

Occurrence of genes encoding spore germination in *Clostridium* species that cause meat spoilage

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Abstract

Members of the genus *Clostridium* are frequently associated with meat spoilage. The ability for low numbers of spores of certain *Clostridium* species to germinate in cold-stored vacuum-packed meat can result in blown pack spoilage. However, little is known about the germination process of these clostridia, despite this characteristic being important for their ability to cause spoilage. This study sought to determine the genomic conditions for germination of 37 representative *Clostridium* strains from seven species (*C. estertheticum*, *C. tagluense*, *C. frigoris*, *C. gasigenes*, *C. putrefaciens*, *C. aligidicarnis* and *C. frigidicarnis*) by comparison with previously characterized germination genes from *C. perfringens*, *C. sporogenes* and *C. botulinum*. All the genomes analysed contained at least one *gerX* operon. Seven different *gerX* operon configuration types were identified across genomes from *C. estertheticum*, *C. tagluense* and *C. gasigenes*. Differences arose between the *C. gasigenes* genomes and those belonging to *C. tagluense/C. estertheticum* in the number and type of genes coding for cortex lytic enzymes, suggesting the germination pathway of *C. gasigenes* is different. However, the core components of the germination pathway were conserved in all the *Clostridium* genomes analysed, suggesting that these species undergo the same major steps as *Bacillus subtilis* for germination to occur.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files. All clostridia isolates that were genome-sequenced in this study were deposited in GenBank (BioProject accession number PRJNA574489).

INTRODUCTION

Spore-forming bacteria are common contaminants of food and the environment, and as such represent a major source of food poisoning and food spoilage. Blown pack spoilage (BPS) typically occurs in red meat that has been vacuum packaged and stored at consistent chilled temperatures (-1.5°C) [1], and has been reported in chilled vacuum-packed meats from many countries, including Brazil, Canada, Iceland, Ireland, Germany and New Zealand. BPS is caused by psychrophilic and psychrotrophic *Clostridium* species, including *Clostridium estertheticum* and *Clostridium gasigenes* [2–4]. The economic losses in New Zealand and worldwide attributed to product spoilage and market access issues are significant [5, 6], in particular BPS of chilled meat (e.g. venison, lamb and beef) [7].

Received 22 October 2021; Accepted 20 December 2021; Published 15 February 2022

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Keywords: *Clostridium*; meat spoilage; spore germination.

Abbreviations: ANI, average nucleotide identity; BPS, blown pack spoilage; CLE, cortex-lytic enzyme; DPA, dipicolinic acid; GR, germinant receptor; ONT, Oxford Nanopore Technologies; PYGS, peptone, yeast extract, glucose, starch broth.

The GenBank BioProject accession number for the updated genome sequence versions of clostridia isolates described in this paper is PRJNA574489.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Three supplementary tables and one supplementary figure are available with the online version of this article.

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Impact Statement

In this work, we present improved genome assemblies for clostridia species associated with meat spoilage. Our comparative analysis of the improved assemblies provided further resolution of the phylogenetic relationships of the genus *Clostridium* with a focus on identification of genes encoding spore germination functions.

Spore germination occurs when spores sense specific molecules in the environment [8]. Following germination, bacterial cells are more susceptible to inactivating processes such as heat, desiccation, chemical sanitization and UV radiation [9, 10]. Currently, inactivation of spores relies on the use of strong disinfectants or toxic chemicals, including 10% bleach solutions and fumigation with oxidating agents [11]. These strategies can be challenging to implement over large areas or on porous surfaces. Historically, due to the lack of genetic systems, our understanding of the mechanisms of spore germination in *Clostridium* species has lagged far behind that of the genus *Bacillus* [12–15].

Genome sequences are rapidly becoming available for many different clostridia [16–18], and recently we have gathered genome sequences of several *Clostridium* species involved in meat spoilage [4, 19–25]. These genomes will yield important information about the modes of germination that will inform the development of germination induction strategies for a group of economically important spoilage organisms for the New Zealand meat industry. Until recently, germination of Gram-positive anaerobic spore-formers was thought to be similar to that of *Bacillus subtilis* [26]. However, studies in *Clostridium sporogenes* and *Clostridioides difficile* have identified two different mechanisms of spore germination. *C. sporogenes* follows a similar germination pathway to that of *B. subtilis*, whereas a different pathway has been observed in *C. difficile* that has not been observed in any other endospore-forming organisms to date [27]. Determination of the genomic conditions for germination in meat spoilage clostridia may allow for the identification of candidate spore germinants.

Triggers of spore germination in *Clostridium* are usually low-molecular-weight biomolecules found in the environment and commonly include amino acids and other molecules (cholesterol-based compounds, organic acids, nucleosides, peptidoglycan fragments, etc.). Once triggered, spore germination is initiated via a germinant receptor (GR) followed by the release of dipicolinic acid (DPA) into the core of the developing spore via the SpoVA proteins [15]. Release of DPA results in partial hydration of the core and subsequent activation of the cortex-lytic enzymes (CLEs) CwlJ, SleB-C or Csp1-C [8, 9]. The spore cortex is then broken down by the CLEs that allows additional core hydration and facilitates core expansion, and thus completion of germination and initiation of spore outgrowth. However, the germinant systems and mechanisms differ within the genus *Clostridium* and in some cases within species.

To identify the known genes responsible for germination, we incorporated long-read (MinION) technology to re-sequence and improve the quality of recent genomes sequenced, combined with an in-depth comparative *in silico* genomics analysis of selected meat-associated clostridia. This study aimed to apply a genomics-based approach to investigate the occurrence of genes encoding germination of clostridia spores associated with meat spoilage. This approach can be adopted to control other spore-forming bacteria associated with other food systems, and human or veterinary health.

METHODS

Bacterial cultivation and growth conditions

The methods for isolation and cultivation of the various meat spoilage-associated *Clostridium* species (strains FP1, FP2, FP3, FP4 and M14) have been previously detailed [4, 28]. *C. estertheticum* strains DSM 14864^T and DSM 8809^T, as well as *C. bowmanii* DSM 14206^T were acquired from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. All cultures were retrieved from storage, grown anaerobically at 10 °C in pre-reduced peptone, yeast extract, glucose, starch broth (PYGS) [29], and culture purity was checked by plating. The PYGS was pre-reduced by boiling to remove oxygen and then adding the reducing agent cysteine-HCl. Following autoclaving the PYGS medium was placed into the anaerobic cabinet to reduce further overnight.

Preparation of genomic DNA and whole-genome sequencing

Genomic DNA was extracted from freshly grown cells using a modification of the phenol–chloroform procedure [4]. Bacterial strain identity was verified by 16S rRNA gene amplification [30]. Total DNA concentrations were determined using a NanoDrop ND-1000 (Thermo Scientific) and a Qubit Fluorometer dsDNA BR Kit (Invitrogen), in accordance with the manufacturer's instructions. Genomic DNA integrity was verified by agarose gel electrophoresis, NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and the Qubit dsDNA broad-range (BR) assay kit (Life Technologies).

Long-read genome sequencing and assembly

Long-read sequencing using the Oxford Nanopore Technologies (ONT) MinIon was carried out on the New Zealand meat spoilage strains FP1, FP2, FP3, FP4 and M14, as well as the *C. estertheticum* strains DSM 14864^T and DSM 8809^T. Libraries were prepared using the ONT rapid barcoding kit (SQK-RBK004) and sequencing was carried out using a MinION Mk 1B device with a SpotON R9 flow cell (version FLO-MIN106D). Sequencing was followed by base-calling using Guppy v.5.0.7. Reads were demultiplexed using qcat v.1.1.0 (ONT), trimmed using Porechop v.0.2.4 and filtered using Filtrlong v.0.2.0. Genome assembly was carried out using Unicycler v.0.4.9b [31] in hybrid mode using previously sequenced Illumina reads [19–25].

Genome sequences

The genome sequences of the seven meat spoilage *Clostridium* strains [five strains previously isolated from New Zealand vacuum packed venison and lamb (FP1, FP2, FP3, FP4, M14) and two strains (*C. estertheticum* DSM 14864^T and DSM 8809^T) from spoiled beef] were compared with the genome sequences of 33 other strains from the genus *Clostridium* previously sequenced by other institutes (Table S1, available in the online version of this article) [32–37]. All these genomes were re-annotated using Prokka v.1.13.3 [38].

Comparative pan genome analysis

The program Roary v.3.8.2 [39], using the Prokka-generated GFF file (this is in GFF3 format and comprises both the annotation and sequence for each CDS [38]) as the input, was used to generate a core-genome nucleotide sequence alignment using PRANK. A neighbor-net was constructed in Splits Tree v.4.14.8 [40], using the core-genome alignment.

Comparative genomics of the germination genes were carried out using the OrthoMCL algorithm from the tool GET_HOMOLOGUES v.3.4.2 [41], where a default E-value of 10^{-05} and a coverage of 20% was used. Previously characterized germination genes (Table S2) concatenated in Geneious v.9.1.8 [42] and exported as a GBK file were used as the reference. Nucleotide and amino acid orthologous clusters were generated. The additional script 'compare_clusters.pl' within GET_HOMOLOGUES was used to generate a pangenome matrix using the amino acid sequence clusters only. A heatmap and dendrogram were generated to visualize the number of homologues for each germination gene using the heatmap.2 function in the gplots library of R (v.4.1.2), visualized in Rstudio (v.2021.09.1). The dendrogram was generated using the euclidean measure to obtain a distance matrix and the complete agglomeration method for clustering. To examine whether there was any correlation between the core genome phylogeny and the dendrogram representing the germination gene presence, a tanglegram was generated using the tanglegram function in the dendextend library of R.

The *gerX* operons were identified using both the annotations generated by Prokka and the orthologous clusters containing the reference *gerX* genes using GET_HOMOLOGUES, then extracted using Geneious (v.9.1.8). The GerA, GerB and GerC proteins were aligned using the default settings of MUSCLE in MEGA v.10.0.5 [43] and a phylogenetic tree was generated using the maximum-likelihood method with the Jones–Taylor–Thorton substitution model.

RESULTS AND DISCUSSION

To gain an understanding of whether genome phylogeny was related to the number of germination gene orthologues and type of germination receptors in *Clostridium* species associated with meat spoilage, a comparative genomics approach was taken. To improve the quality of the assemblies we undertook an illumina short-read and ONT long-read hybrid approach on five strains previously isolated from New Zealand vacuum packed venison and lamb, as well as the type strains *C. estertheticum* DSM 14864^T and DSM 8809^T (Table S3). These strains were compared with 33 strains from ten *Clostridium* species previously associated with meat spoilage [5, 6, 44–49].

Core genome analysis

Roary was used to carry out a pan-genome analysis of the 40 *Clostridium* strains and *B. subtilis* 168, with 111 genes identified in the core genome. To examine the genomic phylogeny of these strains, a phylogenetic neighbour-network was generated using the core genome alignment (Fig. 1). In agreement with previous studies, our analysis grouped these genomes into two distinct groups. *C. estertheticum*, *Clostridium tagluense*, *Clostridium bowmanii* and *Clostridium frigoris* along with strains FP3, CF012, FP4, CM028 and CM027 formed one group (A; (a) in Fig. 1). *Clostridium botulinum*, *Clostridium perfringens*, *C. gasigenes*, *Clostridium frigidicarnis*, *Clostridium algidicarnis* and *Clostridium putrefaciens* along with strain M14 formed a second group (B; (b) in Fig. 1). The New Zealand strains FP1 and FP2 sat within the *C. tagluense* group, whereas strains FP3, FP4 and M14 formed distinct subgroups. FP3 and FP4 were most closely related to *C. tagluense* (type strain A121) and *C. bowmanii* (type strain DSM 14206) respectively. Similarly, M14 was most closely related to *C. gasigenes*. The species boundaries identified in our study (as indicated by the coloured ovals in Fig. 1) agree with those proposed by previous comparative genomics analyses using average nucleotide identify (ANI) [4, 17]. Similarly, in these same studies the three strains FP3, FP4 and M14 have been proposed as representing new taxa. However, phenotypic analyses are required to confirm this proposition.

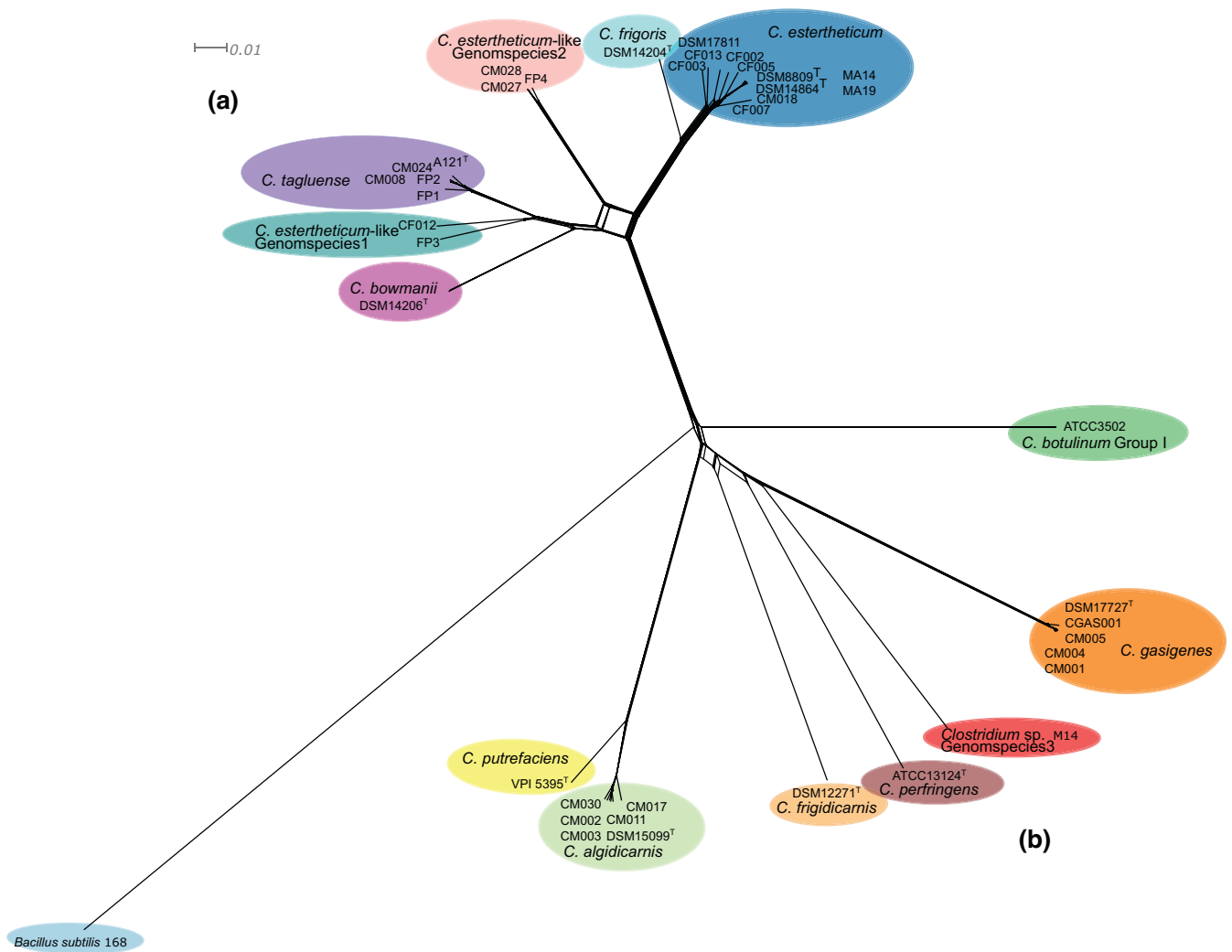


Fig. 1. Core genome comparison of 40 *Clostridium* strains and one *Bacillus subtilis* strain. A phylogenetic network was constructed in SplitsTree using a core-genome alignment generated by Roary (v.3.8.2). Bar, 0.01 nucleotide changes per position.

Conservation of germination genes in meat spoilage *Clostridium* species

Germination comprises five main stages: (1) germination recognition, (2) germination activation, (3) DPA release, (4) cortex hydrolysis and (5) spore hydration [50, 51]. Experimental studies suggest *C. sporogenes* follows the order of this pathway and is similar to *Bacillus* species [52]. By contrast, cortex degradation begins before DPA release in *C. perfringens* and *C. difficile* [53–55]. In our study, differences in the number and type of germination genes conserved by the different meat spoilage *Clostridium* species suggests that there are differences in the germination pathway. However, the number and type of germination genes conserved within each strain did not necessarily relate to its core genome phylogeny. This relationship between core genome phylogeny and the conservation of germination genes was depicted using a tanglegram (Fig. S1), with a cophenetic correlation coefficient of 0.36. All of the germination genes detected were present on the chromosome for those strains sequenced using ONT long-read sequencing.

During the first stage of germination, a germinant is recognized by a germination receptor. Germination receptors are generally made up of three proteins: GerA, GerB and GerC [50]. Strain CF003 had the greatest number of *gerX* genes, with seven *gerXA*, 12 *gerXB* and nine *gerXC* orthologues.

In the model organism *B. subtilis*, DPA uptake during sporulation and DPA release during germination are associated with the *spoVA* operon (*spoVAA*, *spoVAB*, *spoVAC*, *spoVAD*, *spoVAEa*, *spoVAEb*, *spoVAF*) [56–58]. Three of these genes, *spoVAC*, *spoVAD* and *spoVAE*, are conserved in *Clostridium* species [59, 60]. Few studies have investigated the function of SpoVA proteins in *Clostridium*, but one study suggests that SpoVAC is a ‘mechanosensing’ protein, and in *C. difficile* senses changes in osmolarity

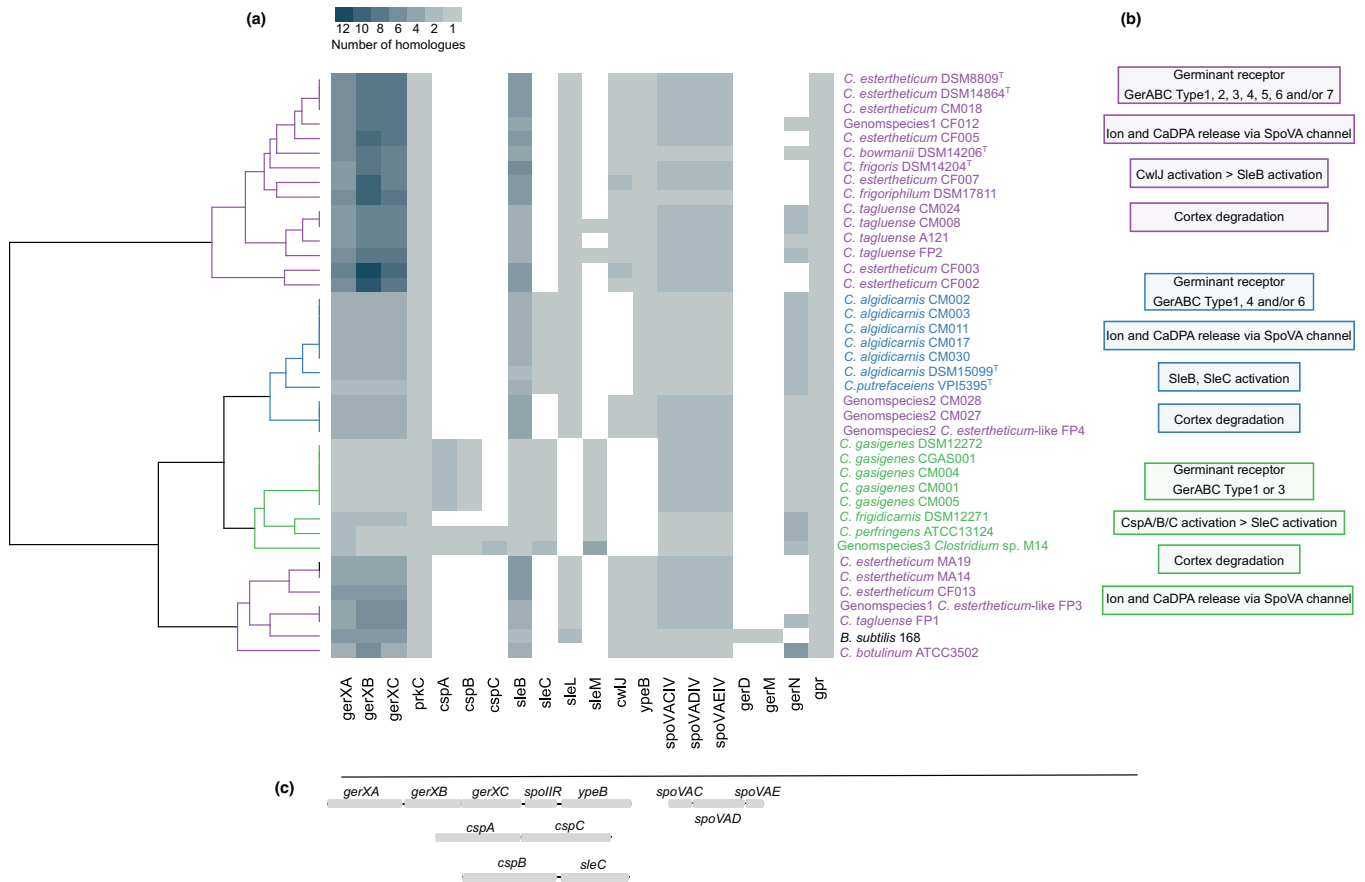


Fig. 2. Conservation of selected germination genes in *Clostridium* species associated with meat spoilage. This heat-map shows 20 genes involved in spore germination (a). The presence of a homologue was determined by using the OrthoMCL algorithm in the GET_HOMOLOGUES tool. No homologues were identified in the *Clostridium* genomes of the additional representative genes from *B. subtilis*: *gerPA*, *gerPB*, *gerPC*, *gerPD*, *gerPE*, *gerPF*, *yfkQ*, *yfkR*, *yfkT*. (b) Three potential pathways as represented by each colour. (c) Those genes that are linked.

resulting in release of DPA [61]. However, in *C. perfringens* the *spoVA* operon is not essential for germination [62]. *C. gasigenes* strains as well those strains belonging to group A (Fig. 1), which included *C. estertheticum*, *C. friginis*, *C. bowmanii* and *C. tagluense* species, all harboured two copies of the *spoVA* operons (Fig. 2). The presence of two *spoVA* operons has been found in some strains of *C. botulinum* and *C. sporogenes* as well as various *Bacillus* species and has been associated with increased heat resistance [63, 64].

In *B. subtilis* and *C. sporogenes*, cortex degradation occurs through activation of the cortex lytic enzymes CwlJ and SleB followed by peptidoglycan degradation [52, 65–67]. Studies suggest that YpeB is required for SleB activation [66, 68]. Orthologues of *ypeB* as well as *sleB* and *cwlJ* were present in all the strains belonging to group A (Fig. 2), suggesting that cortex degradation is initiated via at least the SleB/YpeB pathway. A previous study demonstrated that although some strains of *C. botulinum* harbour both *sleB* and *cwlJ* genes, CwlJ was not crucial for germination [68]. In *C. perfringens*, the cortex lytic enzyme SleC is activated by a protease CspB [69, 70]. In our analysis, strain M14 and all the *C. gasigenes* genomes contained both an *sleC* and *cspA* homologue, suggesting these strains follow a similar germination pathway to that of *C. perfringens*.

Germination gene clusters

To investigate the diversity of germination receptor operons of meat spoilage clostridia, we searched the genomes of the five New Zealand meat spoilage strains along with strain CF003 and the three strains *C. estertheticum* DSM 14864, *C. tagluense* A121 and *C. gasigenes* CGAS001 for *gerX* operons. Five different putative *gerX* configurations were identified (types 1, 3, 4, 5 and 6), along with a lone *gerA* in M14 and a lone *gerB* in strain CF003 (Fig. 3). Three of these configurations (types 1, 3 and 4) have been previously identified in a variety of *Clostridium* species, including *C. botulinum*, *Clostridium kluveri*,

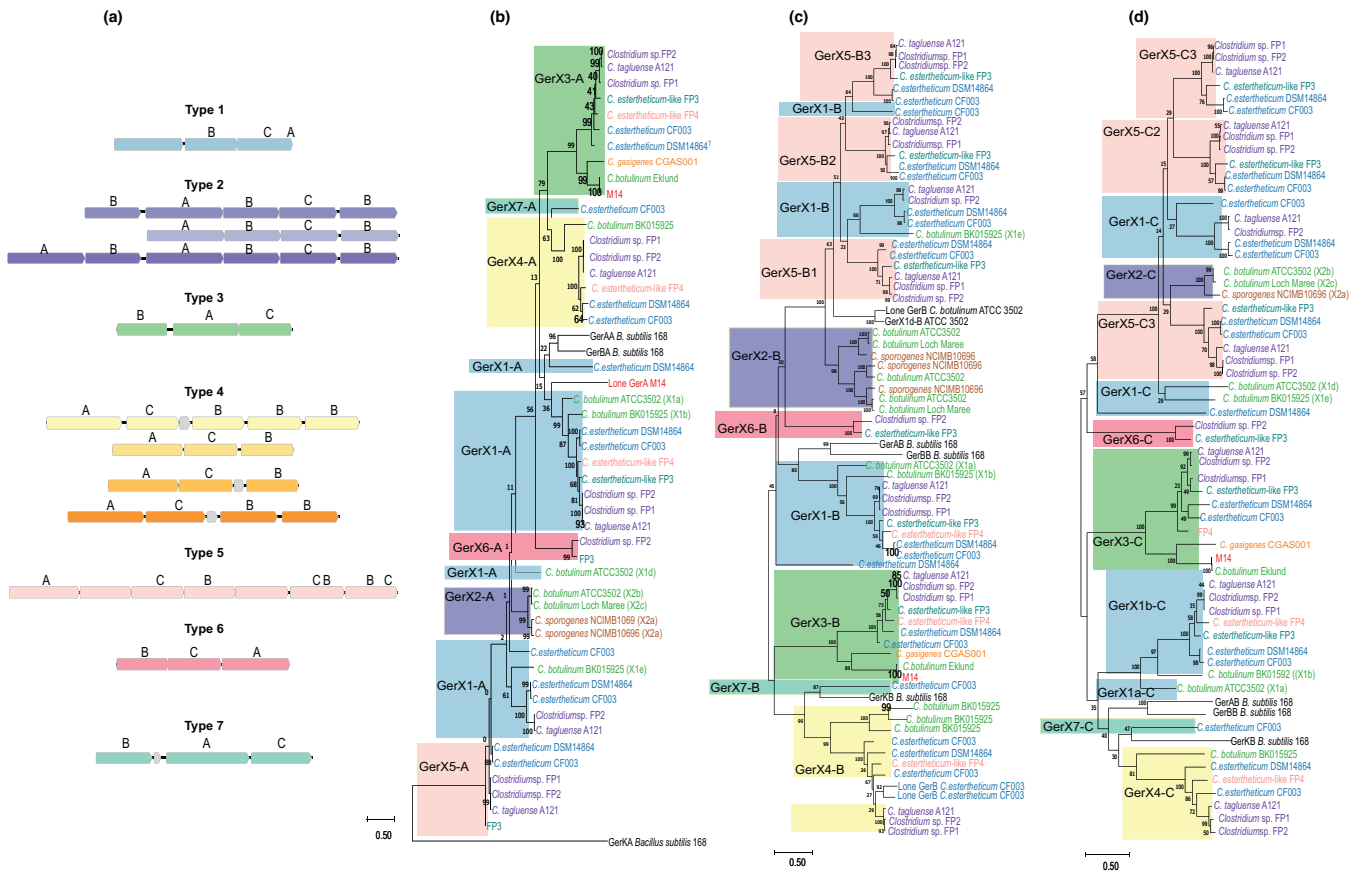


Fig. 3. Germination receptor *gerX* operon configurations in selected strains of *Clostridium* associated with meat spoilage. The *gerX* operon configurations (a) were designated types 1–7 based on the classifications defined previously [59]. Maximum-likelihood phylogenetic trees of the GerA (b), GerB (c) and GerC (d) amino acid sequences from the *C. estertheticum* strains DSM 14864^T and CF003, the *C. tagluense* strains A121, FP1 and FP2, the *C. gasigenes* strain CGAS001, the New Zealand strains FP3, FP4 and M14, as well as the reference strains *C. botulinum* ATCC 3502^T, BKT015925, Loch Maree, Ekland 17-B, *C. sporogenes* NCIMB10696 and *B. subtilis* 168. The GerX type is denoted by the number after the X and the colour block represents each different *gerX* configuration. The scale bar is the number of substitutions per site.

Clostridium tetani and *C. perfringens* [59, 71]. To our knowledge, the configuration types 5 and 6 have not previously been identified. Type 5 has a *gerABCBCCB* configuration and was identified in three of the New Zealand meat spoilage strains (FP1, FP2, FP3), as well as the strains *C. estertheticum* DSM 14864 and *C. gasigenes* CGAS001 (Fig. 3). Multiple *gerB* genes have been identified in other *gerX* configurations, but to our knowledge this is the first time a putative *gerX* operon with multiple *gerC* genes has been identified. Type 6 has a *gerBCA* configuration and was identified in two New Zealand meat spoilage strains, FP2 and FP3, but not in any of the reference strains. Interestingly GerB from the BCA cluster was in the same orthologous group as GerB from the ACB configurations as defined using GET_HOMOLOGUES. Strain M14 was unusual in that it contained one putative *gerBAC* operon and a lone *gerA* gene. A lone GerA in *C. botulinum* has previously been shown to be required for L-alanine-initiated germination [72].

The *gerX* operons have previously been grouped into subtypes based on the similarity of the amino acid sequences [59]. In agreement with previous results [59], amino acid sequences encoding the Ger A, B and C proteins clustered by type or subtype not by strain (Fig. 3b). The *gerX* type 1 configurations ABC were distributed throughout, with them grouping by subtype. All the strains (except for M14) harboured at least one type 1 operon. Type 1 germination receptors have been shown to sense a variety of germinants, including L-alanine, L-cysteine, L-methionine, L-serine and L-phenylalanine (in the presence of L-lactate) [73].

Our findings suggest that *Clostridium* species follow two germination pathways (Fig. 4). The first pathway recognizes germinants through multiple receptors, and cortex degradation occurs via the *cwlJ/sleB* pathway similar to that of *B. subtilis*. The

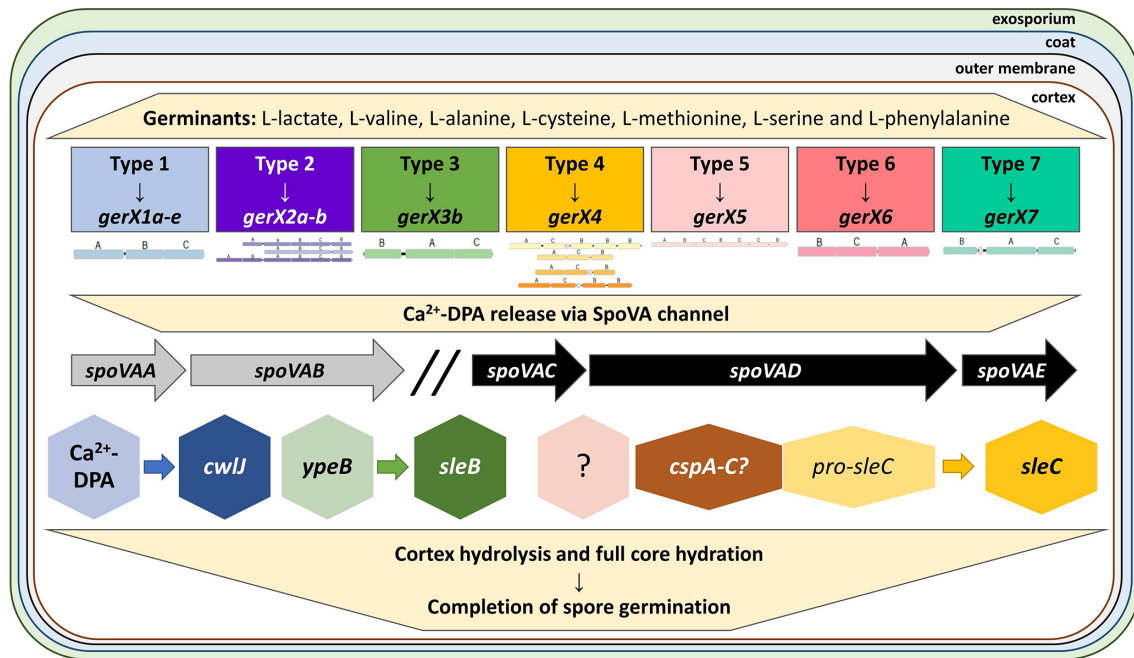


Fig. 4. Schematic depiction for the proposed pathways during the germination of spores of *Clostridium* species associated with meat spoilage.

second pathway recognizes germinants through the GerX1 or GerX3 type receptors with cortex degradation occurring through the *cspA-C/sleC* pathway.

CONCLUSIONS

This study was carried out to determine whether a genomics approach could be used to provide insights into the possible molecular mechanisms of germination in meat spoilage clostridia. The core components of the germination pathway were conserved in these *Clostridium* species, indicating that they undergo the same major steps as *B. subtilis* for sporulation to occur. A key difference between *C. gasigenes* and the *C. estertheticum/C. tagluense* cluster strains was the presence of the gene *sleC* coding for a cortex lytic enzyme in *C. gasigenes* strains but not strains from the *C. estertheticum/C. tagluense* cluster, suggesting *C. gasigenes* strains follow a germination pathway similar to that of *C. perfringens*. Two additional putative *ger* operon configurations were identified in some strains of *C. estertheticum* and *C. tagluense*. Experimental data are needed to determine whether these additional *ger* gene clusters recognize alternative germinants.

Funding information

This work was funded by the AgResearch Ltd Strategic Science Investment Fund (SSIF), contract number A25980.

Acknowledgements

We thank Patrick Biggs for advice on bioinformatics methodologies as well as the Food Integrity Systems Team, AgResearch and the ^mEpiLab, Massey University, for supporting Dr Burgess' post-doctoral research fellowship.

Author contributions

S.B., F.P.P., A.G., J.M., G.B. and N.P. were involved in the investigation; S.B. and N.P. performed the formal analysis; J.M., G.B. and N.P. were involved in the funding acquisition and conceptualization of the study; S.B. and N.P. drafted the manuscript; S.B., F.P.P., A.G., J.M., G.B. and N.P. revised this manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Occurrence of genes encoding spore germination in *Clostridium* species that cause meat spoilage.

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2022-02
