Characterization of *Bacillus subtilis* sporulation and bacteriophage infection via FT-IR spectroscopy

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Abstract. A comparative analysis on cultures from wild-type and sporulation mutants of *Bacillus subtilis* infected with pseudotemperate spore-converting bacteriophages was performed to discern differences in the chemical functionality of the cellular population. Variations in the cell surface chemistry of *B. subtilis* cultures were analyzed using attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectroscopy of dried sample films on optical plates. Comparison of wild-type and phage infected cultures revealed an increase in the amount of aliphatic ester in the infected populations. Spectral analysis of cultures from bacteriophage infected sporulation mutants, *spo0K* and *spo0J* revealed varying levels of ester linkages.

Keywords: *Bacillus subtilis*, FT-IR spectroscopy, sporulation, cell surface composition, bacteriophages

1. Introduction

Bacillus subtilis is an aerobic endospore forming bacterium found in diverse environments [1]. The gram-positive bacterium inhabits soil and participates in the metabolic breakdown of organic matter such as plant and animal debris [2]. *B. subtilis* can undergo three separate but genetically and environmentally linked developmental pathways. *B. subtilis* cells actively grow and metabolize during conditions where nutrient availability is favorable. When nutrients become depleted cells enter into a nonproliferative reversible dormant state, sporulation. When available energy levels are too low to proceed through the sporulation pathway *Bacillus* will enter into a salvage pathway which allows cells a final opportunity to survive until conditions exist that will enable them to enter into either the sporulation or growth pathway. Developmental pathways of *B. subtilis* have been used as a model system to identify changes in prokaryotic gene expression and to study cellular differentiation in response to various environmental stimuli [3].

Spore formation in *B. subtilis* is the result of the differential expression of more than 100 genes. Initiation of this process is controlled by more than a dozen genes including: *spo0A*, *spo0B*, *spo0E*, *Spo0F*, *spo0H*, *spo0J*, *soj* and *spo0K* [4,5]. Sequence data and biochemical studies have identified functions for many of these initiation genes. For example, functional studies have suggested that *spo0H* encodes a sigma factor of RNA polymerase that regulates the expression of early sporulation genes responsible for sensing environmental conditions [6]. The *spo0K* locus contains an operon of five genes that is

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homologous to the oligopeptide permease (*opp*) operon of *Salmonella typhimurium* and plays a role in the development of genetic competence and the initiation of sporulation [7]. Mutations in this operon show defects in sensing extracellular peptides required for the initiation of sporulation and alterations in ability to undergo genetic competence.

Recent data has identified Spo0A, Spo0J and Soj as players that control a key checkpoint for the initiation of sporulation. Sequence data and functional studies have shown that Spo0J is a member of the ParB family of partitioning proteins that bind to specific DNA sequences called *parS* sites that are located near the initiation of chromosomal replication [8,9]. Soj is a member of the ParA family of partitioning proteins and is thought to bind ATP [8,9]. Soj was originally identified as a suppressor of *spo0J* mutations [10]. Studies have suggested that Soj regulates the initiation of sporulation by controlling expression of *spo0A*, a key transcriptional regulator of numerous sporulation genes [8,9]. Several groups have shown using genetic and microscopic analysis that Spo0J and Soj interact to mediate a checkpoint between DNA replication and the initiation of sporulation [8,9]. Spo0J and Soj appear to be required both for chromosome partitioning during vegetative growth and for proper segregation of the chromosome to the developing spore [9,11]. Defects in *spo0J* have severe pleiotrophic effects throughout the developmental pathways of *B. subtilis* [12]. *Spo0J* mutants exhibit a higher rate of anucleated cell division, have a spore-negative phenotype and display dysregulated expression of salvage pathway enzymes [10–12].

Spore-converting bacteriophages have been isolated from several species of spore-forming bacteria, including *B. pumilus*, *B. thuringensis*, *B. subtilis* and *Clostridium perfringens* [13–17]. These bacteriophages form unstable pseudolysogenic carrier states within the host cell and induce sporulation in wild-type cells [13,18]. It has been suggested that spore-converting bacteriophages induce sporulation in wild-type cells so that the bacteriophage DNA becomes trapped within the developing spores [19,20]. When environmental conditions improve, the carrier spore germinates, and trapped bacteriophage DNA can direct lytic development in the progeny of the germinated cell. A selective advantage is provided to the bacteriophage since spores are better resistant to environmental stress than phage particles. It seems likely that spore-converting bacteriophages may have evolved to enhance sporulation so that bacteriophage genome entrapment occurs more frequently [13,21,22]. In addition, infected wild-type cells often sporulate under conditions in which uninfected cells are unable to sporulate [20,21]. This gives the infected host cell an advantage over uninfected cells in that the infected cells can sporulate under conditions where sporulation would not normally occur. In addition to increasing the frequency of wild-type sporulation, spore-converting bacteriophages significantly enhance sporulation in certain spore-negative (*Spo*[−]) mutants [12]. The *B. subtilis* bacteriophages SP10 and PMB12 suppress mutations in *spo0J* and *spo0K* causing infected mutants to sporulate at or near wild-type levels [20]. The ability of PMB12 and SP10 to convert *spo0J* and *spo0K* mutants from a spore-negative to a spore-positive phenotype suggests that the bacteriophages affect the host cell function during the initial stages of sporulation. However, the exact mechanism by which these bacteriophages convert the spore-negative phenotype to spore-positive remains unknown.

The goal of this work was to investigate the biochemical differences among cultures of wild-type and sporulation mutants of *B. subtilis* both uninfected and infected with pseudotemperate spore-converting bacteriophages. An initial hypothesis projected that infected cultures would be chemically identical to non-infected cultures based on the similarities of their phenotypes. Attenuated total reflection (ATR) Fourier transform infrared (FT-IR) spectroscopy was used to detect biochemical and physiological changes induced in cultures infected with bacteriophages. FT-IR spectroscopy has been used to examine chemical changes in *B. subtilis* spore components and demonstrates a nondestructive method to monitor cellular changes [23]. The cell surfaces of the wild type and mutant strain *B. subtilis* have been

studied by differentiating chemical changes measured by infrared absorptions [1]. The effects of mutation and phage infection on the cell cultures from the viewpoint of chemical functionality in *B. subtilis* were explored. Though phenotypically similar, the spores created from phage-infected and non-infected wild-type and mutant cultures of *B. subtilis* exhibited different cell surface chemistry.

2. Materials and methods

2.1. Culture conditions and sample preparations

2.1.1. Bacterial strains and bacteriophages

B. subtilis strains and bacteriophages used in this study are described in Table 1.

2.1.2. Culture conditions

Strains were grown on nutrient broth (NB) agar (Difco) plates for the enrichment of endospores. Percent sporulation was determined by averaging three fields of 400 $\times$ phase contrasting preparations. Liquid NB cultures were grown for 7 days at 37°C on a rotary shaker at 200 rpm. Cell numbers were determined by spectrophotometric analysis at OD_{600 nm} using a BIO-RAD SmartSpec 3000. Samples for FT-IR analysis contained 1 $\times$ 10⁹ cells per milliliter. Cultures were analyzed on days 1, 3 and 7. On sample days, 1 ml of culture was removed and centrifuged at 10,000 rpm for 1 min at 4°C. After three washes with deionized water and subsequent centrifugation, the samples were resuspended in 400 μ l of deionized water for infrared analysis.

2.1.3. Propagation of bacteriophages and cross-streak assay

SP10 and PMB12 bacteriophage lysates were prepared as previously described [13]. Lysates were stored at 4°C.

Cross streak assays were performed as previously described [20]. Briefly, 100 μ l of bacteriophage lysate was applied to agar plates down the center as a vertical band and allowed to dry. Strains were streaked horizontally across the dried lysate and incubated at 37°C for up to 7 days. Sporulation frequencies were determined by calculating the mean percentage of phase bright bodies from three separate fields in wet mounts using a Nikon E100 phase contrasting microscope, 400 $\times$.

2.1.4. DNA extraction

Cells were grown overnight in 100 ml of LB broth. After overnight incubation, cells were concentrated by centrifugation. Cells were spun at 6000 rpm for 15 min and washed by resuspending pelleted cells

Table 1
B. subtilis strains and bacteriophages

	Genotype	Phenotype ^a	Source/reference
Strains			
3-13	<i>his</i> [−]	Spo ⁺	Lovett
MGB3015	<i>his</i> [−] , <i>spo0J-lacZ</i>	Spo ^c	Bramucci
KI246	JH642 <i>spo0K chr::Tn917</i>	Spo [−]	Rudner
3-13A	<i>his</i> [−] <i>spo0K chr::Tn917</i>	Spo ^c	This study
Bacteriophages			
PMB12			Bramucci
SP10			Bott

^aSpo⁺ – wild-type sporulation phenotype, Spo^c – spore-converting phenotype, Spo[−] – spore-negative phenotype.

in 10 ml of $1 \times$ TES (20 mM Tris-HCl, pH 7.5, 5 mM EDTA and 100 mM NaCl). Washed cells were centrifuged again at 6,000 rpm for 15 min. Pelleted cells were resuspended in 10 $1 \times$ TES that contained 200 μ g/ml lysozyme. Treated cells were incubated at 37°C for 30 min. After 30 min, 0.5 ml of RNase (50 μ g/ml) was added and the cells were incubated for an additional 30 min. Finally, 0.6 ml of a 20% SDS solution was added and the sample was incubated at 65°C for 30 min. After the final incubation, the sample volume was adjusted to 20 ml with additional $1 \times$ TES and extracted three times in phenol (equilibrated with Tris-HCl, pH 8.0), phenol/chloroform (24 : 1 chloroform : isoamyl alcohol) and then chloroform. DNA was precipitated after the final extraction with the addition of 2 volumes of 100% ethanol. DNA was isolated as a pellet by centrifugation at 3000 rpm for 5 min at 4°C. Pelleted DNA was washed in 70% ethanol, dried under house vacuum. Dried DNA was resuspended in 1 ml of $1 \times$ TE (10 mM Tris-HCl, pH 8.0 and 50 mM EDTA) and stored at 4°C.

2.1.5. Transformation of *B. subtilis*

Recipient cells were grown overnight in 10 ml of NB. Overnight cultures were diluted 1 : 40 in Spizizen Minimal Glucose media according to the method of Anagnostopoulos [24] and modified according to the method of Bott [25]. Cells became competent between one and two hours after entering stationary phase. Donor DNA (100 μ g DNA) was added to 0.9 ml of competent cells. Transformation mixtures were incubated for 30 min at 37°C with shaking (50 rpm). After 30 min, cells were centrifuged, resuspended in 1 ml of LB with appropriate antibiotics and allowed to recover and express antibiotic genes at 37°C with shaking (200 rpm). After 1 h, 0.1 ml of the transformed cell culture was plated onto LB with appropriate antibiotic selection and incubated at 37°C for 24–48 h. Transformants were assessed for antibiotic resistance and sporulation defects.

2.2. Infrared spectroscopy

For the absorption analysis, the resuspended cultures were deposited onto the entire surface of zinc selenide crystal optical plates. Plates were dried at 60°C for 20 min. Dried plates were visually inspected to ensure uniform biomass distribution. Uneven biomass distribution may result in inconsistent absorption of signal [26]. The air-dried biomass was examined at room temperature via ATR FT-IR spectroscopy using a Nicolet 560 spectrometer over a wavelength range of 4000–800 cm^{-1} . A spectral resolution of 4 cm^{-1} and 128 acquisitions were collected for each spectrum. Thermo Scientific GRAMS/AI version 8 spectral software was used to analyze and compare spectra.

The IR chamber was purged with dry nitrogen for at least 20 minutes prior to the first analysis and during the collection of all spectra. Nitrogen purge minimized spectral absorptions from both CO_2 and water. Backgrounds of dried deionized water were acquired for each of five optical plates in order to provide plate specific background data. Analysis of samples not cross-referenced to respective background plates greatly affected absorption patterns.

3. Results and discussion

Strains of *B. subtilis* were analyzed using FT-IR spectroscopy to compare surface chemical functionality under a variety of culture conditions. An initial comparison of different culture media was made to differentiate the effect of nutrient levels on growth. Samples from cultures of wild-type 3-13 grown in NB and LB media produced the FT-IR spectra comparison in Fig. 1. Absorption band assignments for various functional groups can be referenced in FT-IR studies of differently cultivated *B. subtilis* by

Fig. 1. Comparative FT-IR spectra of wild-type *B. subtilis* 3-13 cultures in NB and LB media (A) day 1 and (B) day 3 of growth in NB media, (C) day 1 and (D) day 3 of growth in LB media.

Chueng et al. [23] and Filip et al. [1]. The absorption peaks in the spectral region between 900 and 1800 cm^{-1} in these two articles are similar to the spectra observed in this study.

B. subtilis grown in NB or LB at different stages of growth were observed to be chemically equivalent by FT-IR analysis. The differences observed in the spectra in Fig. 1 were in the absorption intensities not changes in spectral wavelengths. This result suggests that the observed differences in peak intensities were most likely due to differences in growth rates due to rich medium (LB) versus nutrient poor medium (NB) and not differences in the chemical functional groups on the cell surface.

To investigate possible chemical differences between infected and uninfected cultures of the wild-type 3-13, IR spectra of 3-13 along with two sporulation mutants, MGB3015 (carries a *spo0J* mutation) and 3-13A (carries a *spo0K* mutation) were compared with bacteriophage infected cultures at day 3. The comparative results of spectra for wild-type 3-13, 3-13A and MGB3015 are shown in Fig. 2.

Figure 2 revealed that the greatest difference in the IR spectra between 3-13 and the two mutants cultures was observed for absorptions at 2950 cm^{-1} (R-CH_2 stretch attributed to carbohydrates in the cell wall) and 1080 cm^{-1} (aliphatic esters and polysaccharide ring vibrations). An increase in intensity was observed at both 2950 and 1080 cm^{-1} for 3-13A. To better illustrate changes in functionality of different spectra, peak amplitude ratios relative to the amide I band at 1660 cm^{-1} were determined and presented in Table 2. Relative peak amplitude ratios ($A_{\text{absorption}}$) were calculated using the intensities of the absorptions of interest, for example $A_{\text{R-CH}_2} = \text{intensity}(2950\text{ cm}^{-1})/\text{intensity}(\text{amide I}, 1660\text{ cm}^{-1})$. Peak amplitude ratios in Table 2 comparing uninfected wild-type and mutant strains represent an increase in

Fig. 2. FT-IR spectra of *B. subtilis* comparing (A) wild-type 3-13, (B) MGB3015 and (C) 3-13A. Arrows indicate absorptions at 2950 cm^{-1} (R-CH_2 stretch attributed to carbohydrates in the cell wall) and 1080 cm^{-1} (aliphatic esters and polysaccharide ring vibrations). Noise attributed to atmospheric water is observed in the ranges of $4000\text{--}3500\text{ cm}^{-1}$ and $1900\text{--}1400\text{ cm}^{-1}$.

Table 2

Peak amplitude ratios referenced to the amide I peak for R-CH_2 and ester/ring vibration signals present in the FT-IR analysis of wild-type 3-13 and mutants MGB3015 and 3-13A

	$A_{\text{R-CH}_2}$	A_{ester}		$A_{\text{R-CH}_2}$	A_{ester}		$A_{\text{R-CH}_2}$	A_{ester}
3-13	0.20	0.30	3-13A	0.32	0.56	MGB3015	0.27	0.45
3-13(PMB12)	0.29	0.83	3-13A(PMB12)	0.24	0.53	MGB3015(PMB12)	0.45	0.82
3-13(SP10)	0.24	0.86	3-13A(SP10)	0.23	0.64	MGB3015(SP10)	0.27	0.68

aliphatic character from 3-13 to MGB3015 to 3-13A. Changes in the $A_{\text{R-CH}_2}$ of the wild-type compared to the mutant cultures correlate with those calculated for the A_{ester} suggesting a complementation of structural changes on the cell surface of populations within the culture.

The FT-IR spectra in Fig. 3 revealed a chemical difference between infected and uninfected cultures. For the A_{ester} , wild-type 3-13, 3-13(PMB12), and 3-13(SP10) were 0.30, 0.83, and 0.86, respectively (Table 2). This result suggests an increase in A_{ester} in infected 3-13 as revealed by the increase in intensity at 1080 cm^{-1} compared to uninfected wild-type 3-13. However, no bacteriophage-specific differences in chemical structure were identified. The aliphatic ester or ring vibrations associated with polysaccharides may represent an increase in the presence of glycoproteins which may be associated with endospore formation.

Fig. 3. FT-IR spectra of *B. subtilis* comparing (A) 3-13(PMB12), (B) 3-13(SP10), (C) 3-13.

Strain 3-13A carries a mutation in the sporulation gene, *spo0K*. This mutation in the permease operon (*opp*) confers a spore-negative phenotype. Upon infection with PMB12 or SP10, the spore-negative mutant is converted to spore positive. A comparison of the *spo0K* mutant, 3-13A with 3-13A infected with PMB12 and 3-13A infected with SP10 identified detectable chemical differences in the aliphatic ester absorption at 1080 cm^{-1} (Fig. 4). The A_{ester} in 3-13A, 3-13A(PMB12) and 3-13A(SP10) were 0.56, 0.53 and 0.64, respectively (Table 2). Unlike in the wild-type 3-13 infection which revealed an increased intensity at the ester peak for both bacteriophage cultures, 3-13A was chemically similar to 3-13A infected with PMB12. However, 3-13A infected with SP10 had more aliphatic ester character as indicated by A_{ester} . The difference in A_{ester} between the infected cultures of 3-13A suggests that PMB12 and SP10 although phenotypically similar have differences in the molecular chemistry on the surface of the spore. An additional observation for 3-13A spectra revealed the $A_{\text{R-CH}_2}$ for infected cultures appear lower relative to the uninfected culture (Table 2).

MGB3015 carries a mutation in the *spo0J* gene giving the strain a spore-negative phenotype. Spo0J encodes a protein that has been shown to be important in chromosomal partitioning. Spo0J and Spo0K mutations are suppressed by infection with spore-converting bacteriophages.

Similar to 3-13 and 3-13A, differences between infected and uninfected cultures of MGB3015 are evident in the 1080 cm^{-1} absorption (Fig. 5). The A_{ester} in MGB3015, MGB3015 infected with PMB12 and MGB3015 infected with SP10 were 0.45, 0.82 and 0.68, respectively (Table 2). Based on the absorbance ratios MGB3015 infected with PMB12 resembles 3-13(PMB12) but not 3-13A(PMB12). Absorbance ratios of MGB3015 infected with SP10 appears more similar to the absorbance ratio of 3-13(SP10) than to 3-13A(SP10). Not only were different proportions of aliphatic ester present between the uninfected and infected cultures of MGB3015 but also between MGB3015(PMB12) and MGB3015(SP10). Another

Fig. 4. FT-IR spectra of *B. subtilis* comparing (A) 3-13A(SP10), (B) 3-13A(PMB12), (C) 3-13A.

Fig. 5. FT-IR spectra of *B. subtilis* comparing (A) MGB3015(SP10), (B) MGB3015(PMB12), (C) MGB3015.

example of the phage exerting a phenotypic difference was observed with varying expression levels of α -amylase in SP10 versus PMB12 infected cultures [20].

4. Conclusions

Several conclusions can be made from spectral comparisons among the wild-type 3-13, the mutants and their respective infections with the spore-converting bacteriophages, PMB12 and SP10. The peak ratio comparison in Table 2 revealed a chemical change in wild-type 3-13 as compared to 3-13 infected with either SP10 or PMB12, but no chemical difference based on the type of bacteriophage with which 3-13 was infected. Analysis of the peak ratios of 3-13A cultures showed no change within the A_{ester} between 3-13A and 3-13A infected with PMB12, however, a slight change between A_{ester} for 3-13A(PMB12) and 3-13A(SP10) was identified. In cultures of MGB3015 there appeared to be a change in A_{ester} between uninfected MGB3015 and both infected cultures. Unlike the 3-13 and 3-13A infected cultures, a marked difference in A_{ester} between MGB3015(PMB12) and MGB3015(SP10) was detected.

A spectral comparison of the wild type with the sporulation mutants identified an increase in the amount of A_{ester} in both the *spo0J* and *spo0K* cultures. Cultures from *spo0K* mutants, 3-13A, had a slight increase in A_{ester} compared to that of cultures from the *spo0J* mutant, MGB3015. Mutations in the *spo0J* and *spo0K* genes appear to alter the cell surface by increasing the amount of ester linkages. Cultures of 3-13A infected with bacteriophage did not exhibit an increased A_{ester} as compared to uninfected cultures. Recall this was the case in wild-type 3-13 cultures infected with bacteriophages. The amount of aliphatic ester within the cell membrane of 3-13A was not significantly affected by infection with either SP10 or PMB12. MGB3015 cultures infected with bacteriophage displayed an increase in A_{ester} but to different degrees relative to A_{ester} levels in uninfected MGB3015 cultures (Table 2). MGB3015 cultures infected with PMB12 maintained A_{ester} levels similar to wild-type 3-13 infected with PMB12. However, MGB3015 cultures infected with SP10 demonstrated a level of A_{ester} that was lower than wild-type SP10 infected cultures.

In conclusion, phenotypically similar cultures of *B. subtilis* have been shown to produce spores with chemically different surfaces based on mutation and bacteriophage infection. These chemical differences could indicate that phage infection may be somehow reprogramming or altering gene expression in the sporulation pathway of *Bacillus subtilis*. Results indicate that the bacteriophage infections exert an effect on the mutant cultures but the effect is somewhat different depending on which phage is infecting the mutant cultures. Phage infection on MGB3015 created spore populations that more closely resemble wild-type infected spores. Bacteriophage infection seems to increase levels of ester on the cell surface in wild-type and *spo0J* cultures. This increased level of ester is indicative of more polysaccharide components at the cell surface. The *spo0K* mutation in 3-13A seems to increase levels of ester on the cell surface compared to uninfected wild-type and MGB3015 cultures. However, cultures of 3-13A infected with bacteriophages do not contain significantly increased levels of ester compared to uninfected 3-13A as seen for wild-type and *spo0J* infected cultures. This result suggests that bacteriophage require a functional *spo0K* gene product in order to exert its effect of increased ester levels in infected cultures.

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