Further Characterization of *Clostridium perfringens* Small Acid Soluble Protein-4 (Ssp4) Properties and Expression

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Abstract

**Background:** *Clostridium perfringens* type A food poisoning (FP) is usually caused by *C. perfringens* type A strains that carry a chromosomal enterotoxin gene (*cpe*) and produce spores with exceptional resistance against heat and nitrates. Previous studies showed that the extreme resistance of spores made by most FP strains is mediated, in large part, by a variant of small acid soluble protein 4 (Ssp4) that has Asp at residue 36; in contrast, the sensitive spores made by other *C. perfringens* type A isolates contain an Ssp4 variant with Gly at residue 36.

**Methodology/Principal Findings:** The current study has further characterized Ssp4 properties and expression. Spores made by *cpe*-positive type C and D strains were found to contain the Ssp4 variant with Gly at residue 36 and were shown to be heat- and nitrite-sensitive; this finding may help to explain why *cpe*-positive type C and D isolates rarely cause food poisoning. Saturation mutagenesis indicated that both amino acid size and charge at Ssp4 residue 36 are important for DNA binding and for spore resistance. *C. perfringens* Ssp2 was shown to bind preferentially to GC-rich DNA on gel-shift assays, while Ssp4 preferred binding to AT-rich DNA sequences. Maximal spore heat and nitrite resistance required production of all four *C. perfringens* Ssps, indicating that these Ssps act cooperatively to protect the spore’s DNA, perhaps by binding to different chromosomal sequences. The Ssp4 variant with Asp at residue 36 was also shown to facilitate exceptional spore survival at freezer and refrigerator temperatures. Finally, Ssp4 expression was shown to be dependent upon Spo0A, a master regulator.

**Conclusions/Significance:** Collectively, these results provide additional support for the importance of Ssps, particularly the Ssp4 variant with Asp at residue 36, for the extreme spore resistance phenotype that likely contributes to *C. perfringens* type A food poisoning transmission.

Introduction

*Clostridium perfringens*, a Gram-positive, anaerobic, sporeforming bacterium, can produce at least 17 different toxins. However, individual *C. perfringens* strains never produce this entire toxin repertoire. A commonly used system [1] exploits this variability in toxin production to classify individual *C. perfringens* isolates into types A–E, based upon their production of four typing toxins (alpha, beta, epsilon and iota toxins).

About 1–5% of type A isolates produce another toxin, named *C. perfringens* enterotoxin (CPE), which is responsible for causing the gastrointestinal (GI) symptoms of *C. perfringens* type A FP [2]. This FP currently ranks as the second most commonly reported bacterial foodborne disease in the USA and UK [3,4], where (respectively) over 250,000 or 85,000 cases occur annually. Those cases usually resolve without long-term consequence, but *C. perfringens* type A FP can be fatal in the elderly or debilitated individuals. Consequently, *C. perfringens* ranks among the three or four most common bacterial causes of foodborne death [1,3,4]. In addition to FP, CPE-positive type A *C. perfringens* strains also cause about 5–15% of all cases of nonfoodborne human gastrointestinal diseases, including sporadic diarrhea and antibiotic-associated diarrhea [1,2].

In type A isolates, the gene (*cpe*) encoding CPE can be either chromosomal or plasmid-borne [5,6]. Most (~75%) FP cases are caused by type A isolates carrying a chromosomal *cpe* gene (C-cpe) [5,6,7,8,9,10]. Recent studies have provided at least three [possibly interrelated] explanations for this strong association between C-cpe isolates and FP. First, type A C-cpe isolates were found to be more prevalent than type A plasmid *cpe* (P-cpe) isolates in American retail meat products [11], which are important vehicles for *C. perfringens* type A FP [1]. Second, type A C-cpe isolates usually grow faster, and
over a broader temperature range, than do type A P-cpe isolates [12], which should favor the multiplication of C-cpe isolates in foods so these bacteria can reach the food burden necessary for inducing disease. Finally, compared to the vegetative cells or (particularly) spores of type A P-cpe isolates, the cells/spores of type A C-cpe isolates were shown [12,13,14] to typically exhibit much more resistance against food safety-induced stresses such as heating, cold (refrigerator or freezer temperatures) storage, osmotic stress and food preservatives (e.g. nitrites). Since, i) spores of type A C-cpe isolates are present in retail foods [11] and ii) temperature abuse of foods during cooking or storage is the major underlying factor leading to C. perfringens type A FP outbreaks [1], the spore resistance phenotype of type A C-cpe isolates is likely to facilitate survival of these isolates in foods so they can later cause FP.

We recently identified [15] a major contributor to the exceptional spore resistance phenotype exhibited by the spores of most type A C-cpe isolates. Specifically, C-cpe isolates that produce resistant spores were found to express a variant of a novel small acid soluble protein named Ssp4. Whereas Gly is present at Ssp4 residue 36 in C. perfringens type A isolates producing sensitive spores, the Ssp4 residue 36 is an Asp in most, if not all, type A C-cpe isolates producing resistant spores. Inactivation of the gene (ssp4) encoding Ssp4 was shown to significantly increase the sensitivity of C. perfringens type A spores to both heat and nitrous acid (a fast-killing proxy assay for evaluating spore resistance against nitrite, an often used food preservative), directly demonstrating that Ssp4 plays an important role in spore resistance properties. Furthermore, when spores of those ssp4 null mutants were complemented to express Ssp4 with an Asp at residue 36 (i.e., a Ssp4 Asp variant), they exhibited greater heat and nitrous acid resistance than did spores of the same mutant complemented to express Ssp4 with Gly at residue 36 (i.e., a Ssp4 Gly variant). This result proved that the Ssp4 Asp variant is an important contributor to the exceptional resistance phenotype exhibited by spores made by most type A C-cpe isolates. It was also shown that the exceptional protection afforded spores by the Ssp4 Asp variant apparently involves, at least in part, tighter spore DNA binding by this Ssp4 Asp variant, compared to the Ssp4 Gly variant made by most C. perfringens isolates [15].

The goal of our current study was to characterize further the contributions of the Ssp4 Asp variant to C. perfringens spore resistance properties and to begin examining how C. perfringens ssp4 gene regulates expression of the ssp4 gene during sporulation.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All C. perfringens isolates were stored as stock cultures in Cooked Meat Medium (Sigma) at −20°C. The starter cultures were prepared from those stock cultures by overnight growth at 37°C in fluid thioglycolate broth (FTG) (Difco), as described previously [12,15]. Sporulating cultures of C. perfringens were then prepared by inoculating 0.2 ml of the overnight FTG culture into 10 ml of Duncan-Strong (DS) sporulation medium [12]. After overnight incubation at 37°C, spores in the DS culture were prepared from those stock cultures by overnight growth at 37°C in fluid thioglycolate broth (FTG) (Difco), as described previously [12,15]. Sporulating cultures of C. perfringens were then prepared by inoculating 0.2 ml of the overnight FTG culture into 10 ml of Duncan-Strong (DS) sporulation medium [12]. After overnight incubation at 37°C, spores in the DS culture were purified as described previously [12]. Brain heart infusion (BHI) agar was used for plate count analyses [12].

Determination of the ssp4 sequence in non-type A C. perfringens isolates

DNA was isolated from C. perfringens strains CN1793 (type B), NCTC8533 (type B), JGS1195 (type C), CN5388 (type C), CN4003 (type D), JGS4138 (type D), 853 (type E) and NCIB10748 (type E) using the MasterPure gram-positive DNA purification kit (Epicentre). The primers ssp4proF and ssp4proR [15] were added (at a 5 μM final concentration) to a PCR mixture containing 1 μl of purified DNA template and 25 μl 2×Taq mixture (NEB), with a total volume of 50 μl. Each sample was then placed in a thermal cycler (Techne) and subjected to the following amplification conditions: 1 cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 40 s, and 68°C for 40 sec; and a single extension of 68°C for 5 min. The PCR products were cloned into a TOPO vector PCR2.1-TOPO (Invitrogen), which was sent for sequencing to the University of Pittsburgh Genomics Core Sequencing Facility. The ssp4 genes sequences were deposited in GenBank under accession numbers GQ222061 (CN11793); GQ222062 (NCTC8533); GQ222063 (JGS1195); GQ222064 (CN5388); GQ222065 (CN4003); GQ222066 (JGS4138); GQ222067 (strain 853) and GQ222068 (NBIC107481).

Measurement of spore resistance against heat and nitrous acid

The heat and nitrous acid resistance of spores were determined as described previously [13,14]. Briefly, an aliquot of a DS spore culture was serially diluted in ddH2O, heated at 70°C for 20 min (to kill vegetative cells and promote spore germination), plated onto BHI agar, and incubated anaerobically overnight at 37°C to determine the initial Colony Forming Units (CFU)/ml of spores in the culture. Aliquots of the remaining DS culture were then heat-treated at 70°C for 20 min to kill vegetative cells, followed by a second heating at 100°C. At specified times, aliquots of those heated cultures were diluted and plated onto BHI agar. In other experiments, aliquots of DS cultures were heat-treated at 70°C for 20 min and then suspended in 100 mM NaNO2, 100 mM Na Acetate (pH 4.5) at room temperature for 1 h. Aliquots of those nitrous acid-treated cultures were diluted and plated on BHI agar. After overnight anaerobic incubation (BD GasPack EZ Anaerobe Container system) at 37°C, the CFU/ml was counted and those results were used to calculate the decimal reduction times (D values), which is the treatment time needed to cause a 90% reduction in spore CFU/ml.

Site-directed mutagenesis of the ssp4 gene

Three site-directed mutations (D36K, D36E and D36N) were individually introduced into the ssp4 gene that we had separately cloned [15] into both pJR751 (a C. perfringens-E. coli shuttle plasmid encoding erythromycin-resistance [Em]) and pTrcHis A (Invitrogen). Each of those mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The reaction parameters were in accordance with the manufacturer’s instructions. Each mutation was confirmed by DNA sequencing at the University of Pittsburgh Genomics Core Facility.

Mutated plasmid DNA resulting from each site-directed mutagenesis reaction was then transformed into XL1-Super-competent Blue E. coli. The shuttle plasmids (named pJIR751-D36K, pJIR751-D36E, and pJIR751-D36N) were separately electroporated into the SM101::ssp4 null mutant. Transformants were selected on BHI plates containing 40 mg/L of Em. Spore heat and nitrous acid resistance were then determined, as described above.

pTrcHisA-D36K, pTrcHisD36E and pTrcHisD36N were separately transformed into E. coli DH5α. Transformants were selected by growth on LB containing Amp (50 mg/L). The presence of the desired mutated ssp4 gene in each transformant was then confirmed by nucleotide sequencing. Overproduction and nicked affinity purification of each recombinant, His6-tagged
rSsp4 mutant was performed as previously described [15]. The purified rSsp4 mutants were then used for Electromobility shift assay (EMSA) analyses as described later.

Comparison of low temperature survival for spores made by wild-type SM101 or F4969, their isogenic ssp4 null mutants, or complementing strains

The cold temperature (4°C or −20°C) resistance of spores produced by wild-type, ssp4 null mutants, or complementing strains of those mutants were determined as described previously [12]. Briefly, sporulating cultures were prepared for each isolate by overnight growth in DS medium. After determining the total number of spores present in an aliquot of each DS medium culture at the start (day 0) of the experiment, the remainder of the DS culture was divided into small tubes, half of which were incubated at 4°C and the other half at −20°C. Aliquots were removed from these small tubes after 6 months and surviving spore numbers (determined as described in a preceding section) were used to calculate the log reduction after each treatment.

Transformation of pDR81 into wild-type SM101 and an isogenic ssp4 mutant

Plasmid pDR81 [16], which encodes an ssp2 antisense gene that can inhibit ssp1, ssp2 and ssp3 transcription [16], was introduced by electroporation [2] into wild-type SM101 or SM101::ssp4. Erm<sup>+</sup> transformants were then selected. The resultant SM101 and SM101::ssp4 transformants were designated as SM101 (pDR81) and SM101::ssp4 (pDR81), respectively.

Heat and nitrous acid resistance of spores made by SM101 (pDR81) and SM101::ssp4 (pDR81) were determined as described earlier.

Small acid soluble proteins (SASPs) extraction and Western blotting

To evaluate SASPs presence in spores, C. perfringens SASPs were extracted, as described previously [15], from 50 mg of dry washed spores produced by specified C. perfringens strains. The extracted proteins were subjected to SDS-PAGE and the separated proteins (determined as described in a preceding section) were used to calculate the log reduction after each treatment.

Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Sources or Refs |
|-------------------|--------------------------|-----------------|
| C. perfringens    |                          |                 |
| SM101             | Food poisoning type A isolate, carries a chromosomal cpe gene | [18] |
| F4969             | GI disease type A isolate, carries a plasmid borne cpe gene | [6] |
| CN1793            | Type B isolate, toxicogenic | UK, 1947 |
| NCTC8533          | Type B isolate, lamb dysentery | UK, 1950s |
| JGS1495           | Type C isolate, porcine | unknown |
| CN5388            | Type C isolate, human pigbel | unknown |
| CN4003            | Type D isolate, lamb | USA, 2002 |
| JGS4138           | Type D isolate, goat | North America |
| rSsp4             | Type E isolate, calf with enteritis | Institut Pasteur, reference strain |
| IH101             | spo0A knock-out mutant derivative of SM101 | [17] |
| SM101::ssp4       | ssp4 knock-out mutant derivative of SM101 | [15] |
| F4969::ssp4       | ssp4 knock-out mutant derivative of F4969 | [15] |
| Plasmids          |                          |                 |
| pDR81             | ssp2 fragment in the antisense direction to the ssp2 promoter | [16] |
| pMR5123           | spo0A ORF and ~200 bp upstream sequence in pJIR751 | [17] |
| pJIR751           | C. perfringens/E. coli shuttle vector; Erm<sup>+</sup> | [17] |
| pCS               | SM101 ssp4 ORF and ~300 bp upstream sequence in pJIR751 | [15] |
| pCF               | F4969 ssp4 ORF and ~300 bp upstream sequence in pJIR751 | [15] |
| pD36E             | pCS 36 amino acid site-directed mutagenesis D to E | This study |
| pD36N             | pCS 36 amino acid site-directed mutagenesis D to N | This study |
| pD36K             | pCS 36 amino acid site-directed mutagenesis D to K | This study |

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Spo0A production

To evaluate Spo0A production, C. perfringens strains were grown for 8 h at 37°C in DS medium and those cultures were then sonicated until >95% of the cells had lysed. Each culture lysate was then analyzed for the presence of Spo0A by Western blot using an antibody specific for B. subtilis Spo0A [17].

Electromobility shift assays (EMSA)

A 3'-biotin-labeled, AT-rich (72.8% AT) C. perfringens DNA sequence was prepared using a biotin 3'-end DNA labeling kit (Pierce) as described previously [15]. Similarly, a 3'-biotin-labeled,
GC-rich probe was prepared consisting of a 55 bp sequence from a GC-rich (69.1% GC) *C. perfringens* genomic DNA sequence. For this purpose, the following two oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA): Label-D2 (5'CTG-GCGACTCACAGGGGCTCGAACCTCCG-GC-GTG-ACAGGCCGGCACT-3') and Label-R2 (5'AGTGCG-CGCCCTGTCAAGCCGGAG-GTCCAGGGTGGC-GC- TCTGAGTCGCCAG-3') and 3'-biotin-end-labeled by the manufacturer's instructions using a biotin 3'-end DNA labeling kit (Pierce).

The AT-rich probe was used in a modified EMSA to compare the DNA binding of the three site-directed, His6-tagged rSsp mutants. AT-rich and GC-rich probes were used in a modified EMSA, as described previously [15], to compare rSsp4 and rSsp2 binding preferences. Briefly, 1 μl of probe was incubated with 50, 100 or 200 ng of purified His6-tagged rSsp at 37°C for 1 h. Bound rSsp was then fixed to the DNA probe by the addition of glutaraldehyde (final concentration of 0.01% (v/v)) by 15 min incubation at 37°C. Those mixtures were loaded into a 6% polyacrylamide gel and electrophoresed in 0.5 mM EDTA buffer at 4°C for 1 h. DNA-protein complexes were then transferred to a positive charge nylon membrane (Roche Applied Science), UV crosslinked and detected with a LightShift Chemiluminescent EMSA kit (Pierce).

**Results**

**Comparison of the Ssp4 sequence and spore heat- and nitrite-resistance properties amongst non-type A *C. perfringens* strains**

Our initial Ssp4 study [15] found that the Ssp4 protein produced by 13 different type A strains shares an identical sequence, except for variations at amino acids 36 and 72. Those 15 type A isolates produced an Ssp4 with either Gly or Asp at residue 36 and either Asn or Lys at residue 72. As described in the Introduction, the presence of Asp at Ssp4 residue 36 was shown to be important for helping to mediate the exceptional spore resistance properties exhibited by most type A *C. perfringens* isolates.

To further evaluate Ssp4 sequence diversity amongst *C. perfringens* isolates, the current study sequenced the *ssp4* ORF carried by eight strains belonging to *C. perfringens* type B, C, D or E (Tables 1 and 2). Those analyses revealed the presence of an identical *ssp4* ORF in all eight surveyed non-type A isolates. Furthermore, the *ssp4* ORF sequence present in these eight type B, C, D and E isolates identically matched the *ssp4* sequence found in type A isolates (e.g. F4969) producing an Ssp4 with Gly present at residue 36 and Lys present at residue 72.

The presence of the same *ssp4* sequence in F4969 and the eight surveyed non-type A isolates suggested that the spores produced by non-type A isolates might resemble the spores made by F4969 in terms of their heat- and nitrous acid-sensitivity. This hypothesis was tested by phenotyping the spores produced by a type B, C and D isolate for their ability to withstand boiling and nitrous acid (no type E isolate in our collection produced suitable levels of spores to conduct phenotype analyses). Results of those experiments showed that, relative to the resistant spores made by most type A *C. perfringens* isolates (e.g. SM101), the spores of the three tested non-type A isolates of *C. perfringens* exhibited significantly more sensitivity to heat and nitrous acid (Table 2). Furthermore, the resistance properties determined for spores made by non-type A isolates closely matched those of spores produced by *P. aeruginosa* isolates (e.g. F4969) (Table 2).

**Saturation mutagenesis of the SM101 *ssp4* gene at the codon encoding Asp residue 36 and phenotyping the spore resistance properties of those Ssp4 mutants**

The Table 2 results supported previous results [15] demonstrating that variations at Ssp4 residue 36 are important for the heat- and nitrous acid-resistance properties of *C. perfringens* spores. Specifically, those *C.-cpe* isolates (e.g. SM101, a transformable derivative of FP isolate NCTC 8798 [18]) forming exceptionally resistant spores make an Ssp4 with Asp at residue 36, while this Ssp4 residue is Gly in heat- and nitrous acid-sensitive *C. perfringens* spores, including F4969 and the non-type A isolates phenotyped in Table 2.

To evaluate which amino acid properties at Ssp4 residue 36 are important for helping to mediate the exceptional resistance phenotype of spores produced by most *C.-cpe* FP isolates, site-directed mutagenesis was performed on the SM101 *ssp4* gene cloned into either the pJIR751 shuttle plasmid (to allow testing of spore phenotypes) or the pTrcHis plasmid (to allow testing of DNA binding properties of rSsp4 mutants, which can be easily purified by nickel affinity chromatography due to their N-terminal, vector-encoded His6 sequence). These mutagenesis reactions created Ssp4 or rSsp4 variants where the natural Asp (D) present at residue 36 of the SM101 Ssp4 had been replaced by Glu (E), Lys (K) or Asn (N).

| Strain   | Types | Toxin gene       | 3 6 res | 72 res | Heat resistance (min) | Chemical resistance (log reduction) |
|----------|-------|------------------|---------|--------|-----------------------|-------------------------------------|
| SM101    | A (FP) | cpe (chrom)      | D       | N      | 59.1 ± 1.3            | 1.1 ± 0.4                           |
| F4969    | A (NFP)| cpe (plasmid)    | G       | K      | 0.5 ± 0.0             | 4.0 ± 0.5                           |
| CN1794   | B      | cpe, etx, cpeb2  | G       | K      | ND                    | ND                                  |
| NCTC8533 | B      | cpe, etx, cpeb2  | G       | K      | 1.4 ± 0.5             | 4.2 ± 0.7                           |
| JGS1495  | C      | cpeb2            | G       | K      | NA                    | NA                                  |
| CN5388   | C      | cpe, cpe, cpeb2  | G       | K      | 2.3 ± 0.3             | 4.0 ± 0.1                           |
| CN4003   | D      | etx, cpe, cpeb2  | G       | K      | ND                    | ND                                  |
| JGS4138  | D      | etx, cpe, cpeb2  | G       | K      | 2.7 ± 0.6             | 5.3 ± 0.1                           |
| 853      | E      | iota, cpe        | G       | K      | ND                    | ND                                  |
| NBI10748 | E      | iota, cpe, cpeb2 | G       | K      | ND                    | ND                                  |

Table 2. Ssp4 sequence and spore resistance in various *C. perfringens* types.
The pTrcHis plasmids carrying each rSsp4 mutant were separately transformed into E. coli, while the pJIR751 plasmids carrying each mutant ssp4 gene were separately transformed into a previously-created SM101 ssp4 null mutant (SM101::ssp4) [15]. The presence in each transformant of a plasmid carrying mutated ssp4 sequences was demonstrated by PCR (Fig. 1A) and the presence of the desired ssp4 ORF mutation in the transformant was confirmed by sequencing (not shown). Production of each Ssp4 mutant (Fig. 1B) or rSsp4 mutant (not shown) was demonstrated by Ssp4 Western blotting.

Experiments were then performed to evaluate the resistance properties of spores produced by SM101::ssp4 mutants after those null mutants had been complemented to express each Ssp4 variant with a residue 36 mutation. Control phenotypic comparisons first confirmed our previous observations [15] that inactivating the ssp4 gene in SM101 reduced spore heat and nitrous acid resistance properties (Table 3). As we had also reported previously [15], complementation of the SM101::ssp4 mutant to enable expression of Ssp4 with a wild-type Asp at residue 36 (SM101::ssp4-pCS) resulted in spores exhibiting exceptional resistant to both heat and nitrous acid. Similar complementation of this SM101::ssp4 mutant so it expressed wild-type F4969 Ssp4 with a Gly at residue 36 (SM101::ssp4-pCF) resulted in a much more limited increase in spore heat and nitrous acid resistance properties.

When the resistance properties of spores made by SM101::ssp4 transformants expressing the Ssp4D36E or Ssp4D36K mutants were tested (Table 3), those spores exhibited similar heat and nitrous acid resistance properties as SM101::ssp4-pCS spores containing the wild-type Ssp4 Asp variant. However, the spores produced by SM101::ssp4 transformants expressing the Ssp4D36N mutant had lower resistance against both heat and nitrous acid. Spores containing the Ssp4D36N mutant exhibited similar resistance properties to SM101::ssp4-pCS spores expressing the wild-type Ssp4 Asp variant. However, the spores produced by SM101::ssp4 transformants expressing the Ssp4D36N mutant had lower resistance against both heat and nitrous acid. Spores containing the Ssp4D36N mutant exhibited similar resistance properties to SM101::ssp4-pCS spores expressing the wild-type Ssp4 Asp variant.

| Table 3. Heat and chemical resistance of SM101 transformants producing site-directed mutants. |

|                  | SM101 | SM101::ssp4 | SM101::ssp4 - pCS | SM101::ssp4 - pD36E | SM101::ssp4 - pD36N | SM101::ssp4 - pD36K |
|------------------|-------|-------------|-------------------|---------------------|--------------------|----------------------|
| Heat resistance  |       |             |                   |                     |                    |                      |
| (D value) (min)  | 59.1±1.3 | 8.7±1.9    | 44.7±1.8          | 16.4±0.6            | 41.9±2.6           | 24.0±1.7             | 40.8±1.3            |
| Chemical Resistance | 4.1±0.4 | 4.0±0.1    | 1.1±0.6           | 3.2±0.1             | 1.2±0.3            | 2.2±0.2              | 1.6±0.2             |

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properties as spores made by SM101::ssp4 transformed to produce the wild-type F4969 Ssp4 Gly variant.

To assess whether the resistance properties of spores containing Ssp4 with a site-directed mutation at residue 36 correlated with the DNA binding properties of their Ssp4 variant, wild-type SM101 rSsp4, the SM101 rSsp4 site-directed mutants, and wild-type F4969 rSsp4 were each purified and tested (Fig. 1C) for their DNA binding properties using an EMSA assay. Control EMSA analyses confirmed our previous report [15] that wild-type SM101 rSsp4 (with an Asp at residue 36) binds strongly to an AT-rich DNA probe, while F4969 rSsp4 (with a Gly at residue 36) binds less well to this DNA probe (Fig. 1C). Amongst the three SM101 rSsp4 variants with a mutated residue 36 amino acid, as created by site-directed mutagenesis for this study, Ssp4D36E and Ssp4 D36K exhibited similar binding to the AT-rich DNA probe as did native SM101 rSsp4 with an Asp at residue 36, while Ssp4D36N exhibited weaker DNA binding.

Ssp4 preferentially binds to AT-Rich DNA

Results from Fig. 1 and our previous study [15] have demonstrated that Ssp4 can bind to an AT-rich probe mimicking C. perfringens DNA, which has a high (~72%) overall AT content. Interestingly, the small acid soluble protein named SspC made by Bacillus spp. (another Gram-positive, sporeforming bacteria with low-GC% DNA) reportedly binds better to GC-rich DNA compared to AT-rich DNA [19,20].

Therefore, the current study performed an EMSA analysis to compare the binding of C. perfringens SM101 rSsp4 to AT-rich vs. GC-rich DNA probes. The current study also examined the binding of C. perfringens SM101 rSsp2 (which has a very similar sequence to Bacillus Ssp, as well as C. perfringens Ssp1 and Ssp3) to the same AT-rich vs. GC-rich DNA probes. These EMSA analyses revealed (Fig. 2) that purified rSsp2 binds preferentially to a probe containing a GC-rich (69.1% GC) sequence of C. perfringens DNA vs. a probe containing an AT-rich (72.8% AT) sequence of C. perfringens DNA. In contrast, purified rSsp4 exhibited a binding preference for the AT-rich vs. the GC-rich C. perfringens DNA sequence (Fig. 2).

Ssp4 works in concert with other small acid soluble proteins (Ssp1, Ssp2, Ssp3) for maximal protection of C. perfringens spores against heat and nitrous acid

As mentioned above, C. perfringens produces at least four small acid soluble proteins. These include three proteins, named Ssp1–3, that differ from Ssp4 but share substantial sequence similarity with one another [16,21,22]. To explore why this bacterium produces so many different SASPs, an ssp2 antisense plasmid was transformed into our SM101::ssp4 null mutant. This antisense plasmid was shown previously to simultaneously block expression of Ssp1, Ssp2 and Ssp3 [16]. Consistent with those previous observations, Western blot analyses showed that neither SM101::ssp4 (pDR81) nor SM101(pDR81) produced Ssp1, Ssp2 or Ssp3 proteins (Fig. 3), although SM101(pDR81) still produced Ssp4.

When heat and nitrous acid resistance were compared, wild-type SM101 spores exhibited about 7-fold higher resistance than spores of the ssp4 null mutant (Table 4). Although the SM101(pDR81) transformant still produced Ssp4, its spores showed a reduced heat resistance compared against wild type spores, indicating that (in addition to Ssp4) Ssp1, Ssp2 and Ssp3
are also important for the full development of SM101 spore heat resistance. Importantly, SM101::*sp4*(pDR81), which does not produce any of the four known *C. perfringens* Ssp4, produced spores with virtually no heat resistance at 100°C.

Spore nitrous acid resistance properties for these *C. perfringens* strains showed a similar pattern of differences as described above for spore heat resistance differences (Table 4). Spores of wild-type SM101 exhibited a nitrous acid-induced log reduction in viability of only 1.1, while nitrous acid caused a 4 log reduction in spore viability for SM101::*sp4*, confirming a role for Ssp4 in SM101 spore nitrous acid resistance. Decreased production of Ssp1, Ssp2 and Ssp3 also reduced SM101 spore resistance against nitrous acid, although this 2.5 log reduction in spore viability was less than observed after *sp4* gene inactivation. The strongest reduction in SM101 spore nitrous acid resistance (a 4.8 log reduction) was observed for SM101::*sp4* (pDR81), which does not produce any of the known *C. perfringens* Ssp4. Collectively, these results (Table 4) indicate that Ssp4 works in combination with the other Ssp4s to protect *C. perfringens* spores against heat and nitrous acid treatment.

### Table 4. Heat and chemical resistance of SM101 or SM101::*sp4* with or without the pDR81 antisense plasmid.

|                     | SM101       | SM101(pDR81) | SM101::*sp4* | SM101::*sp4* (pDR81) |
|---------------------|-------------|--------------|--------------|----------------------|
| Heat resistance D value (min) | 59.1±1.3    | 18.1±2.7     | 8.7±1.9      | 1.4±0.1              |
| Chemical Resistance (log reduction) | 1.1±0.4     | 2.5±0.3      | 4.0±0.1      | 4.8±0.7              |

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Low temperature survival of spores produced by wild-type SM101 and F4969, their *sp4* null mutants and complemented strains

As confirmed in Table 2, inactivation of the *sp4* gene in SM101 or F4969 causes these isolates to produce spores with considerably less heat- and nitrous acid-resistance than their corresponding wild-type spores. In addition to heating and use of preservatives, storage of foods at low temperature (in refrigerators or freezers) is a very important food safety approach for controlling storage of foods at low temperature (in refrigerators or freezers) is a very important food safety approach for controlling *C. perfringens* spore survival at 4°C and −20°C.

This study first confirmed previous conclusions [12] that wild-type SM101 spores exhibit exceptional survival at low temperatures, with only a 0.35 and 0.58 log reduction in spore viability measured after 6 months of storage at 4°C or −20°C, respectively (Table 5). Table 5 also shows, for the first time, that specific inactivation of the *sp4* gene in SM101 reduced spore viability upon low temperature storage, with 0.82 or 1.91 log reduction in spore viability measured after 6-month storage of *sp4* null mutant spores at 4°C or −20°C, respectively. These spore survival differences between wild type SM101 and its isogenic *sp4* null mutant were statistically significant (P<0.01) at both 4°C and −20°C. Demonstrating that the decreased low temperature resistance of spores made by the SM101 *sp4* null mutant was specifically due to inactivation of the *sp4* gene, complementation of this mutant with a shuttle plasmid carrying the wild-type SM101 *sp4* gene was able to substantially increase 6-month spore survival at both 4°C and −20°C. In contrast, transformation of the SM101::*sp4* mutant with a shuttle plasmid carrying the wild-type F4969 *sp4* gene more modestly increased 6-month spore survival of the SM101 *sp4* null mutant upon storage at low temperatures.

Consistent with previous observations, wild-type F4969 spores exhibited poorer survival at 4°C or −20°C compared to wild-type SM101 spores (Table 5). Table 5 also shows that inactivation of the *sp4* gene in strain F4969 substantially decreased 6-month spore survival at both 4°C and −20°C. Spores of the F4969 *sp4* null mutant exhibited much better 6-month survival at these low temperatures when they were complemented with a shuttle plasmid carrying the SM101 *sp4* gene (Table 2). In contrast, complementation with the same shuttle plasmid carrying the wild-type F4969 *sp4* gene caused a lesser increase in 6-month spore survival of the F4969 null mutant upon storage at either 4°C or −20°C.

Collectively, these results (Table 5) demonstrate that Ssp4 is important for spore survival at low temperatures and that the SM101 Ssp4 variant is better at protecting spores against low temperature-induced lethality than F4969.

### The role of Spo0A in regulating *sp4* expression

Our previous studies [21,22] have shown that production of Ssp1–5 requires a *C. perfringens* isolate to possess a functional *spo0A* gene. Therefore, the current study investigated whether Ssp4 expression is also Spo0A-dependent.

We first confirmed that *C. perfringens* IH101, a previously prepared *spo0A* null mutant of SM101 that cannot form spores [17], does not produce Spo0A. When Western blotting was used to compare by wild-type SM101 versus IH101 grown in Duncan-Strong (DS) sporulation medium (Fig. 4), the results obtained showed that DS cultures of SM101 and SM101::*sp4* both produce Spo0A, but DS cultures of IH101 do not produce Spo0A. Confirming that this phenotype was specifically due to inactivation of the *spo0A* gene in IH101, complementation of IH101 with a plasmid carrying the wild-type *spo0A* gene restored Spo0A expression.

### Table 5. Cold resistance of wild-type, *sp4* null mutants and complementing strains of SM101 and F4969.

| Stains             | 4°C (log reduction after 6 month) | −20°C (log reduction after 6 month) |
|--------------------|----------------------------------|-------------------------------------|
| F4969              | 0.88±0.13                        | 1.23±0.11                           |
| F4969::*sp4*       | 2.02±0.15                        | 3.12±0.10                           |
| F4969::*sp4*(pCS)  | 0.70±0.10                        | 1.01±0.33                           |
| F4969::*sp4*(pCF)  | 1.30±0.20                        | 1.81±0.10                           |
| F4969::*sp4*(pJIR751) | 1.82±0.10                      | 2.70±0.40                           |
| SM101              | 0.35±0.10                        | 0.58±0.17                           |
| SM101::*sp4*       | 0.82±0.18                        | 1.91±0.35                           |
| SM101::*sp4*(pCS)  | 0.40±0.13                        | 0.65±0.20                           |
| SM101::*sp4*(pCF)  | 0.50±0.14                        | 1.22±0.56                           |
| SM101::*sp4*(pJIR751) | 1.03±0.42                     | 1.86±0.67                           |

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As expected from our previous studies [15], Ssp4 was produced by DS cultures of wild-type SM101 but not by SM101::ssp4 [15]. As also reported previously [15], expression of Ssp4 by DS cultures of SM101::ssp4 was restored by complementation with a shuttle plasmid carrying the wild-type ssp4 gene. We now show that DS cultures of IH101 failed to produce Ssp4. However, DS cultures of the complementing strain IH101 [MRS123] did show Ssp4 expression, thereby demonstrating that Spo0A is specifically required for Ssp4 production.

Discussion

*C. perfringens* FP isolates generally possess two complimentary virulence traits, i.e., production of CPE and the ability to form spores that are highly resistant to food environment stresses, such as heat, low temperatures and preservatives such as nitrites [1]. Since our previous study [15] had identified a novel Ssp4 variant as a major contributor to the spore resistance properties of FP isolates, the current study sought to better characterize Ssp4 proteins and to begin exploring the regulation of Ssp4 expression.

Our previous study [15] had determined that the *C. perfringens* type A FP isolates forming resistant spores usually produce an Ssp4 with Asp at residue 36, while other type A *C. perfringens* isolates, including both P-cpe isolates and cpe-negative isolates, typically make an Ssp4 with Gly at residue 36. Cross-complementation approaches with ssp4 null mutants have also directly demonstrated that, 1) Ssp4 is an important mediator of spore resistance against heat and nitrous acid, and 2) the Ssp4 variant with Asp at residue 36 is better than the Ssp4 variant with Gly at residue 36 at protecting spores against heat and nitrous acids. In another previous study [12], we had shown that the spores of type A C-cpe isolates also exhibit exceptional survival at low temperatures, i.e., at 4°C and −20°C. The current study now reports that the strong low temperature resistance phenotype of spores made by FP isolates also involves Ssp4 and that the Ssp4 Asp variant is more against low temperature than the Ssp4 Gly variant. The exceptional low temperature resistance exhibited by spores containing the Ssp4 Asp variant is a likely contributor to *C. perfringens* FP transmission since meat and poultry products, common food vehicles for *C. perfringens* FP, are known to be contaminated with resistant spores of C-cpe isolates and are typically stored in refrigerators or freezers.

Determining that type A, C-cpe FP isolates typically produce spores whose resistance phenotype is mediated, in large part, by the Ssp4 Asp variant, while type A, P-cpe isolates produce spores whose sensitivity involves the Ssp4 Gly variant provided one explanation for the strong association between type A C-cpe isolates and FP. However, about 15% or 25% (respectively) of *C. perfringens* type C and D isolates also carry a cpe gene [23,24] from which they produce similar amounts of an identical CPE protein as type A cpe-positive isolates. Interestingly, those cpe-positive type C or D isolates rarely, if ever, cause human FP. Results from the current study suggest that cpe-positive type C or D isolates may not commonly be involved in FP, at least in part, because they form spores that are sensitive to food environment stresses such as heating and preservatives. The cpe-positive type C and D isolates surveyed in this study also produce the same Ssp4 Gly variant as found in type A isolates producing sensitive spores. Since this same Ssp4 variant has been established as a major contributor to the sensitivity of type A isolates producing sensitive spores, the Ssp4 Gly variant is also likely to be an important factor behind the spore sensitivity of cpe-positive type C and D isolates, although this should be experimentally confirmed.

The current study also demonstrated that Ssp4 works in combination with Ssp1–3 to produce maximal spore resistance properties for *C. perfringens* type A C-cpe FP isolates. Furthermore, this work found that Ssp4 exhibits a preference for AT-rich DNA sequences, in contrast to Ssp2 (and most likely Ssp1 and 3 based...
upon their sequence similarities to Ssp2), which prefers binding to GC-rich DNA. This diversity in sequence binding preferences may help to explain why C. perfringens makes several Ssps, i.e., by producing multiple Ssps that bind to different chromosomal regions depending on their local AT% ratio, the entire chromosome may be maximally protected from damage induced by stresses such as heat or preservatives.

The properties of Ssp4 residue 36 that mediate spore resistance were also examined in the current study. These analyses revealed that spores retain exceptional heat and nitrous acid resistance if Lys or Gln were substituted for the Asp naturally found at Ssp4 residue 36 in C. perfringens type A. FP isolates forming highly resistant spores. However, changing Ssp4 residue 36 from Asp to Asn produced sensitive spores resembling those made by isolates producing Ssp4 with Gly at residue 36. These results suggest that both the side chain length and presence of a charge at Ssp4 residue 36 may be important for mediating spore resistance properties. The strong resistance phenotype exhibited by spores carrying the mutant Ssp4 correlated with DNA binding properties of the corresponding purified rSsp4 mutant, supporting previous suggestions [15] that the DNA binding properties of Ssp4 variants are important determinants of spore resistance properties.

A bioinformatics search of Genbank revealed that other Clostridium spp. naturally carry ORFs encoding putative Ssp homologues with Glu (Cac4 of Clostridium acetobutylicum ATCC824, Cnos of Clostridium novyi NT), Asp (Cac2 of Clostridium acetobutylicum ATCC824, Ssp2 of C. perfringens SM101, Cno2 of Clostridium novyi NT, Cte2 of Clostridium tetani), Lys (C1 of Clostridium novyi NT) or Gly (Ssp4 of C. perfringens ATCC3624) at the equivalent position as SM101 Ssp4 residue 36. The site-directed mutagenesis results of the current study might suggest that C. acetobutylicum ATCC824, C. novyi NT and C. tetani E88 spores would exhibit substantial resistance against stresses such as heat, low temperature and food preservatives. However, this should be evaluated experimentally for two reasons. First, these isolates all carry ORFs encoding several different putative Ssps and it is clear from the current and previous studies [15] that no single Ssp fully determines spore resistance properties. Second, it is clear that Ssp4 variants are not the only contributor to variations in C. perfringens spore resistance properties. For example, the size of the spore core may also influence variations in C. perfringens spore resistance [25,26]. It would also be of interest to evaluate whether other clostridial species exhibit intraspecies Ssp4 variants that influence spore resistance phenotypes, as occurs with C. perfringens.

Finally, the current results [22] revealed that, like Ssp1–3, expression of Ssp4 requires Spo0A. Spo0A is a master regulator of many genes expressed during sporulation [27] and late-stationary phase, so its involvement in Ssp4 expression is consistent with our previous finding that Ssp4 production is strongly sporulation-associated. Some Spo0A-regulated genes are regulated by Spo0A binding to sequences (0A boxes) located upstream of the ORF [27,28]. A bioinformatics search detected potential 0A boxes upstream of all four C. perfringens ssp4 genes, including ssp4A, in SM101. However, Ssp expression in C. perfringens may not only involve Spo0A regulation, as these bioinformatics searches also identified potential SigK binding sites upstream of the ssp4A and ssp4B genes of SM101. The presence of those SigK boxes could suggest that SigK, an alternative sigma factor, is also involved in the sporulation-associated regulation of some ssp genes. Further studies are underway to better understand how C. perfringens regulates Ssp expression.

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Author Contributions

Conceived and designed the experiments: JL BAM. Performed the experiments: JL DPS. Analyzed the data: JL DPS BAM. Contributed reagents/materials/analysis tools: JL DPS MRS. Wrote the paper: JL DPS MRS BAM.

References

1. McClane BA (2007) Clostridium perfringens in Food Microbiology Fundamentals and Frontiers (3rd Edition); Doyle MP, Beuchat LR, eds. Washington D.C.: ASM press. pp 423–444.
2. Sarker MR, Carman RJ, McClane BA (1998) Inactivation of the gene (cpe) encoding Clostridium perfringens enterotoxin eliminates the ability of two osp-positive C. perfringens type A human gastrointestinal disease isolates to affect rabbit ileal loops. Molec Microbiol 33: 946–950.
3. Adak GK, Long SM, O’Brien SJ (2002) Trends in indigenous foodborne disease in England and Wales: 1992 to 2000. Gut 3: 832–841.
4. Lynch M, Painter J, Woodruff R, Braden G (2006) Surveillance for foodborne- disease outbreaks - United States, 1998–2002. Morbidity and Mortality Weekly Report, CDC 55: 1–42.
5. Cornillot E, Saint-Joanis B, Daube G, Katayama S, Granum PE, et al. (1995) The enterotoxin gene (cpe) of Clostridium perfringens can be chromosomal or plasmid-borne. Molec Microbiol 15: 639–647.
6. Collee RE, McClane BA (1998) Evidence that the enterotoxin gene can be episomal in Clostridium perfringens isolates associated with foodborne human gastrointestinal diseases. J Clin Microbiol 36: 30–36.
7. Sparks SG, Carman RJ, Sarker MR, McClane BA (2001) Genotypying of enterotoxigenic Clostridium perfringens isolates associated with gastrointestinal disease in North America. J Clin Microbiol 39: 885–888.
8. Miyamoto K, Wen Q, McClane BA (2004) Multiplex PCR genotyping assay that distinguishes between isolates of Clostridium perfringens: Type A carrying a chromosomal enterotoxin gene (cpe) locus, a plasmid cpe locus with an IS4470-like sequence or a plasmid cpe locus with an IS151 sequence. J Clin Microbiol 41: 1552–1558.
9. Grant K, Kenyon S, Nwafor I, Plosman J, Ohai C, et al. (2008) The identification and characterization of Clostridium perfringens by real-time PCR, location of enterotoxin gene, and heat resistance. Foodborne Pathog Dis 5: 629–639.
10. Lahni P, Heikinheimo A, Johannson T, Korkeala H (2007) Clostridium perfringens Type A isolates carrying the plasmid-borne enterotoxin gene (genotypes IS151-ps or IS1470-like-ps) are a common cause of food poisonings. J Clin Microbiol 46: 371–373.
11. Wen Q, McClane BA (2004) Detection of enterotoxigenic Clostridium perfringens type A isolates in American retail foods. Appl Environ Microbiol 70: 2935–2941.
12. Li J, McClane BA (2006) Further comparison of temperature effects on growth and survival of Clostridium perfringens type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. Appl Environ Microbiol 72: 4561–4568.
13. Li J, McClane BA (2006) Comparative effects of osmotic, sodium nitrite-induced, and pH-induced stress on growth and survival of Clostridium perfringens type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. Appl Environ Microbiol 72: 7620–7625.
14. Sarker MR, Shawers RF, Sparks SG, Janeja VK, McClane BA (2000) Comparative experiments to examine the effects of heating on vegetative cells and spores of Clostridium perfringens isolates carrying plasmid versus chromosomal enterotoxin genes. Appl Environ Microbiol 66: 3234–3240.
15. Li J, McClane BA (2000) A novel small acid-soluble protein variant is important for spore resistance of most Clostridium perfringens food poisoning isolates. PLoS Pathogens 4: e1000656.
16. Rajo D, Setlow P, Sarker MR (2007) Antisense-RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased resistance of Clostridium perfringens spores to moist heat and UV radiation. Appl Environ Microbiol 73: 2048–2053.
17. Huang HY, Watters M, Grau RR, Sarker MR (2004) Disruption of the gene (spo0A) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic Clostridium perfringens type A. FEMS Microbiol Let 233: 233–240.
18. Zhao Y, Melville SB (1999) Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (cpe) of Clostridium perfringens. J Bacteriol 180: 136–142.
19. Setlow P (2007) I will survive: DNA protection in bacterial spore. Trends Microbiol 15: 172–180.
20. Temmen R, Setlow R, Davis KL, Leshon CA, Setlow P (2000) Mechanisms of killing of spores of Bacillus subtilis by iodine, glutaraldehyde and nitrous acid. J Appl Microbiol 89: 330–338.
21. Raju D, Waters M, Setlow P, Sarker MR (2006) Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of Clostridium perfringens spores to heat. BMC Microbiol 8: 50.
22. Raju D, Sarker MR (2007) Production of small, acid-soluble spore proteins in Clostridium perfringens non-foodborne gastrointestinal disease isolates. Can J Microbiol 53: 514–518.
23. Fisher DJ, Fernandez-Miyakawa ME, Sayeed S, Poon R, Adams V, et al. (2006) Dissecting the contributions of Clostridium perfringens type C toxins to lethality in the mouse intravenous injection model. Infect Immun 74: 5200–5210.
24. Sayeed S, Li J, McClane BA (2007) Virulence plasmid diversity in Clostridium perfringens type D isolates. Infect Immun 75: 2391–2398.
25. Novak JS, Juneja V, McClane BA (2003) An ultrastructural comparison of spores from various strains of Clostridium perfringens and correlations with heat resistance parameters. Int J Food Microbiol 86: 239–247.
26. Orsborn B, Melville SB, Popham D (2008) Factors contributing to heat resistance of Clostridium perfringens endospores. Appl Environ Microbiol 74: 3328–3335.
27. Moller V, Fujita M, Jensen ST, Eichenberger P, Gonzalez-Pastor JE, et al. (2003) The Spo0A regulon of Bacillus subtilis. Molec Microbiol 50: 1683–1701.
28. Greene AE, Spiegelman GB (1996) The Spo0A protein of Bacillus subtilis inhibits transcription of the ahpB gene without preventing binding of the polymerase to the promoter. J Biol Chemistry 271: 11435–11441.