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Clostridial spore germination versus bacilli: Genome mining and current insights

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ABSTRACT

Bacilli and clostridia share the characteristic of forming metabolically inactive endospores. Spores are highly resistant to adverse environmental conditions including heat, and their ubiquitous presence in nature makes them inevitable contaminants of foods and food ingredients. Spores can germinate under favourable conditions, and the following outgrowth can lead to food spoilage and foodborne illness. Germination of spores has been best studied in *Bacillus* species, but the process of spore germination is less well understood in anaerobic clostridia. This paper describes a genome mining approach focusing on the genes related to spore germination of clostridia. To this end, 12 representative sequenced *Bacillus* genomes and 24 *Clostridium* genomes were analyzed for the distribution of known and putative germination-related genes and their homologues. Overall, the number of *ger* operons encoding germinant receptors is lower in clostridia than in bacilli, and some *Clostridium* species are predicted to produce cortex-lytic enzymes that are different from the ones encountered in bacilli. The *in silico* germination model constructed for clostridia was linked to recently obtained experimental data for selected germination determinants, mainly in *Clostridium perfringens*. Similarities and differences between germination mechanisms of bacilli and clostridia will be discussed.

1. Introduction

Spore-forming bacteria play an important role in food spoilage and disease (Setlow and Johnson, 2007) and food industries actively employ strategies to ensure adequate inactivation of spores and control outgrowth, both for species that potentially lead to spoilage and foodborne pathogens. With regard to bacterial foodborne disease, two major bacterial spore-forming species, namely *Bacillus cereus* and *Clostridium perfringens*, are accountable for an estimated 1.3% and 4.0% of cases, respectively (EFSA report, 2005).

Survival and persistence of *Bacillus* and *Clostridium* species largely depends on their ability to produce endospores under conditions that are unfavourable for growth, whereas their pathogenicity resides in the fact that the spores can germinate under favourable conditions. The very first stage of the germination process involves sensing specific compounds, named germinants, or can be due to physical factors. Subsequent events required for full germination include the hydrolysis of the cortex peptidoglycan, rehydration of the core, and resumption of metabolic activity and the degradation of small acid-soluble proteins (SASPs) by germination protease GPR (Sussman and Setlow, 1991; Moir, 2006; Setlow, 2008).

The process of spore germination is irreversible and eventually results in a complete and viable vegetative cell. The first detectable events of germination are the release of Zn^{2+}, K^+, Na^+, dipicolinic acid and Ca^{2+} (Ca-DPA) and a rise in spore internal pH (Paidhungat et al., 2002). Potassium ions are subsequently reabsorbed by an energy-dependent process (Swerdlow et al., 1981). The initial events are accompanied by a loss of spore heat resistance and dormancy, and can be recognized microscopically by the transition from phase bright to phase dark. Nutritional inducers of germination include L-alanine and a combination of L-asparagine, D-glucose, D-fructose, K^+ (AGFK) for *Bacillus subtilis* (Moir and Smith, 1990), and L-alanine and inosine for *B. cereus* (Barlass et al., 2002; Hornstra et al., 2005). Non-nutritional germinants include chemicals such as dodecylamide and Ca-DPA. Also, peptidoglycan fragments have recently been shown to induce germination (Shah et al., 2008). Other non-nutritional physical factors that can trigger germination include high hydrostatic pressure (HHP), heat, abrasion and ageing (Raso et al., 1998; Moir et al., 2002). Spore germination in clostridia often involves a combination of nutrient germinants and generally proceeds more slowly than *Bacillus* species (Peck, 2009). Similar to

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germination in bacilli, non-proteolytic *Clostridium botulinum* spores can germinate in response to L-alanine and various other amino acids and nutrients such as sugars, lactate and nicotinamide (Plowman and Peck, 2002). For *C. perfringens* spores it has been established that the following compounds can trigger germination: L-asparagine, KCl, a mixture of L-asparagine and KCl, Ca-DPA and a mixture of Na^+ and inorganic phosphate (NaPi) (Paredes-Sabja et al., 2008b, 2009d). The cholate derivatives and the amino acid glycine in bile act as cogerminants of *Clostridium difficile* spores (Sorg and Sonenshein, 2008). *Clostridium tetani* spore germination has been reported to be triggered by a mixture of methionine, lactate, nicotinamide and Na^+ in phosphate buffer (Shoesmith and Holland, 1972).

At a molecular level the process of spore germination is better understood in *Bacillus* species than in their anaerobic relatives, thanks to decennia of research on model organisms such as *B. subtilis* and *B. cereus*, which are genetically accessible and for which whole genome sequences have been available, with *B. subtilis* being amongst one of the first bacterial genome sequences to be completed (Kunst et al., 1997). In the last decennium, the complete genomes of a number of *Clostridium* species have been sequenced and annotated, enabling comparisons of genes involved in spore germination of *Bacillus* and *Clostridium* using comparative genomics approaches. In the present study, the occurrence of known and putative *Bacillus* germination-related genes was analyzed in *Clostridium* species, and in a representative number of *Bacillus* species. The presence of genes involved in germination will be discussed with a particular focus on the clostridia.

2. Materials and methods

The occurrence of known and putative *Bacillus* germination-related genes was analyzed in *Clostridium* species by searching for homologues in 24 sequenced *Clostridium* genomes and 12 sequenced *Bacillus* genomes, representing a total of 47 *Bacillus* genomes (Table 1). For *Bacillus*, 12 genomes were selected; these selected genomes generally cover two main *Bacillus* groups, namely, the *B. subtilis* group and *B. cereus* group, according to a taxonomy review on aerobic spore-forming bacteria (Fritze, 2004) and an article on the ecological diversity of *B. cereus* group strains (Guinebretière et al., 2008). Another selection criterion was the way in which gene annotation of sequenced genomes had been performed, with a preference for manually verified annotations (not merely performed by software).

A list of genes encoding germination-related proteins of *Bacillus* species was generated based on published literature (Table 2). The amino acid sequences of these proteins were used to identify putative homologues in the *Bacillus* and *Clostridium* genomes listed in Table 1. The data were extracted using the genetic analysis platform ERGO (Overbeek et al., 2003) and using the BLAST cut-off scores listed in Table 2. Meanwhile, domain analysis was performed via Pfam (Finn et al., 2008) and transmembrane helices were identified using TMHMM (Krogh et al., 2001). The identified sequences were aligned using Clustal ×2 (Larkin et al., 2007). Bootstrapped Neighbour Joining Trees were generated via Clustal ×2 and the sequences were classified using LOFT (van der Heijden et al., 2007). When necessary, sequences were edited using Jalview (Waterhouse et al., 2009). Finally, the genomic context of the related gene clusters, (i.e. the *ger* clusters or *csp* clusters), was used to further classify the identified homologues.

3. Results and discussions

The distribution of germination-related genes in sequenced genomes of *Bacillus* and *Clostridium* species has been investigated in this study and the outcome has been linked with experimentally obtained data that is available for only a sub-set of the related species (as discussed in detail below). Based on intensive studies on the model species *B. subtilis* and *B. cereus*, a model of physiological germination was put forward that includes interaction between germinants and specific receptors (germination receptors), ion fluxes during germination and cortex enzymatic lysis (Moir, 2006). Spores of *Bacillus* and *Clostridium* species have comparable multilayer structures and their germination and outgrowth shows similar morphological events. The genes and their encoded proteins can be categorized into three main function groups, involving (1) environmental sensing, (2) cortex degradation and (3) hydrolysis of core proteins. In the following sections, the function and role of each member of these distinct functional groups will be described with emphasis on similarities and differences in their presence in *Clostridium* versus *Bacillus*.

3.1. Environmental sensing

3.1.1. Specific spore germination receptors

Germination in response to nutrients is mediated by receptors that reside in the inner spore membrane of *B. subtilis* spores (Hudson et al., 2001; Paidhungat and Setlow, 2001). The germination receptor is composed of 3 proteins (A, B and C), normally encoded within a tricistronic operon e.g. the *gerA* operon. Screening of *B. subtilis* mutants defective in germination identified the presence of three homologous gene clusters: the *gerA* operon (L-alanine response) (Moir et al., 1979), the *gerB* operon and the *gerK* operon (AGFK response) (Corfe et al., 1994). Secondary structure analyses of the gene products of these operons suggested that GerAA contains five to six transmembrane helices, that GerAB is a member of the amino acid/polyamine/organo-cation (APC) superfamily of transporters (subfamily of spore germination proteins) containing ten membrane-spanning regions (Jack et al., 2000), while GerAC is a lipoprotein (Zuberi et al., 1987). In *Bacillus* species, all three subunits are believed to be essential for receptor function.

We used a component A–C crossing approach to identify putative *ger* operons in sequenced genomes and took the genetic context of gene clusters into account. Thereby only operons containing component A or C were considered as germinant receptor operons. Spores of *Bacillus* species can contain 4 to 8 different germinant receptor operons with different organisation of genes. Our analysis of the sequenced whole genomes of *Clostridium* species revealed lower numbers of genes encoding germination receptors than in *Bacillus* species, while in addition many incomplete operons or operons with unusual structures were found compared with the *Bacillus* ABC tricistronic operons (summarized in Fig. 1).

Recent functional studies on *ger* mutants in *C. perfringens* SM101 demonstrated that not all three subunits are required for receptor functionality. This food-poisoning strain, which produces *C. perfringens* enterotoxin (CPE), contains a bicistronic operon, *gerKA-KC*, and oriented in the opposite direction, an upstream monocistronic gene *gerKB*. In addition, this strain contains an orphan GerA homologue, named *gerA*. Deletion or interruption of these genes in strain SM101 demonstrated an essential role for *gerKA* and *gerKC* in potassium-initiated spore germination (Huang et al., 2007; Paredes-Sabja et al., 2008b), whereas *gerKB* and *gerA* only played an auxiliary role in spore germination under some conditions (Paredes-Sabja et al., 2008a, 2009c). Our *in silico* analysis showed that these four genes are conserved in the other two available *C. perfringens* genomes (gas gangrene strains strain 13 and ATCC 13124), but their exact function in these non-food-poisoning strains has not been established experimentally. The divergon-like structure of the *C. perfringens gerK* operon has been found in nine other