For the other fluorescent signals (cFDA, PI) we used the pulse Area parameter. Finally, 50,000 events were collected, and analysis was done using software FlowJO 10 (FLOWJO, LLC and BD Biosciences, USA; https://www.flowjo.com/solutions/flowjo). The sorted-out populations (A1, A2, A3 and A4) were observed under microscope and further heat treated at 85 °C for 15 min to verify the expected populations.

3. Results and discussion

3.1. Microscopic characterization of P. thermosuccinogenes

P. thermosuccinogenes, is a thermophilic, anaerobic and spore-forming bacterium. The genome of *P. thermosuccinogenes* DSM 5809 was sequenced by Illumina shotgun sequencing [11]. The potential sporulation proteins of *P. thermosuccinogenes* was revealed based on bioinformatic analysis with the best blastp hits as described in Table S1, Supplementary data. These proteins were selected based on sporulation and germination protein data obtained from other clostridia and bacilli [37,38]. To further characterize *P. thermosuccinogenes*, we performed phase contrast microscopy and Transmission Electron Microscopy (TEM) of cultures in the late exponential phase (20 h). Microscopic analysis showed that *P. thermosuccinogenes* DSM 5809 exhibited diverse populations, including a) vegetative cells, b) bright phase endospores, c) dark phase endospores and d) mature free spores (data not shown). The morphology of the vegetative cells and mature spores were evaluated with TEM (Fig. 1B). Thin sections of fixed spores showed a highly structured, multi-layered surface as also indicated by Drent et al. [39]. The rigid unique structural layers of the spores provide resistance to these organisms against adverse conditions [40]. The innermost spore part, called the spore core (Fig. 1A) comprises the spore DNA, RNA and other enzymes [40]. The core is enclosed by an inner membrane protein with similar phospholipid composition like vegetative cells [41]. This membrane safeguards the core DNA from damaging chemicals [42]. Adjacent to the spore's inner membrane in *P. thermosuccinogenes* is the germ cell wall, composed of a peptidoglycan layer and at a later stage the cell wall of the bacterium. The peptidoglycan layer is surrounded by a spore cortex derived from the mother cell. This protects the spore against cellular cortex hydrolases but confers no resistance properties. The surrounding spore cortex has a proteinaceous coat that prevent spore lysis by protozoal enzymes [42–45]. The spore coat is the outer layer in spore-formers such as *Bacillus subtilis*. In contrast, some pathogenic and thermophilic species like *Bacillus anthracis*, *Bacillus cereus*, *C. difficile*, *H. thermocellum* and *P. thermosuccinogenes*, the exosporium (Fig. 1C) is the outermost layer [34,46–50]. The spore structure was defined based on similarity to pathogenic strain of *C. difficile* [48,51].

3.2. Different fluorescence dyes for detecting the culture heterogenity

In earlier studies the discrimination between free spores, endospores, viable and damaged/dead cells within a sample has been done using fluorochromes, based on respiratory activity, membrane integrity, enzymatic action and DNA/RNA content. These assays have been used extensively as a viability marker for several bacterial species, which differentiates live and damaged/dead cells or spores under limiting conditions [26,52–54]. For detecting cell viability and sporulation heterogeneity, *P. thermosuccinogenes* DSM 5809 was grown in CP medium starting from spore suspension (t0). Samples were taken at 5 and 20 h referred as t5 and t20, respectively. The population heterogeneity of the strain was analysed using combinations of two dyes either Carboxyfluorescein diacetate (cFDA)/Propidium Iodide (PI) or Syto9/PI dyes. Application of the cFDA/PI dyes enabled the division of *P. thermosuccinogenes* DSM 5809 into different populations with overlapping areas. The predicted populations based on microscopy and uptake of cFDA/PI dyes were grouped as mature spores (H3), endospores (H2) and vegetative cells (H1) as differentiated by BD Accuri C6 flow cytometry (Supplementary data, Fig. S1). At stationary phase, a clear extra population appeared suggesting dead/damaged cells. Patakova et al. discussed the double staining (PI and cFDA)

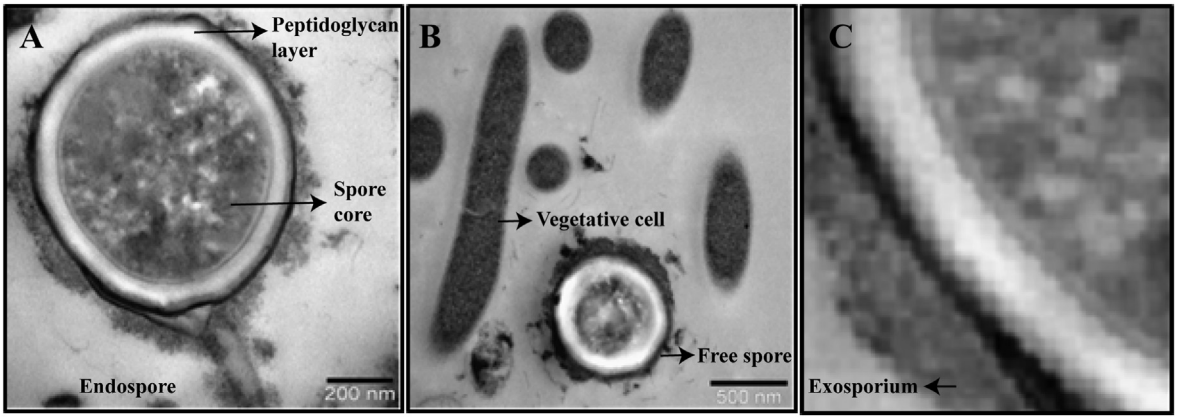

Fig. 1. Morphology of vegetative and sporulating cells of *P. thermosuccinogenes* DSM 5809 grown in CP medium for 20 h. Transmission Electron Microscopy (TEM) pictures of A) Endospore-containing cells with spore core and peptidoglycan layer; B) Vegetative cell and free mature spores; C) Ultrastructural morphology of spore with the exosporium. The spore structure was described based on similarity to *C. difficile* [48,51].

technique, suitable for assessing the number of non-sporulating vegetative cells during exponential phase of *C. beijerinckii* and *C. tetanomorphum* and concluded no distinct relation between cFDA/PI staining and morphologically diverse cell forms could be obtained [55]. Another study displayed different morphological states of *C. pasteurianum* and *C. beijerinckii* inclusive of active cells, doubly stained cells, damaged cells and spores were discriminated based on combination of light scattering and fluorescent staining (PI/cFDA) [36]. Successful application of the similar methodology was done for phenotypic characterization of C. *beijerinckii* NRRL B-598 strains induced by different culture conditions [56]. Likewise, for *P. thermosuccinogenes* our initial results of flow cytometry hinted at better separation of the sporulating populations by cFDA/PI double staining. Conversely, when a combination of Syto9 and PI dyes was used no clear separation of populations was observed, conceivably due to unspecific binding and/or interference of signals between Syto9 and PI dyes. Therefore, we selected the combination of cFDA/PI dyes as an activity/viability marker to distinguish different populations including metabolically active vegetative cells and mature dormant spores.

3.3. cFDA/PI double staining allows identification of *P. thermosuccinogenes* phenotypes at different stages of sporulation

P. thermosuccinogenes DSM 5809 was cultured from a spore suspension to synchronize growth. Three different time points of cultivation were analysed from pure spores at the start (t0), early growth stages (t5) and late exponential phase (t20). These samples were then examined by flow cytometry analysis (FACSAria III, BD Biosciences, USA) for their LS characteristics including FSC-H and SSC-H in combination with cFDA and PI double staining. FSC is the scattering of light axial to the laser and is mostly due to light diffraction around the cell. The intensity of the signal obtained via photodiode can often be used as an indicator of particle size. SSC is perpendicular light scattering measured in an orthogonal direction to the laser showing particle properties related to cell morphology, cellular content and granularity [57]. The bacterial vegetative cell and spore populations were gated to make a clear separation from cell debris. Notably, the spores could be separated from cell debris, based on their increased intensity in the FSC-H plot as shown in Fig. 2A (IV). Inside the gated region for FSC-H/SSC-H plot (I) and cFDA/FSC-H (IV), the first time point t0 containing a pure spore suspension showed a high FSC-H, minor side scattering and no cFDA fluorescence. Notably, cFDA/PI dyes stained vegetative cells but not spores as shown in SSC-H/cFDA (II) and SSC-H/PI (III) plots (Fig. 2A), in line with previous results from Kolek et al. [36]. Therefore this methodology offering options for discrimination of sporulation heterogeneity of *P. thermosuccinogenes*.

At t5 a shift along the SSC-H axis for FSC-H/SSC-H (I) was observed in Fig. 2B. The SSC-H higher intensity illustrates complexity or altered diffraction of light of the cell in comparison to spores. Fig. 2B displays the SSC-H/cFDA plot (II) with three divergent populations and two subpopulations. This includes a 1) lower region: cFDA low/SSC-H low with another extended subpopulation (high SSC-H), 2) intermediate region: SSC-H middle/cFDA middle 3) upper region: SSC-H high/cFDA high involves overlapping metabolically active subpopulations, respectively. The SSC-H/PI (III) plot presents unstained PI populations together with an extra PI stained population containing dead/damaged cells or spores. FSC-H/cFDA (IV) showed three cFDA based populations without any FSC subpopulations. The mature free spore population was predicted based on their increased autofluorescence. These spores had lower signal intensities than stained cells but greater than debris or non-stained vegetative cells. The exact mechanism for spore autofluorescence is unknown, but it could be due to the composition of spore coat or its oxidase or peroxidase activity [58]. Spore autofluorescence as a sorting condition was used for *Bacillus* spores [59] or via a combined strategy of LS and fluorescence staining discriminated *Paenibacillus* spores from vegetative cells [60].

Furthermore, after cultivating *P. thermosuccinogenes* DSM 5809 for 20 h (t20, Fig. 2C) the various populations were better defined compared to the pattern seen at t5 (Fig. 2B). An extra population appeared with high FSC-H/SSC-H (Fig. 2C, I) revealing longer cells and significantly different diffraction of light showing changed cell complexity. FSC-H/cFDA (IV) stain showed the same five major populations composed of metabolically active/inactive cells of different sizes or intensities in their signals. This will be further discussed in details in the next section. Therefore, all populations reflecting the different cell morphologies and stages during sporulation of *P. thermosuccinogenes* DSM 5809 were identified.

3.4. FACS analysis for detailed insights on various populations of *P. thermosuccinogenes*

Analysis of the spore populations during late exponential phase provided insights into population diversity with the best separation using SSC-H/cFDA contour plots (Fig. 3, main plot). The FSC-H/SSC-H plot showed overlapping populations based on both size and LS effects. In this case, LS only does not allow differentiation of spores, vegetative cells and other populations. Thus, it was necessary to use the metabolic dye cFDA adding a new dimension to the analysis. SSC-H/cFDA main plot revealed five populations with overlapping regions. To characterize these groupings we decided to sort them using FACSAria III. We also compared this main plot with other subplots FSC-H/cFDA (I), FSC-H/PI (II) and SSC-H/FSC-H (III), respectively, to get a correlation between them. The various distinguished populations were as follows (Fig. 3, main plot):

A1 - SSC-H low/CFDA high: The uppermost left corner was shown to represent vegetative cells with high metabolic activity as shown in Fig. 3 (main plot A1). We sorted one million cells, observed them using phase contrast microscopy and heat-treated them to select for the presence of spores. This resulted in killing of all cells confirming it to be a vegetative cell population absent of spores. The subplots FSC-H/cFDA highlighted the A1 (I) region with high cFDA uptake. The absence of PI staining (subplot, A1 (II)) for this population indicated active cells with intact membranes.

A2 - SSC-H high/CFDA high (overlap area): Two overlapping populations based on the refractive index of light due to similar SSC properties were identified as shown in Fig. 3 (main plot A2). Interestingly, the sorted populations depicts dark/bright phase endopores and forespores, confirmed by microscopy. The main plot SSC-H/cFDA gave a better separation of overlaying populations between forespores and vegetative cells when compared to FSC-H/cFDA (Fig. 3, subplot A1 (I)). This shows no significant size differences but alterations in light diffraction due to cellular complexity. Moreover, this specific population apart from the dormant and germinating spores had (partial) outgrowth after heat inactivation. Partial due to the existence of spores that are still in the germination process and did not acquire (complete) heat resistance yet.

A3 - SSC-H middle/CFDA middle: The middle area when sorted displayed populations consisting of germinating spores (Fig. 3, main plot A3). We observed under the phase contrast microscope germinating dark swollen spores slightly stained with cFDA dye. They are similar to spores based on size with no shift in FSC-H but move in SSC-H axis to the mid region (Supplementary data, Fig. S2, subplot A3 (I)).

A4 - SSC-H low/CFDA low: This single population resembles the starting spore suspension with mature free spores which are dormant (Fig. 3, main plot A4). Particles do not stain with cFDA or PI and are smaller in size than the endospores. Previous studies

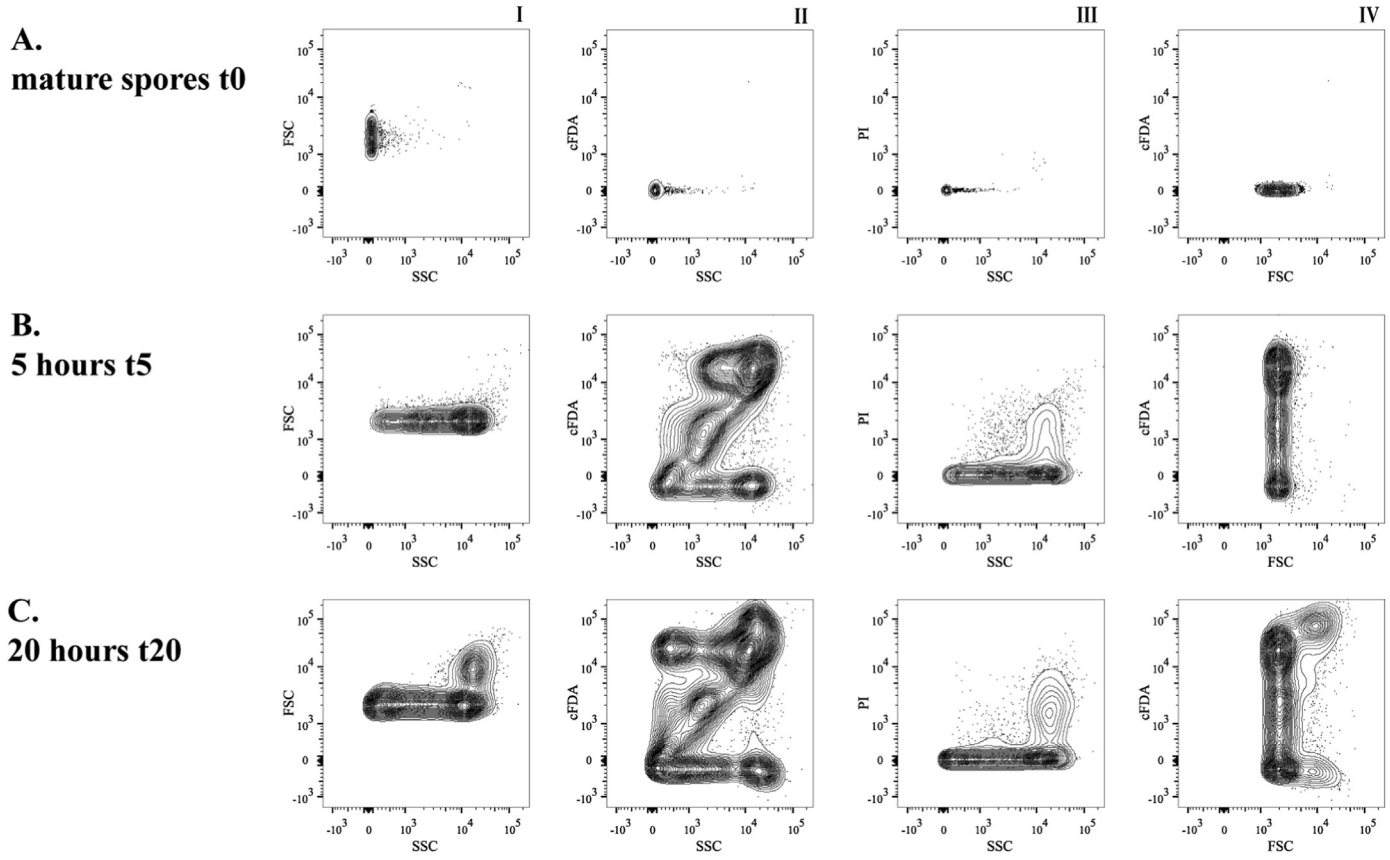

Fig. 2. LS (FSC-H/SSC-H) investigation in comparison to cFDA/PI dyes at start t0 (0 h), early t5 (5 h) and late exponential phase t20 (20 h) cultures of *P. thermosuccinogenes* DSM 5809. Contour plots FSC/SSC (I), SSC/cFDA (II), SSC/PI (III) and FSC/cFDA (IV) show population heterogeneity starting from pure spore suspension to five different populations at three time points (t0, t5 and t20). A) pure spore suspension (t0): high FSC-H scattering distinguishes from cell debris, minor shift in SSC-H axis and no uptake of cFDA or PI dyes; B) t5: SSC/cFDA (II) plot showed three divergent populations and two subpopulations in contrast to FSC-H (I and IV) plots which lack two populations. The SSC-H/PI (III) plot shows an additional population containing dead/damaged cells or spores and C) t20: SSC/cFDA (II) and FSC-H/cFDA (IV) plots displayed better defined populations (metabolically active/inactive cells and spores) compared to t5 time point. Another population appeared with high FSC-H/SSC-H (I) revealing longer complex form of cells probably endopores/forespores.

showed similar outcomes with mature free spores stained with cFDA/PI to understand culture heterogeneity in *C. beijerinckii* NCIMB 8052 and *C. pasteurianum* NRRL B-598 during solvent production [36].

A5 - SSC-H high/CFDA low: The last population in the rightmost lower corner which are extending from the mature spores has two overlying areas as depicted in Fig. 3 (main plot, A5). It has no cFDA staining indicating the presence of inactive cells (Supplementary data, Fig. S2, subplot A5 (I)). One of the populations is PI stained showing dead or damaged cells with high side and forward scatter (Supplementary data, Fig. S2, subplot A5 (II)). We could not obtain clear microscopical conformation of this population due to its low population size resulting in low sort counts. Based on the parameters, we assume it is a mixed population of dead/damaged cells and spores.

Finally, to give a supporting overview FSC-H/cFDA and SSC-H/FSC-H contour plots were obtained by overlaying the five individual populations (Supplementary data, Fig. S3). Looking at the FSC-H/cFDA plot A1 (red) and A2 (light blue), containing cells versus forespores/endospores, largely overlap. Furthermore, A4 (light green) partially overlaps with A5 (dark green) subpopulations based on FSC-H. For SSC-H/FSC-H plot does not provide a better representation as the A1 population is partially hidden due to overlap with A4 population. Therefore, when these two FSC based plots were compared to SSC-H/cFDA, we concluded that the highest resolving power for all the diverse populations was observed with the latter one. Hence, these results showcase the use of flow cytometry using that a SSC-H/cFDA parameter acts as a potential tool to effectively assess the complex culture heterogeneity of thermophilic clostridia.

4. Conclusions

Our study using succinate producer *P. thermosuccinogenes* as a model, shows that FCM can be an efficient and quick method for assessment of population heterogeneity and sporulation dynamics of anaerobic spore-forming bacteria. The optimized flow cytometry-based method adapted from Kolek et al. [36] was applied in combination with SSC-H/cFDA parameters to quantify culture heterogeneity of *P. thermosuccinogenes*, discriminating mature spores, dark and bright phase endospores, forespores and vegetative cells. It can be applied to study sporulation dynamics during the fermentation, optionally after sorting, to determine physiological parameters that may provide leads for further optimization of the process.

Declaration of competing interest

The authors declare no conflict of interest.

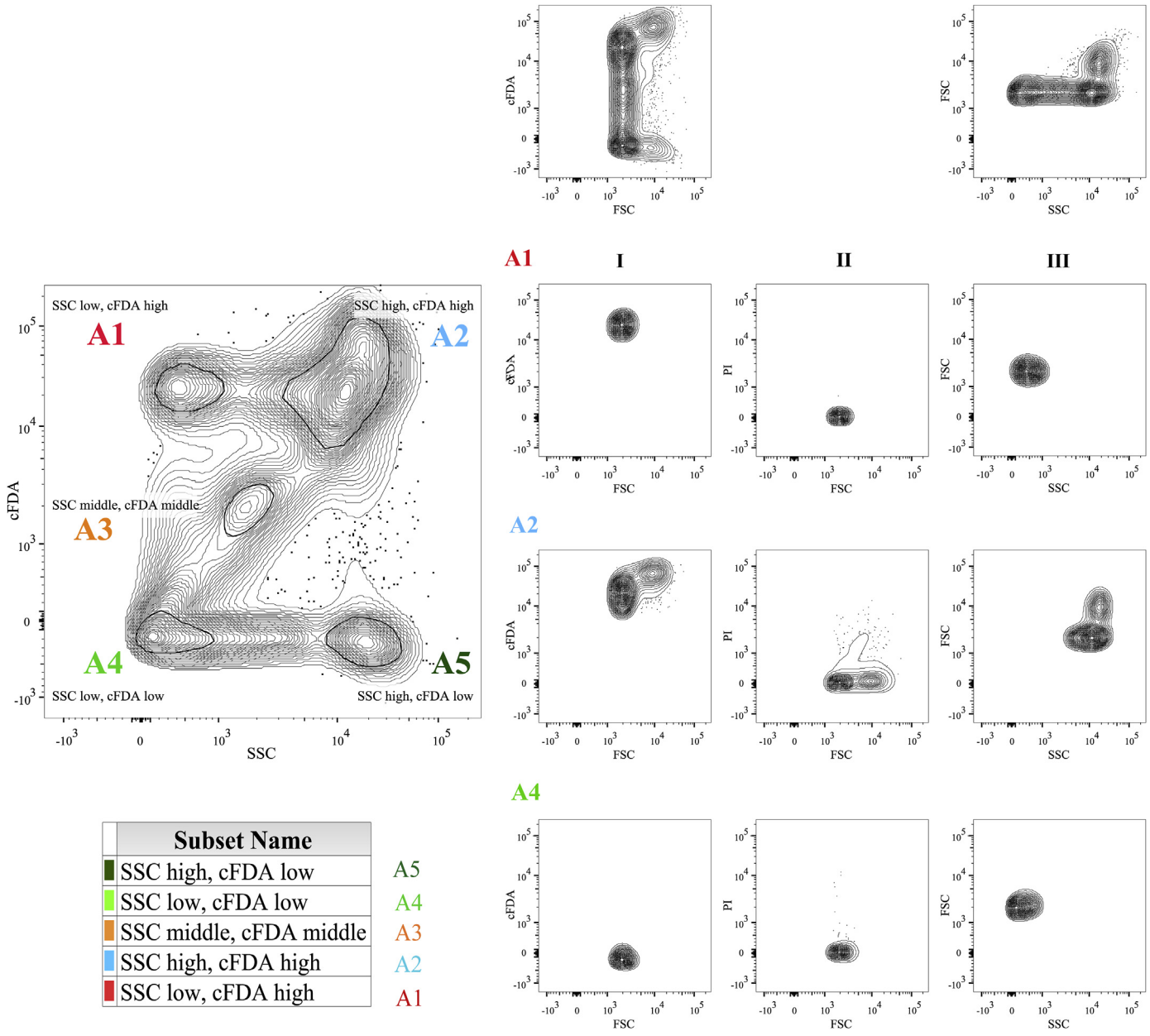

Fig. 3. Detailed insights of LS investigation with cFDA/PI staining for late exponential phase cultures of *P. thermosuccinogenes* DSM 5809. Populations were sorted based on the combination of SSC-H and cFDA staining characteristics. Five distinct populations were sorted by FACS in the main plot: A1 - SSC-H low/cFDA high, vegetative cells; A2 - SSC-H high/cFDA high, endospores and forespores; A3 - SSC-H middle/cFDA middle, germinating spores; A4 - SSC-H low/cFDA low, mature free spores and A5 - SSC-H high/cFDA low, dead or damaged spores. SSC-H and cFDA plot were correlated with cFDA/FSC-H (I), PI/FSC-H (II) and SSC-H/FSC-H (III) contour subplots to get a clear picture of major morphologies of each sorted population. *P. thermosuccinogenes* DSM 5809 was grown in CP medium at 60 °C for 20 h. At t20 samples were sorted and analysed using a FACSAria III, BD Biosciences, USA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2020.102208.

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