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C0013 The Genus *Geobacillus* and Their Biotechnological Potential

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Abstract

The genus *Geobacillus* comprises a group of Gram-positive thermophilic bacteria, including obligate aerobes, denitrifiers, and facultative anaerobes that can grow over a range of 45–75 °C. Originally classified as group five *Bacillus* spp., strains of *Bacillus stearothermophilus* came to prominence as contaminants of canned food and soon became the organism of choice for comparative studies of metabolism and enzymology between mesophiles and thermophiles. More recently, their catabolic versatility, particularly in the degradation of hemicellulose and starch, and rapid growth rates have raised their profile as organisms with potential for second-generation (lignocellulosic) biorefineries for biofuel or chemical production. The continued development of genetic tools to facilitate both fundamental investigation and metabolic engineering is now helping to realize this potential, for both metabolite production and optimized catabolism. In addition, this catabolic versatility provides a range of useful thermostable enzymes for industrial application.

A number of genome-sequencing projects have been completed or are underway allowing comparative studies. These reveal a significant amount of genome rearrangement within the genus, the presence of large genomic islands encompassing all the hemicellulose utilization genes and a genomic island incorporating a set of long chain alkane monooxygenase genes. With G + C contents of 45–55%, thermostability appears to derive in part from the ability to synthesize protamine and spermine, which can condense DNA and raise its *T*m.

s0010

1. INTRODUCTION

p0010

Geobacillus spp. are Gram-positive thermophilic aerobic or facultatively anaerobic spore forming bacilli. Prior to 2001, they were grouped together as thermophilic variants of *Bacillus* spp., but accumulating evidence for clustering of many of the thermophiles in a separate subgroup (group 5) supported by 16SrRNA analysis led to their reclassification as a separate genus, *Geobacillus* gen. nov. (Nazina et al., 2001), with *Geobacillus stearothermophilus* as the type strain (note that the moderate thermophiles *Bacillus*

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caldotenax and *Bacillus smithii*, which fell into group 1, are excluded from this group). The majority of *Geobacillus* strains grow over the temperature range 45–70 °C and, being catabolically diverse, they are readily isolated from active communities growing in compost, hot springs and deep geothermal sites, including oil wells and deep sediments. However, it has long been known that *Geobacillus* spp. can be isolated from a wide range of moderate- and low-temperature environments including temperate soils, and have also been isolated from low-temperature environments such as the Bolivian Andes, deep sea water, and even the Mariana Trench. Indeed, they can be isolated in significant numbers from sites that rarely, if ever, exceed 30 °C. This paradoxical distribution has recently been analyzed by Zeigler (2014) who attributes it largely to the properties of *Geobacillus* spp. spores. It is well established that *Bacillus* and *Geobacillus* spores are extremely resistant to ultraviolet (UV) light, desiccation, and thermal inactivation (Setlow, 2006), the latter property lending itself to the use of these spores as viability indicators for the effectiveness of heat sterilization methods. Furthermore, once released into the lower atmosphere, their small size allows the spores to remain airborne for considerably longer than a typical bacterium. Indeed, there is known to be a "scavenging gap" that allows particles of about 1 μm in diameter to remain suspended in the atmosphere for disproportionately long times (Burrows, Elbert, Lawrence, & Poschl, 2009), which leads to an intriguing suggestion that the size of bacterial spores may have been under evolutionary pressure for their ability to disperse more effectively. The ability to remain suspended for long periods, certainly sufficient for intercontinental transit, can readily explain the widespread distribution of *Geobacillus* spp. spores to regions where they are incapable of reproduction. However, the relatively high abundance of *Geobacillus* spp. in these environments (e.g., 10^4–10^5 m^{-2} in some Northern Ireland soil; Marchant et al., 2008) is a little more difficult to explain. Clearly, spores can remain viable for a considerable time and, assuming they can withstand the accumulated UV damage, an estimation of their thermal inactivation time in temperate climates based on extrapolating the effect of temperature on their D-value (decimal reduction time) gives a figure in excess of a billion years (Nicholson, 2003)! So a picture is gradually emerging of a genus that probably reproduces explosively on composting vegetation and more gradually in other geothermal environments, and then forms spores that can be easily distributed over wide distances and remain dormant but viable for extensive periods.

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2. PHYLOGENY AND GENOMIC ANALYSIS

p0015 Nazina et al. (2001) divided the genus *Geobacillus* into eight species, based on a combination of 16SrRNA gene sequence analysis and a variety of physical and biochemical characteristics. Two were novel species, namely, *Geobacillus subterraneus* and *Geobacillus uzenensis*, while six were transferred from group 5 of the genus *Bacillus*, namely, *G. stearothermophilus*, *Geobacillus thermoleovorans*, *Geobacillus thermocatenulatus*, *Geobacillus kaustophilus*, *Geobacillus thermoglucosidasius*, and *Geobacillus thermodenitrificans*. Over the subsequent years, a number of new species have been proposed based primarily on 16SrRNA gene sequence differences, but this has been shown to be a poor discriminator for detailed classification, with the *recN* gene (or a combination of 16SrRNA and *recN* gene sequences) being a better phylogenetic marker of closely related species because of its higher rate of divergence (Zeigler, 2005). Based on *recN* analysis, nine distinct groups with strong bootstrap support were recognized. Five of these corresponded unambiguously with *G. stearothermophilus*, *Geobacillus toebii*, *Geobacillus caldoxylosylitus*, *G. thermoglucosidasius*, and *G. thermodenitrificans* while one group comprised a single strain of (*Geo*)*Bacillus thermoantarcticus*, which Ziegler has argued should be reclassified as *G. thermoglucosidasius*. The classification of the remaining three groups is a little more contentious with one group covering all three of the previously described species *G. thermoleovorans*, *G. thermocatenulatus*, and *G. kaustophilus*, which DNA–DNA hybridization has previously suggested should form a single species. The other groups cover *G. subterraneus* and *G. uzenensis*, which may be subspecies of *G. thermodenitrificans* and *G. stearothermophilus* NUB3621, which is probably a subspecies of *G. caldoxylosylitus*. Studholme (2014) has attempted to resolve the "kaustophilus clade" by core genomewide phylogenetic analysis, but given the high level of horizontal gene transfer evident in the noncore-genome, the reliability of this approach is open to question. The division into monophyletic groups based on *recN* sequence comparison shown in Figure 1 represents the most robust phylogenetic analysis available to date.

p0020 A number of genome sequences of *Geobacillus* spp. have been reported since 2004 (Studholme, 2014) allowing both analysis of potentially interesting metabolic characteristics and analysis of genome structure and dynamics. The G + C content varies between species with a clear difference between the "kaustophilus clade" of *G. thermoleovorans* (52%), *Geobacillus vulcani* (53%), *Geobacillus lituanicus* (52.5%), *G. kaustophilus* (51.9%) and

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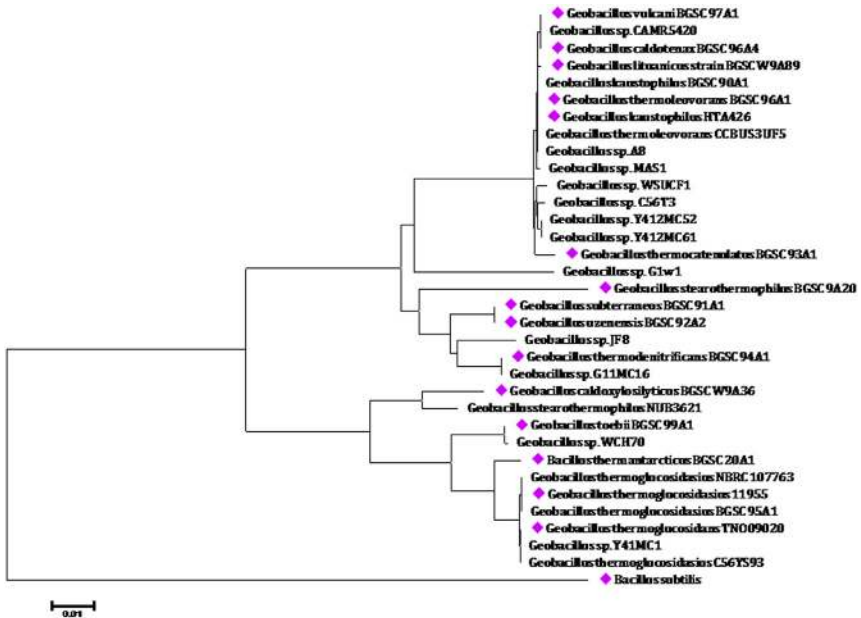

f0010 **Figure 1** Evolutionary relationships of taxon *Geobacillus*. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in [Q29] MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

G. thermocatenulatus (55%), the denitrifying *Geobacillus denitrificans* (48.9%) and the facultative anaerobes *G. caldoxylosylitus* (44%), *G. toebii* (43.9%) and *G. thermoglucosidasius* (43.9%). *Geobacillus stearothermophilus sensu strictu* [Q1] also has a ~~gas chromatography~~ content of 43.8%, close to that of *G. thermoglucosidasius* even though it groups more closely with *G. thermodenitrificans*. While it is clear that *Geobacillus* spp. do not have a high G + C content, unlike their mesophilic counterpart, they have genes for the production of protamine and spermine, small cationic histone-like proteins, which allow chromosome condensation and increase the *T*m of DNA (Takami, Takaki, et al., 2004).

p0025 The average genome size for *Geobacillus* spp. ranges from 3.5 to 3.9 Mbp. The smallest genomes can be found in the "kaustophilus clade" and the highest in the "thermoglucosidasius clade." This might reflect the additional coding requirements associated with anaerobic growth, CRISPR regions as well as genes of unassigned function found between transposable elements in the genome of *G. thermoglucosidasius*. Analysis of the number of transposases

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in the genomes of *G. kaustophilus* HTA46 (Takami, Nishi, Lu, Shimamura, & Takaki, 2004), *G. thermodenitrificans* NG80-2 (Feng et al., 2007), *G. thermoglucosidasius* C56-YS93 and *G. thermoleovorans* CCB_US.3_UF5 (Muhd Sakaff, Abdul Rahman, Saito, Hou, & Alam, 2012) showed that the highest number of IS/Transposable elements were present in the "kaustophilus clade." *Geobacillus kaustophilus* encodes 95 transposases (of which 19 are associated with IS-like elements: ISBst12, IS654, IS5377, IS642, IS604, and IS3) and *G. thermoleovorans* encodes 105 transposases with IS elements: IS204, IS5377, IS1001, IS1096, IS1165, IS605, IS654, IS605, and IS116. Clade "kaustophilus" shares the IS elements IS654 and IS5377 among all members. The *G. thermodenitrificans* genome is annotated with 14 transposases, six of them associated with the IS elements: IS426 and IS605, which indicates that *G. thermodenitrificans* and *G. thermoleovorans* both have IS605 elements. The *G. thermoglucosidasius* genome encodes 41 transposases where 39 are associated with the IS elements: IS4, IS3, IS911, IS116, IS110, IS902, and IS653. These four strains have also been analyzed for the presence of CRISPR-related sequences. Here, we found that the highest numbers of annotated CRISPR motifs are present in the *G. thermoglucosidasius* C56–YS93 genome with over 112 CRISPR-associated proteins (Cas5, Cas6, Cst1, Cas8a1, Cas3, Cas1, Cas2, Cmr3, Cmr5, Crm2, Crm1, Csx1, Csm6, Cas4, and Csh2). In comparison, *G. kaustophilus* HTA46 encodes only one annotated CRISPR-associate helicase, which is also found in *G. thermoleovorans* CCB_US.3_UF5. *Geobacillus thermodenitrificans* NG80-2 was found to encode the same helicase as these two strains together with a Cas2 protein.

s0020 2.1 Genome Organization

p0030 Comparison of the genome organization (Figure 2) of these four well-studied *Geobacillus* species (*G. kaustophilus* HTA46 (Takami, Nishi, et al., 2004), *G. thermodenitrificans* NG80-2 (Feng et al., 2007), *G. thermoglucosidasius* C56-YS93, and *G. thermoleovorans* CCB_US.3_UF5 (Muhd Sakaff et al., 2012)) shows a major rearrangement that appears to be associated with the early divergence into obligate aerobes (plus denitrifiers) and facultative anaerobes (although the latter is only represented by a single species in this analysis). Clade "kaustophilus" (*G. thermoleovorans and G. kaustophilus*) has the highest conservation of genome arrangement (Figure 2(a)) consistent with the *recN* analysis. However, when comparing *G. thermoglucosidasius* with *G. kaustophilus* (Figure 2(b)), a significant genome rearrangement is evident with maximum conservation found at the beginning, end, and a fragment of

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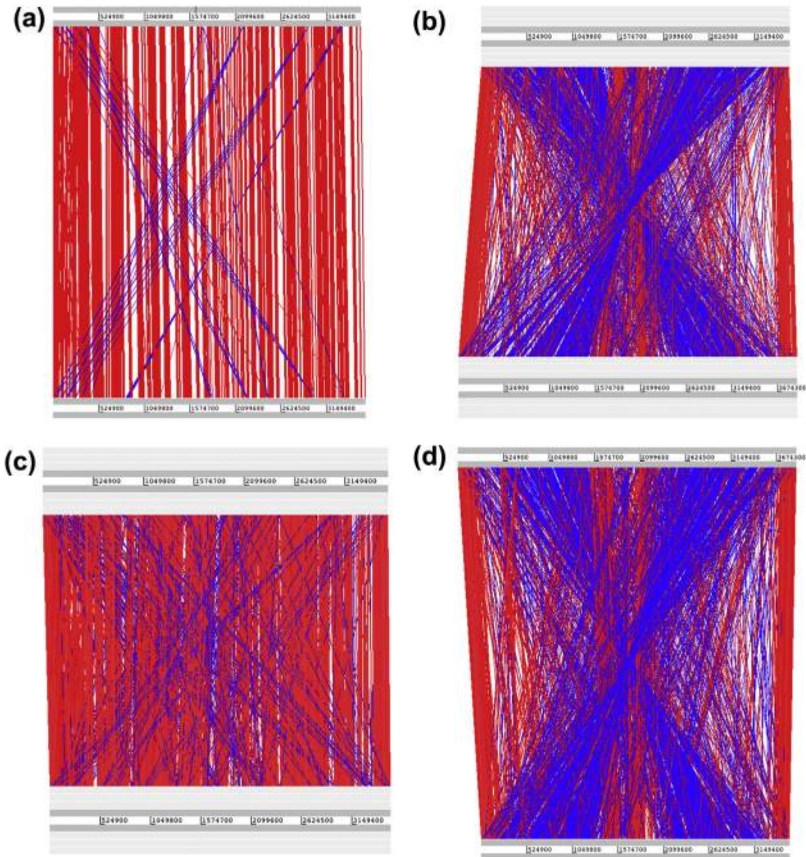

f0015 **Figure 2** Comparison of the genome arrangement between *Geobacillus kaustophilus* HTA46 (Takami, Nishi, et al., 2004), *Geobacillus thermodenitrificans* NG80-2 (Feng et al., 2007), *Geobacillus thermoglucosidasius* C56-YS93 and *Geobacillus thermoleovorans* CCB_US.3_UF5 (Muhd Sakaff et al., 2012) using ACT software. All genomes start at the origin of replication with the same orientation. Red (gray in print versions) lines link orthologous genes in the same orientation, blue (black in print versions) lines link orthologs in the reverse orientation. The images show comparison (first named at the top) between (a) *G. kaustophilus* and *G. thermoleovorans*, (b) *G. kaustophilus* and *G. thermoglucosidasius*, (c) *G. thermoleovorans* and *G. thermodenitrificans*, and (d) *G. thermoglucosidasius* and *G. thermodenitrificans*.

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800 kbp localized in the middle of the genome sequences. Comparison between *G. thermoleovorans* and *G. thermodenitrificans* (Figure 2(c)) shows that despite their greater phylogenetic separation based on the *recN* analysis, there is a relatively high degree of conservation in genome order (although less than within the "kaustophilus clade") between the two species. Finally,

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comparison between *G. thermoglucosidasius* with *G. thermodenitrificans* (Figure 2(d)) is consistent with the argument that the major genome rearrangement is associated with the separation of facultative anaerobes from the rest.

s0025
2.2 Bacteriophage

p0035 Complete prophage sequences can be found within the genomes of *G. kaustophilus*, *G. thermoleovorans*, and *G. thermodenitrificans* but not in the genome of *G. thermoglucosidasius*. The prophage found in the genome of *G. thermoleovorans* is a Thermus sp phage phiOH2, which is 45.7 kb in length [Q2] and contains 150 open reading frames (ORFs). *Geobacillus kaustophilus* has in its genome a sequence that derives from phage phiIBB_Pl23, which comes from *Paenibacillus larvae* (48 ORFs with a size of 52.6 kb). *Geobacillus thermodenitrificans*, on the other hand, encode within its genome sequences for the [Q3] *Geobacillus* virus E2 of a length 61 kb. Thus, there are no common prophage sequences present in different strains and the position of the phage sequence in the genome differs depending on the strains. The abundance of CRISPR sequences and Cas genes found in the genome *G. thermoglucosidasius* might explain the lack of prophage sequence within its genome.

s0030
2.3 Plasmids

p0040 Some of the sequenced strains of *Geobacillus* spp. have one or more large plasmids in their genomes. Mega plasmids have been found and characterized for the following strains: *G. kaustophilus* HTA426 (pHTA426), *G. stearothermophilus* STK (pSTK1), *G. thermoglucosidasius* NCIMB 11955 (pGTH11955-1, pGTH11955-2), and C56-YS93 (pC56-YS93) along with *G. thermodenitrificans* NG80 (pNG8O-2).

s0035
3. PHYSIOLOGY AND MESSAGES FROM THE GENOME

s0040
3.1 Fermentative Metabolism

p0045 *Geobacillus* spp. are recorded as being facultative anaerobes, with anaerobic (nondenitrifying) metabolism involving mixed acid fermentation producing lactate, formate, acetate, ethanol, and succinate. However, a few strains have also been found to produce R,R 2,3-butanediol (Xiao et al., 2012). Based on the presence of genes encoding pyruvate–formate lyase (PFL; or formate acetyltransferase) and the PFL-activating enzyme, the genus clearly divides into an obligately aerobic cluster, comprised of the "kaustophilus clade" of *G. thermoleovorans*, *G. thermocatenulatus*, and *G. kaustophilus*,

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the denitrifying but nonfermentative "*G. denitrificans* clade" and the truly fermentative *G. caldoxylosylitus*, *G. toebii*, and *G. thermoglucosidasius* clade, and this is consistent with physiological studies. However, *G. stearothermophilus* forms an exception to this rule; the partial genome sequence of *G. stearothermophilus* ATCC7953 (the original Donk isolate from the 1920s) which, based on *recN* analysis is closely related to the type strain, BGSC9A20, does encode these genes, despite *G. stearothermophilus sensu strictu* being phylogenetically close to the "kaustophilus" and "denitrificans" clades. *G. stearothermophilus* NUB3621 is probably a strain of *G. caldoxylosylitus* based on both its *recN* sequence, and it also has genes encoding PFL and the activating enzyme. Based on sequenced genome annotations, the ability to produce butanediol (based on the presence of a gene encoding R,R-butanediol dehydrogenase), appears to be found only in the truly fermentative *G. toebii* and *G. thermoglucosidasius* and not *G. caldoxylosylitus*. However, there is one anomalous record of a butanediol dehydrogenase gene in *Geobacillus* sp. WSUCF1, a strain that clusters with the "kaustophilus clade" based on *recN* phylogenetic analysis. A blastp search suggests that this shows a much greater similarity to homologs from mesophilic *Bacillus* spp. than the genes from the fermentative *Geobacillus* spp., which have a high degree of sequence identity; hence, the identity and role of the gene in *Geobacillus* sp. WSUCF1 need confirmation.

p0050 [Q4] Lactate dehydrogenase appears to be common to all *Geobacillus* spp. Typically, strains encode both FAD-linked and NAD-linked enzymes, with the latter being linked to lactate production under limiting oxygen concentrations, where respiration is unable to reoxidize NADH sufficiently fast. Given the relatively low oxygen solubility over the normal growth temperature range of *Geobacillus* spp., lactate production may be envisaged as a redox sink to allow continued metabolism. In fermentative strains, this forms the primary fermentation pathway under reduced oxygen tensions, with the PFL pathway predominating under fully anaerobic conditions. The FAD-linked enzymes probably function primarily in lactate catabolism, although some lactate production has been observed in strains where the gene encoding the NAD-dependent enzyme has been deleted, suggesting that this may also act as a sink for reducing equivalents.

p0055 Unlike the situation in *Escherichia coli*, succinate production does not appear to be part of a dedicated fermentation pathway and probably reflects the simple reversal of flux from oxaloacetate as an additional redox sink. Typically, succinate forms a very small part of the fermentation products in a wild-type strain.

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s0045
3.2 Can *Geobacillus* spp. Grow Fully Anaerobically?

p0060 While some *Geobacillus* spp. clearly have functional fermentation pathways, and can be grown at low cell densities under seemingly anaerobic conditions, our work with the company TMO Renewables Ltd has clearly demonstrated that a small amount of oxygen is essential for fermentative growth of *G. thermoglucosidasius* (this is particularly obvious at high cell densities), and we normally produce fermentation products in cultures grown at low redox potential (−230 to −280 mV) by supplying a small amount of air. The amount of oxygen being supplied to achieve this is too low to be involved in central metabolism, but inspection of the genome sequence of the fermentative *Geobacillus* spp. shows that the production of thiamine involves the oxygen requiring glycine to iminoglycine (EC 1.4.3.19) route rather than the tyrosine to iminoglycine route (4.1.99.19) typically found in obligate and some facultative anaerobes. Supplementation of oxygen limited cultures of *G. thermoglucosidasius* 11955 with thiamine certainly enhances the growth rate, but it is clear that this is not the only process requiring oxygen in these cells and studies continue.

s0050
3.3 Growth, Lysis, and Sporulation

p0065 *Geobacillus* spp. can grow extremely rapidly and aerobically in rich growth media, with *G. thermoglucosidasius* 11955 growing at rates approaching 2 h^{-1} at 65 °C (TMO Renewables, personal communication), which is marginally below its optimum growth temperature. Anaerobic growth is slower but still impressive. Growth can also be obtained both aerobically and anaerobically on a mineral salts medium, but some strains require supplementation with biotin. However, in our experience, it is difficult to grow directly by inoculation in a mineral salt medium, so this tends to be reserved for growth in bioreactors, where strains are initially grown in a richer medium, then adapted to growth on the leaner mineral salt medium. A mineral salt medium supplemented with a small amount of yeast extract and tryptone works reliably and is a good compromise.

p0070 Where it has been measured, *Geobacillus* spp. appear to have a relatively high maintenance energy at 60−65 °C, so maintenance becomes a dominant feature of growth at low growth rates, particularly in minimal media. Although cells can be maintained without problem for extended periods in nutrient-limited continuous culture (although there is a question as to whether to the high maintenance reflects a certain amount of cell lysis, they tend to lyse as a result of rapid nutrient starvation typical of the late

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stages of batch culture (Pavlostathis, Marchant, Banat, Ternan, & McMullan, 2006). While this partly reflects the rate of transition from growth to starvation, it is clear that there are, as yet unexplained, regulatory factors involved that trigger an irreversible loss of viability and lysis. This feature may also explain why it is difficult to grow cultures directly from inoculation in mineral salts media. Like their mesophilic counterparts, all *Geobacillus* spp. encode the majority of the core sporulation genes (Zeigler, 2014), so this response may be connected with sporulation, even if excessively rapid starvation fails to produce viable spores (as has been observed). The ability to inactivate *G. stearothermophilus* spores is a well-established test for the efficiency of heat sterilizing equipment. Comparison of sporulation gene homologs in the sequenced genomes shows that 67 of the 75 core sporulation gene orthologs (Galperin et al., 2012; Zeigler, 2014) are present in *Geobacillus* spp. and sporulation is commonly observed, suggesting that the other functions are either not essential in *Geobacillus* spp. or provided by nonorthologous proteins.

p0075 Natural competence is a feature that is an integral part of the regulatory cascade, which typically precedes sporulation in *Bacillus* spp. As with the sporulation genes, many of the genes involved in the development of competence are present in the genome sequences of *Geobacillus* spp. but not the complete set identified in *Bacillus subtilis* (Kovacs, Smits, Mironczuk, & Kuipers, 2009). Like *Bacillus cereus*, *Geobacillus* spp. (with the exception of *G. caldoxylosilyticus* [Q5] NBRC107762 which has *comGE* and *comGG* orthologs) appear to lack *comFB*, *comGE*, and *comGG* genes (and also uniquely lack the *nin* and *bdb* genes). Although natural competence was difficult to demonstrate in *B. cereus*, artificial induction of the master regulator ComK was shown to produce a low level of competence (Mironczuk, Kovacs, & Kuipers, 2008), so it is reasonable to assume that natural competence is also a feature of *Geobacillus* spp., but conditions have not been established for induction. Interestingly, there has been a single report where natural competence has been used (Zhang, Yi, Pei, & Wu, 2010), but no conditions were provided.

s0055
4. CATABOLISM

s0060
4.1 Polysaccharide and Oligosaccharide Hydrolysis

s0065
4.1.1 Hemicellulose

p0080 In a recent analysis, De Maayer, Brumm, Mead, and Cowan (2014) have shown that most of the sequenced and partially sequenced strains of

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Geobacillus spp. have a range of hemicellulose utilization genes present in a genomic island, typically although not exclusively located between *echD*, [Q6] an enoyl coenzyme A (CoA) hydratase-encoding gene, and *npd*, a nitropropane dioxygenase-encoding gene. Comparison of the G + C content between the genes in the island and the rest of the chromosome suggests that the island was acquired from an organism with lower G + C content, and this has occurred in at least two independent events in different strains. Based on the extensive work characterizing hemicellulose utilization in *G. stearothermophilus* T6 (Alalouf et al., 2011; Salama et al., 2012; Shulami et al., 2011; Tabachnikov & Shoham, 2013), identification of orthologous genes in other strains shows that both the gene complement and arrangement of clusters in the genomic island are highly variable. A pattern emerges that suggests that *Geobacillus* spp. fully secrete only a small number of glycoside hydrolases, but these degrade noncrystalline polymeric substrates to short oligomers that can be transported inside the cell. These are then further hydrolyzed to monomers by nonsecreted glycoside hydrolases and glycosidases inside the cell. This catabolic strategy reveals a notable metabolic efficiency, employing a minimal set of secreted enzymes together with enhanced energy gain through transporting (then internally hydrolyzing) oligomers rather than monomers.

p0085 There is no evidence for true cellulolytic activity (ability to degrade crystalline cellulose) in *Geobacillus* spp., although extracellular enzymes showing endoglucanase activity (probably low specificity GH5; Aspeborg, Coutinho, Wang, Brumer, & Henrissat, 2012) have been detected. However, GH10 xylanases are secreted by many strains (Balazs et al., 2013; Liu et al., 2012) and GH43 endo α-1,5-arabinanases (pectin-derived arabinan degrading) by a few (De Maayer et al., 2014; Shulami et al., 2011).

p0090 In *G. stearothermophilus*, T6 the island encodes 13 gene clusters covering 76.1 kb (De Maayer et al., 2014). One "cluster" comprises the single extracellular xylanase gene, which is functionally complemented by a cluster encoding xylooligosaccharide transport, two clusters encoding intracellular xylooligosaccharide degradation, and a cluster encoding xylose utilization. This strain is also capable of pectin degradation, with a gene cluster encoding arabinan utilization that includes *abnA*, encoding the GH43 endo α-1,5-arabinanase, and *abnEFG*, which encodes an arabinosaccharide transporter. This is functionally complemented by clusters encoding intracellular arabinofuranose metabolism (a single "cluster" of *abnF* encoding arabinofuranosidase), arabinose transport, and arabinose metabolism. For metabolism of the glucuronic acid-rich backbone of pectin, the T6 genomic

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island also has clusters that encode aldotetrauronic acid transport, intracellular aldotetrauronic acid degradation, and glucuronic acid metabolism. Some strains that express a GH5 endoglucanase also encode an oligosaccharide transporter, although its substrate specificity is not known and an intracellular β-glucosidase, which is consistent with the extracellular breakdown of amorphous cellulose, transport of β-glucan oligosaccharides, and subsequent intracellular hydrolysis.

s0070

4.1.2 Starch

p0095 The main commercial α-amylases derive from *Bacillus amyloliquefaciens* and *Bacillus licheniformis* and are particularly thermostable (Termamyl, a modified version of the *B. licheniformis* enzyme is active at 110 °C; Nielsen & Borchert, 2000). It is therefore not surprising to find that many *Geobacillus* spp. also produce α-amylases with excellent thermostability (Offen, Viksoe-Nielsen, Borchert, Wilson, & Davies, 2015; Suvd, Fujimoto, Takase, Matsumura, & Mizuno, 2001). As α-amylases tend to be specific for cleavage of the α1-4 linkage, the complete breakdown of starch requires a debranching enzyme to cleave the 1-6 linked side chains. In commercial starch breakdown, this is normally done after high-temperature (to aid liquefaction) α-amylase ~~treatment using~~ a fungal glucoamylase. Bacteria tend to use a 1-6 specific pullulanase for this step, and a few *Geobacillus* spp. also express this activity (Kuriki, Okada, & Imanaka, 1988), although accumulating evidence suggests that the enzyme involved might actually belong to a novel class of enzyme known as a neopullulanase, which cleaves both 1-4 and 1-6 linkages and also have high activity against cyclodextrin (Lee et al., 2002; Takata et al., 1992).

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4.2 Hydrocarbons

s0080

4.2.1 Alkanes

p0100 The ability of *Geobacillus* spp. to use aliphatic and aromatic hydrocarbons as carbon substrates and transform hydrocarbon substrates such as steroids has been frequently reported, but has only been systematically studied in a few cases. A conventional alkane-degrading *alkBFGHJKL* operon has been elucidated (Wentzel, Ellingsen, Kotlar, Zotchev, & Throne-Holst, 2007), with the first ORF encoding a membrane-bound alkane monooxygenase (AlkB), which is functional against midchain length (C_6—C_{18}) alkanes. An *alkB* homolog has been amplified from *G. thermoleovorans* strain T70, and demonstrated to be induced in a temperature-dependent manner in the presence of 1% *n*-hexadecane (Marchant, Sharkey, Banat, Rahman, & Perfumo, 2006). To date, *alkB* homologs have been found in *Geobacillus* sp.

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MH-1 (Liu et al., 2009) and *G. subterraneus* K (Korshunova et al., 2011), as well as in 11 alkane-degrading *Geobacillus* isolates in a single study (Tourova et al., 2008).

Geobacillus denitrificans NG80-2 has also been shown to grow on long chain (C_{15}—C_{36}) alkanes employing a novel, plasmid-encoded monooxygenase, LadA. Clearly, the ability to metabolize very long chain hydrocarbons is assisted by high temperatures, which should keep the substrate liquid and improve solubility Although LadA was originally thought to be extracellular and functions without cofactors, it is now known to contain FMN and require an NADPH-dependent FMN reductase for activity. The crystal structure of *G. thermodenitrificans* NG80-2 LadA has since been elucidated in complex with FMN, which suggests that hydroxylation goes via a C4a-hydroperoxyflavin intermediate, rather than the classic haeme or non-haeme iron mechanisms (Feng et al., 2007; Li et al., 2008; Wang et al., 2006). LadA orthologs have subsequently been described in *G. thermoleovorans* B23, *Geobacillus* sp. GHH01, G11MC16, Y4.1MC1, and *G. thermoglucosidasius* C56-YS93 (Boonmak, Takahashi, & Morikawa, 2014). In these strains, the *ladA* orthologs are present on a genomic island, which also includes the gene encoding the FMN reductase; however, in *G. thermoleovorans* B23 at least one of these genes is more similar to an alkanesulphonate monooxygenase from the same SsuD bacterial luciferase family.

4.2.2 Aromatics

The capacity of *Geobacillus* strains to metabolize aromatic compounds has been studied since the mid-1970s, then generally referred to as thermophilic *Bacillus* spp. (Buswell & Twomey, 1975). Since then, several phenol-degrading *Geobacillus* spp. have been isolated and characterized, including *G. stearothermophilus* DSM 6285 (Omokoko, Jäntges, Zimmermann, Reiss, & Hartmeier, 2008) and *G. thermoglucosidasius* A7 (Duffner, Kirchner, Bauer, & Müller, 2000).

The phenol degradation pathway in *G. stearothermophilus* DSM 6285, encoded by 20.2 kb of DNA, has been elucidated as 15 ORFs residing on a low-copy megaplasmid (Omokoko et al., 2008). Ten genes encode proteins that are directly linked with the meta-cleavage pathway, including a phenol hydroxylase (PheA), a catechol 2,3-dioxygenase, a 4-oxalocrotonate tautomerase, a 2-oxopent-4-dienoate hydratase, a 4-oxalocrotonate decarboxylase, a 4-hydroxy-2-oxovalerate aldolase, and an acetaldehyde dehydrogenase (Duffner et al., 2000; Omokoko et al., 2008). The largest ORF, *pheR*, displays a strong similarity to transcriptional regulators associated

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with phenol metabolism in *Geobacter lovleyi* SZ, and is thought to be the first example of a transcriptional regulator of phenol metabolism identified in Gram-positive bacteria (Omokoko et al., 2008).

p0120 Notable work has been done on the characterization of the first enzyme in the degradation pathway, PheA, which catalyzes the ortho-hydroxylation of phenol to catechol. PheA, a two-component enzyme encoded by the *pheA1* and *pheA2* genes, is strictly FAD dependent (Kirchner, Westphal, Müller, & van Berkel, 2003). Intriguingly, a function of PheA2 is in the catalysis of the NADH-dependent reduction of free flavins according to a Ping-Pong Bi—Bi mechanism, and it represents the first member of a newly recognized family of short-chain flavin reductases that use FAD both as a substrate and as a prosthetic group. The reduced FAD is subsequently used by the oxygenase component PheA1, which hydroxylates phenol and its derivatives to the corresponding catechols (van den Heuvel et al., 2004).

p0125 Many *Geobacillus* strains have chromosomal gene clusters encoding benzoate and phenylacetate metabolism, via initial conversion to the CoA derivative and 4-hydroxyphenylacetate and naphthalene metabolism via ring monohydroxylation and dihydroxylation together with downstream steps to join with central metabolism. *G. thermodenitrificans* also encodes a 3-hydroxyanthranilate dioxygenase, but the downstream pathway is either missing or not annotated. Marchant, Banat, Rahman, and Berzano (2002) isolated strains of *G. caldoxylosylitus*, *G. toebii*, and *G. oleovorans* from soil using a range of polyaromatic hydrocarbons as sole carbon sources, but there was no obvious pattern of usage between species.

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5. CENTRAL METABOLISM

p0130 [Q10] All *Geobacillus* spp. appear to use a combination of the Emden—Meyerhof—Parnas pathway of glycolysis and the oxidative pentose phosphate pathway for carbohydrate metabolism. There is no evidence for a functional Entner—Doudoroff pathway in any of the sequenced strains, due the lack of ability to produce phosphogluconate dehydratase (Alm [Q11] et al., 2005; Tang et al., 2007). They also have a classical TCA cycle, glyoxylate cycle and can use both pyruvate carboxylase and PEP carboxylase as anaplerotic reactions to top up the TCA cycle as well as PEP carboxykinase for gluconeogenesis. So, in many ways, they look like classical *Bacillus* spp. However, there are some subtle differences, particularly in pentose

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phosphate interconversions. Unlike *Bacillus* spp., *Geobacillus* spp. do not encode a 6-phosphogluconolactonase, but they do have a gene for glucose-6-phosphate dehydrogenase and transcriptomic studies (manuscript in preparation) have shown that this and the other pentose phosphate pathway enzymes are expressed during growth on glucose. 6-Phosphogluconolactone is known to undergo spontaneous hydrolysis at room temperature, although the rate is not sufficient to allow the growth of mesophiles without a lactonase (Miclet et al., 2001), but may be sufficient in thermophiles. However, some *Geobacillus* spp. may have an alternative oxidation route for production of ribulose-5-phosphate as they encode 6-phospho-3-hexuloisomerase and 3-hexulose-6-phosphate synthase, two key enzymes in the ribulose monophosphate pathway of formaldehyde fixation (Ferenci, Strom, & Quayle, 1974; Kato, Yurimoto, & Thauer, 2006). Given that these steps can act reversibly (Kato et al., 2006) and some *Geobacillus* spp. also express a formaldehyde dehydrogenase, an alternative oxidative route may be available.

p0135 While the lack of 6-phosphogluconolactonase is clearly an interesting piece of cellular economy, it does impose a fixed (presumably temperature-dependent) rate on this step, which may explain the changes in flux [Q12] through the ~~phosphoprotein phosphatase~~ with growth rate, observed by Tang et al. (2009). These authors ascribed the changes in flux to the lack of a transhydrogenase activity in *Geobacillus* spp.

p0140 Finally, some members of the genus are uniquely equipped with 2-oxoglutarate synthase (2-oxoglutarate:ferredoxin oxidoreductase (decarboxylat- [Q13] ing)) (Lisowska et al., in preparation), which facilitates the conversion of [Q14] 2-oxoglutarate to succinyl-CoA. Interestingly, this enzyme was originally found and characterized in photosynthetic bacteria (Buchanan & Evans, 1965) and later in the hypothermophile *Thermococcus litoralis* (Mai & Adams, 1996). It is not found in the mesophilic close relative *B. subtilis* or *E. coli*.

s0095

6. THE *GEOBACILLUS* GENETIC TOOL KIT

p0145 The wealth of potential applications arising from the engineering of *Geobacillus* spp., together with the importance of gene deletion and complementation in physiological studies, leads to a requirement for robust and seamless genetic manipulation of their genomes. The promise of these tools is augmented by the relatively close phylogenetic relation of *Geobacillus* spp. to the workhorse *B. subtilis*.

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p0150 However, the thermophilic nature of *Geobacillus* spp. means that potentially translatable genetic tool kits used in *Bacillus* spp. and other mesophiles are limited due to thermal instability of proteins and commonly used antibiotics. This is illustrated by the adaptation of the *Lactococcus lactis* group II intron-based Targetron technology for use in *Clostridium acetobutylicum* (Heap, Pennington, Cartman, Carter, & Minton, 2007), yet technologies developed for *B. subtilis* are incompatible for use in *Geobacillus* spp. Therefore, the development of novel thermoactive tools for the genetic engineering of *Geobacillus* spp., and other thermophilic bacteria, requires the exploitation of native genetic machinery.

s0100 6.1 Plasmid Vectors

p0155 The initial developments in the genetic manipulation of *Geobacillus* spp. were the characterization and development of plasmids capable of self-replication and selection markers for plasmid maintenance through multiple generations (Table 1). Plasmids that replicate via the rolling-circle (RC) mechanisms and theta-replicating mechanisms have been described (reviewed by del Solar, Giraldo, Ruiz-Echevarría, Espinosa, & Díaz-Orejas, 1998). Initially, multiple vectors based on different replicons were constructed, but with drawbacks that made them inconvenient for use as genetically malleable shuttle vectors. The *G. stearothermophilus* shuttle vector pBST22 (derived from the natural *G. stearothermophilus* plasmid pBST1) lacked a multiple cloning site and the facility for β-galactosidase-mediated blue—white screening in *E. coli*, and pNW33N (derived from a *Bacillus coagulans* cryptic plasmid pBC1) is maintained in *Geobacillus* spp. using chloramphenicol, which is only moderately thermostable (Taylor, Esteban, & Leak, 2008).

p0160 To improve versatility, pUCG18 was constructed by introducing the evolved kanamycin resistance gene and origin of replication (theta) from pBST22 with pUC18, retaining all the cloning and selection benefits of the latter (Taylor et al., 2008). This has subsequently been further improved as pUCG3.8 by a reduction of size (Bartosiak-Jentys, Hussein, Lewis, & Leak, 2013) and more recently converted into a modular format that allows ready replacement of parts, such as origins of replication and antibiotic resistance genes [Q15] (Klimova et al., in preparation).

p0165 It has been argued that RC plasmids usually have a broader host range and sometimes a higher plasmid copy number than their theta-replicating alternatives (Heinl, Spath, Egger, & Grabherr, 2011), leading to efforts to isolate, sequence, and characterize new RC replicating plasmids from *Geobacillus* spp. (Kananavičiūtė, Butaitė, & Čitavičius, 2014).

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t0010 **Table 1** *Escherichia coli—Geobacillus* shuttle vectors

Year of Publication	Plasmid name	Size	Selective marker	Origins of replicon	Mechanism of replication	References
2013	pUCG3.8	3.8	Kan^R *(TK101)*	pBST1	Θ	Bartosiak-Jentys et al. (2013)
2009	pTMO31	5.1	Kan^R (pUB110)	pUB110	RC	Cripps et al. (2009)
2008	pUCG18*	6.3	Kan^R *(TK101)*	pBST1	Θ	Taylor et al. (2008)
2001	pNW33N	3.9	Cam^R (pC194)	pBC1	RC	Zeigler (2001)
2001	pBST22	7.6	Kan^R *(TK101)*, Cam^R (pC194)	pBST1	Θ	Liao and Kanikula (1990)
1993	pSTE33*	5.7	Kan^R *(TK101)*	pSTK1	Θ	Narumi et al. (1993)
1992	pSTE12	5.8	Tet^R (pTHT15)	pTHT15	–	Narumi et al. (1992)

* Conjugation-mediated transfer has been reported with derivatives of these shuttle vectors incorporating an *incP* origin of transfer.

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s0105 6.2 DNA Transfer

p0170 Several procedures to transfer plasmid DNA into *Geobacillus* spp. have been developed, which can be categorized into protoplast transformation (Imanaka, Fujii, Aramori, & Aiba, 1982; Liao, McKenzie, & Hageman, 1986; Wu & Welker, 1989), electroporation (Cripps et al., 2009; Zeigler, 2001), and conjugation protocols (Suzuki, Wada, Furukawa, Doi, & Ohshima, 2013).

p0175 The first successful transformation procedure for the transfer of DNA into *Geobacillus* spp. involved the preparation of protoplasts by the removal of the peptidoglycan cell wall (Imanaka et al., 1982). The protoplast transformation procedure, modified from an established procedure for *B. subtilis*, involves the treatment of cells with lysozyme in an osmotically buffered media to prepare the protoplasts. DNA transfer into the 'naked' cells is subsequently mediated by the addition of polyethylene glycol as a transforming agent (Liao et al., 1986). The main drawbacks of this method are its inconvenience, as each new transformation requires the preparation of fresh protoplasts, and unreliability, due to the fragility of the prepared protoplasts. Nevertheless, frequencies of 10^3-10^4 transformants per microgram of plasmid DNA have been reported, and the procedure was used consistently for over a decade (Imanaka et al., 1982).

p0180 During the early 1990s, less time-consuming electroporation protocols were developed to introduce plasmid DNA into *Bacillus* spp. without preparing protoplasts, and extended to *G. stearothermophilus* transformations (Narumi et al., 1993, 1992). Frequencies of up to 2.8×10^6 transformants per microgram of pSTE33 DNA were published for ~~the isolated~~ *G. denitrificans* K1041 (originally classified as *Bacillus stearothermophilus*), which are the highest transformation frequencies among a large collection of *Geobacillus* strains (Zeigler, 2001). For instance, the transformation efficiencies observed by electroporation of pUCG18 DNA in *G. thermoglucosidasius* DL44 were over two orders of magnitude lower at 9.8×10^3 (Taylor et al., 2008). Still, where high transformation frequency is not critical, electroporation procedures have replaced protoplast transformation as the preferred method for transferring DNA to *Geobacillus* spp., not only due to the relative ease of the procedure but also for the facility for long-term storage of electrocompetent cell preparations.

p0185 Recent demonstrations of efficient plasmid transfer into *G. kaustophilus* HTA426 (Hirokazu, 2012; Suzuki, Wada, et al., 2013) and *G. thermoglucosidasius* [Q16] (A. Pudney, personal communication) using conjugative transfer

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look set to establish an even simpler method for the routine transformation of *Geobacilli*. Conjugative transfer is typically performed by incubating mixtures of recipient *Geobacillus* spp. cells and donor *E. coli* cells harbouring mobilization genes found on the chromosome (*E. coli* S-17) or on helper plasmids (pRK2013 and pUB307). Exploiting their inherent thermophilicity, the recipient *Geobacillus* spp. are readily distinguished from donor cells after incubation at 60 °C, and conjugative transfer has been reported to result in transfer efficiencies as high as 1.2×10^{-3} and 2.83×10^{-4} transformants per recipient *G. kaustophilus* and *G. thermoglucosidasius*, respectively.

p0190 One problem encountered with the transfer of foreign DNA into some *Geobacillus* spp. is native restriction-modification (R-M) systems that cleave specific sites in double-stranded exogenous DNA, which probably provide a natural defense mechanism against invasion by bacteriophage DNA. Four types of R-M systems have been characterized, all of which involve methylation of target sites on host DNA and restriction activity of exogenous unmethylated DNA. *Geobacillus kaustophilus*, for example, has been reported to harbour two sets of type I R-M genes, one set of type II R-M genes and three type IV R-M genes (Suzuki, Wada, et al., 2013). Global deletion of the type I and IV R-M system gene clusters in *G. kaustophilus* MK72, a derivative of strain HTA426 produced strain MK244, which was able to receive pUCG18T DNA transferred from *E. coli* DH5α with a transformation efficiency of 1.3×10^{-5} recepient^{-1}, whereas strains HTA426 and MK72 could not (Suzuki, Wada, et al., 2013). An alternative strategy is to methylate the DNA at the appropriate site before transfer into the recipient. This can either be done enzymatically, or more conveniently by expressing the cognate methylase in the host strain from which DNA is to be transferred.

s0110 6.3 Positive-Selection Markers

p0195 All endeavors in genetic engineering require indication of successful delivery of foreign DNA into the recipient strain, and selection for maintenance of the genetic construct through subsequent generations. In the research laboratory, this is conventionally done using antibiotic resistance genes that confer resistance to supplemented growth inhibitors. The thermophilic nature of *Geobacillus* spp. limits the use of established selection markers, with few antibiotic resistance proteins or antibiotics currently available with sufficient thermostability at 60–70 °C (Table 2).

p0200 The chloramphenicol acetyltransferase gene (Cam^R), derived from the *Staphylococcus aureus* plasmid pC194, confers resistance to the bacteriostatic

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t0015 **Table 2** Degradation constants ($k = -1/t_{1/2}$ in $days^{-1}$ assuming first-order decay) based on loss/gain of potency, assuming degradation products are inactive, for a variety of antibiotics (Peteranderl, Shotts, & Wiegel, 1990)

	Average k value at			
Antibiotic	**72 °C (pH 7.3)**	**50 °C (pH 7.3)**	**72 °C (pH 5.0)**	**50 °C (pH 5.0)**
Lasalocid	0.57	ND	−0.16	0
Neomycin	0.15	ND	−0.15	−0.23
Monensin	0.13	ND	−0.37	0.15
Cycloheximide	0.05	ND	ND	1
Kanamycin	0	ND	−0.55	−0.06
Trimethoprim	0	ND	0.17	ND
Erythromycin	−0.31	−0.75	−1.56	−0.75
Chloramphenicol	−0.59	0.22	0	ND
Novobiocin	−0.9	−0.18	−0.04	−0.19
Polymyxin	−0.92	0.25	0.01	0.81
Bacitracin	−0.93	−0.08	−0.02	ND
Streptomycin	−1.34	−0.45	0	ND
Vancomycin	−1.5	−0.04	−1	−0.04
Penicillin	−1.58	−0.82	−3.33	−0.43
Tetracycline	−4.27	−0.3	−3.48	−0.38
Ampicillin	−7.26	−1.56	−3.79	−0.41

antibiotic chloramphenicol. As a selective marker, it has been shown to be functional in *Geobacillus* spp. at growth temperatures of at least 60 °C (Blanchard, Robic, & Matsumura, 2014), and the ~~gene present~~ on four *Geobacillus* shuttle vectors (pBST22, pRP9, pIH41, and pNW33N) (Liao & Kanikula, 1990; Zeigler, 2001). However, above 60 °C, both the antibiotic and acetyltransferase are thermolabile. Furthermore, direct selection of transformants with chloramphenicol has been shown be inefficient, with the transformation efficiencies of pIH41 in *G. thermodenitrificans* K1041 decreasing from $1.4 \times 10^5/\mu g$ using tetracycline selection to $7.2 \times 10^4/\mu g$ using chloramphenicol selection, both at 48 °C (Liao & Kanikula, 1990; Narumi et al., 1992).

p0205 Of the commonly used antibiotics, kanamycin has the highest thermostability, so Liao and colleagues selected (an early example of forced evolution) a thermostable variant of the kanamycin nucleotidyltransferase gene (KNT-ase), conferring resistance to the bacteriocidal antibiotic kanamycin at temperatures up to 70°C (Liao & Kanikula, 1990; Liao et al., 1986). Using the *E. coli* mutD5 mutator strain to introduce mutations and selection in *G. stearothermophilus*, a thermostable KNT-ase TK101 mutant

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(D80Y, T130K) of the mesostable KNT-ase gene from pUB110 was developed, and has been shown to function as a selection marker in both *Geobacillus* spp. and *E. coli* (Bartosiak-Jentys et al., 2013; Taylor et al., 2008). A similar approach, but relying on "error-prone" *G. kaustophilus*, has been used to increase the thermotolerance of the thiostrepton resistance protein [Q17] TsrR from *Streptomyces* ~~*aureus*~~ by 5 °C (Suzuki, Kobayashi, Wada, Furukawa, & Doi, ~~2015~~).

p0210 Antibiotic-free selection systems based on the complementation of auxotrophy are more desirable for industrial uses. Recently, a thermophilic version of the eukaryotic ura3—FOA (5-fluoroorotic acid) counterselection system has been developed in *Geobacillus* spp. The bacterial equivalent of the eukaryotic *ura3*, the gene encoding orotidine 5′-phosphate decarboxylase is *pyrF*. PyrF is essential for de novo synthesis and metabolism of pyrimidines [Q18] (uridine monophosphate, uridine diphosphate, and uridine triphosphate), and has been successfully applied for marker-free selection of uracil-prototrophic transconjugants using the plasmid pGAM46 (Suzuki, Murakami, & Yoshida, 2012). Like its eukaryotic counterpart, the *pyrF* encoded orotidine 5'-phosphate decarboxylase converts 5-FOA into toxic metabolites, facilitating the development of a robust *pyrF*-based counterselection system (i.e., allowing selection for loss of *pyrF*) that has been demonstrated in *G. kaustophilus* HTA426 (Hirokazu, 2012; Suzuki et al., 2012,; Suzuki, Wada, et al., 2013; Suzuki, Yoshida, et al., 2013).

s0115 6.4 Allelic Replacement

p0215 Plasmid vectors serve well for studies of recombinant gene expression, but for long-term genetic manipulation of *Geobacillus* spp., it is preferable to stably integrate genes into the bacterial chromosome. Furthermore, the permanent silencing of chromosomal genes and regulatory components serves as a powerful tool for manipulating metabolic fluxes and phenotypes. All current methods of integrating genetic components into the *Geobacillus* spp. chromosome involve the use of a transient DNA transfer system to deliver an integration cassette that site specifically inserts into the chromosome via homologous recombination.

s0120 *6.4.1 Integration Cassettes*

p0220 To facilitate site-specific integration, DNA fragments identical to the target chromosomal locus must flank a specific genetic insert. For permanent inactivation of genetic components, deletion cassettes are commonly generated by amplification and ligation of two noncontiguous fragments (typically

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≥300 bp) from each end of the intended deletion site (Cripps et al., 2009). Depending on the need to remove or reuse selection markers, the marker may be placed between or outside these two fragments. The latter arrangement facilitates selection of a second crossover event, which either removes the integrated vector entirely or (preferably) removes the bulk of the vector but replaces the region between the two flanking arms. An example of this was the permanent inactivation of the *ldhA* gene in *G. thermoglucosidasius* NCIMB 11955 by the amplification of two gene fragments from each end of the Ldh coding sequence, replacing a 42-bp central region with a 7-bp section containing a diagnostic restriction site (Cripps et al., 2009).

p0225 Since any DNA inserted between the flanking sequences designed for homologous recombination is cointegrated into the chromosome, knockin/knockout plasmids containing functional or conditionally functional genetic components can be constructed. For example, the knockout of the endogenous adenylate kinase gene from *G. stearothermophilus* NUB3621-R involved the introduction of a promoter-less chloramphenicol transacetylase (*cat*) selectable marker, so that true recombinants could be readily screened (Couñago & Shamoo, 2005).

s0125 6.4.2 Short Life-Span Vectors

p0230 Cassettes designed for homologous recombination require an efficient DNA transfer system for delivery into the cell, but with the capacity to be selectively eliminated after successful integration of the cassette. To this end, integrative vectors have been constructed that either have restricted or no replicative stability in *Geobacillus* spp. Once plasmid DNA is transferred into the recipient *Geobacillus* strain, conditional or absolute replicative instability facilitates the elimination of these plasmids from future generations; hence, they are commonly referred to as "suicide vectors."

p0235 Replicative stability in *Geobacillus* spp. has been limited by two methods. The first example is the plasmid pSTE12, which is very segregationally unstable in the absence of selective pressure (Narumi et al., 1992). Therefore, growth under nonselective conditions leads to the loss of pSTE12 from *Geobacillus* spp. transformants. This method was used for chromosomal replacement of the adenylate kinase gene in *G. stearothermophilus* NUB3621-R using pSTE12 containing a homologous recombination cassette (Couñago & Shamoo, 2005).

p0240 The high optimal growth temperature of *Geobacillus* spp. has also been exploited to limit plasmid replicative stability by the construction of vectors containing temperature-sensitive origins of replication. The mesophilic

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plasmid pUB110, isolated from *S. aureus*, is capable of replicating in a wide range of Gram-positive bacteria, but is only stable in *Geobacillus* spp. at temperatures <55 °C (Gryczan, Contente, & Dubnau, 1978; Imanaka et al., 1982; Liao et al., 1986; Matsumura, Katakura, Imanaka, & Aiba, 1984). This thermolability was exploited in the construction of the conditional suicide vector pTMO31, an *E. coli*—*Geobacillus* shuttle vector containing the kanamycin resistance marker and replicon from pUB110 (Cripps et al., 2009). Plasmid transformants can been selected and maintained through antibiotic selection in *Geobacillus* spp. at temperatures <55 °C. Raising the growth temperature to 65 °C leads to plasmid elimination through failure to replicate, but any components of the vector that have integrated into a thermostable replicon, such as the chromosome are retained (Cripps et al., 2009). This powerful temperature-mediated system has been utilized in *G. thermoglucosidasius* NCIMB 11955 to eliminate the lactate dehydrogenase and PFL pathways by disruption of the *ldh* and *pflB* genes, respectively (Cripps et al., 2009).

p0245 By exploiting the increased transformation efficiency achievable with conjugative transfer of plasmid DNA into *Geobacillus*, integrative vectors have recently been used that lack a replicon that functions in *Geobacillus* spp. altogether. These pGAM plasmids utilize the effective *pyrF* counterselection system described above. The uracil-auxotrophic mutant *G. kaustophilus* strain MK72 (Δ*pyrF* Δ*pyrR*) is resistant to 5-FAO (Suzuki et al., 2012). When pGAM46 is transferred into the MK72 mutant, a plasmid-borne *pyrF* gene adjacent to the integration cassette complements the *pyrF* knockout, rendering single-crossover mutants prototrophic for uracil. Subsequent rounds of passaging leads to a second crossover event, removing the supplemented *pyrF* gene, and yielded uracil-auxotrophic mutants that are 5-FOA resistant in the presence of uracil, and 5-FOA sensitive in the absence of uracil (Boeke, LaCroute, & Fink, 1984; Suzuki et al., 2012).

p0250 Using this technology, chromosomal integration and heterologous expression of *G. stearothermophilus* IAM11011 *bgaB* and *G. stearothermophilus* CU21 *amyE* genes, encoding β-galactosidase and α-amylase, respectively, were demonstrated. Although successful, the counterselection system yielded 50% uracil-prototrophic false positives, which were 5-FOA resistant in the presence of uracil, and 5-FOA sensitive in the absence of uracil (Suzuki et al., 2012). Since mutations observed in the *pyrF* and *pyrE* regions of false positives were negligible, it is hypothesized that *G. kaustophilus* may potentially repress pyrimidine biosynthesis and/or incorporation under uracil-rich conditions independently of *pyrR* (Suzuki et al., 2012).

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s0130
6.5 Reporter Genes

p0255 Fundamental physiological studies and biotechnological applications involving single or multiple gene expression depend on investigation and application of promoter- and ribosome-binding site operation and strength. Recently, there has been a rapid increase in the characterization of promoters, particularly inducible promoters that may be used for conditional expression and easily assayable reporter genes are useful tools for characterization of these promoters. GFP (green fluorescent protein) is a commonly used reporter in mesophiles and a useful thermostable variant, superfolder GFP (sfGFP) (Pédelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006) has been shown to work in *Thermus* spp. and *Geobacillus* spp. (Blanchard et al., 2014) where it has been used for the assessment of various promoters. However, like the majority of fluorescent proteins, the maturation of the fluorescent chromophore requires molecular oxygen; therefore, sfGFP cannot be used in oxygen-deprived environments (e.g., under fermentative conditions).

p0260 An alternative transcriptional reporter gene that can be used to circumvent this drawback is the *pheB* gene from *G. stearothermophilus* DSM 6285, which encodes a thermostable catechol 2,3-dioxygenase. The expression of *pheB* in the presence of 100 mM catechol results in the formation of the yellow-colored 2-hydroxymuconic semialdehyde, which can be detected at an absorbance of 375 nm (Bartosiak-Jentys, Eley, & Leak, 2012). Enzymes associated with carbohydrate metabolism have also been exploited as expression reporters in *Geobacillus* spp., including α-amylase, β-galactosidase, and α-galactosidase (Blanchard et al., 2014; Lin et al., 2014; Suzuki et al., 2012).

s0135
6.6 Recombinant Gene Expression

p0265 A limited number of constitutive or inducible promoters have been characterized as suitable for controlled heterologous protein expression in *Geobacillus* spp. For strong constitutive expression in *G. kaustophilus* HTA426, the native promoter P_{sigA}, found immediately upstream of two housekeeping genes (Suzuki et al., 2012), has been characterized in β-galactosidase assays. The promoter for ribonuclease H III, P_{RHIII}, isolated from *G. stearothermophilus* NUB3621, has −10 and −35 regions closely matching the consensus and has been used for constitutive expression of the fluorescent sfGFP reporter (Blanchard et al., 2014).

p0270 Due to the recent focus on engineering fermentation pathways in *Geobacillus* spp., promoters that can be induced under anaerobic conditions are

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of interest. In particular, promoters of the lactate dehydrogenase genes, P_{ldh}, isolated from *G. stearothermophilus* NCA1503 and *G. thermodenitrificans* DSM 465^T have been characterized and applied in the production of ethanol and isobutanol, respectively (Cripps et al., 2009; Lin et al., 2014). However, it has been suggested that P_{ldh} activity is more active under oxygen-limiting conditions than during fully anaerobic growth, and potentially induced as a result of the transitory change in redox conditions between aerobic and anaerobic growth (Bartosiak-Jentys et al., 2013).

p0275 A wider range of ligand-inducible promoters functional in *Geobacillus* spp. have been characterized, especially carbohydrate-inducible promoters. Inducible promoters are essential for the controlled heterologous expression of "toxic" proteins, which may require strong transcriptional silencing to facilitate sufficient growth of the host organism. Although several positively regulated promoters have been shown to facilitate controlled protein expression, many of these promoters are functional under various other conditions (Bartosiak-Jentys et al., 2013; Suzuki, Yoshida, et al., 2013). However, the *G. kaustophilus* HTA426 promoters P_{gk704}, P_{gk1859}, P_{gk1894}, and P_{gk2150} have been identified as being inducible by maltose, lactose, myoinositol, and D-galactose, respectively (Suzuki, Yoshida, et al., 2013). Notably, expression under promoter P_{gk704} increased 4.5- and 12-fold by the addition of either soluble starch or maltose to culture medium. Growth on the pentose sugars D-xylose or L-arabinose inhibited gene expression, but there were negligible effects on expression during growth on D-glucose, D-galactose, sucrose, melibiose, lactose, myoinositol, cellobiose, or fructose. The promoter $P_{\beta glu}$, isolated from the cellobiose-specific phosphotransferase system operon of *G. thermoglucosidasius* NCIMB 11955, enhanced expression of *pheB* in the presence of cellobiose, but was also activated by glucose and xylose (Bartosiak-Jentys et al., 2013). The native promoter of the sucrose-utilization operon in *G. stearothermophilus* NUB3621, P_{surP}, has been demonstrated to increase α-galactosidase expression fivefold in the presence of sucrose, although characterization in the presence of other sugars has not been reported (Blanchard et al., 2014).

s0140 6.7 Secretion

p0280 The utilization of polymeric substrates such as proteins and polysaccharides, as well as the breakdown of oils/fats, requires secretion of the relevant hydrolytic enzymes into the extracellular milieu. Understanding and control of this process should increase the potential of *Geobacillus* spp. as an expression host for commercial useful proteins (e.g., lipases, amylases) as secreted

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proteins are simpler to recover than intracellular proteins. It will also allow modification of the catabolic potential of *Geobacillus* spp., which is particularly useful for metabolic engineering. Nevertheless, there have been reports of secretion of heterologous proteins in *Geobacillus* spp. Secretion is mediated by N-terminal signal peptides, which typically are of a variable length and amino acid sequence. Recently, the secretion of a *G. stearothermophilus* α-amylase and a truncated-cellulase from *Pyrococcus horikoshii* has been achieved in *G. kaustophilus* HTA426 using their native signal sequences (Suzuki, Yoshida, et al., 2013). However, systematic screening of native signal peptides for the secretion of heterologous enzymes in *B. subtilis* showed that the optimal signal peptide for one protein can be inefficient for another and vice versa, and therefore multiple signal peptides need to be tested for efficient secretion of different recombinant proteins (Brockmeier et al., 2006). With this in mind, Bartosiak-Jentys et al. have created a modular system to bring various genetic component parts together, including signal peptides, in different combinations. This was demonstrated by the heterologous expression and secretion of a glycosyl hydrolase from *Thermotoga maritima* MSB8 using a signal peptide from a *G. thermoglucosidasius* xylanase (Bartosiak-Jentys et al., 2013).

s0145

7. BIOTECHNOLOGICAL APPLICATIONS

p0285 The ability to secrete commercially useful enzymes such as hemicellulases and amylases and catabolic versatility of *Geobacillus* spp. are being exploited for both biocatalysis and metabolic engineering. However, a [Q19] number of other diverse applications are also being investigated from the production of sweeteners, production of therapeutics, and exploitation of S-layer-based proteinaceous nanostructures.

s0150

7.1 Metabolic Engineering for Production of Fuels and Chemicals

p0290 The most widely researched biotechnological application of *Geobacillus* spp. is for fermentation to produce second-generation biofuels, an endeavor that has focussed ~~largel~~y on *G. thermoglucosidasius* largely due to the industrial impetus provided by companies such as Agrol Ltd and TMO Renewables. Growth at 60–70 °C facilitates the continuous removal of volatile fermentation products (e.g., the boiling point of ethanol is 78 °C) while not causing excessive attrition of mechanical equipment (Cripps et al., 2009). Furthermore, higher growth temperatures reduce potential contamination issues

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from mesophilic contaminants (Cripps et al., 2009). However, it is the catabolic promiscuity of *Geobacillus* spp., particularly their ability to take up and degrade a wide range of oligomeric carbohydrates, which sets them apart in terms of second-generation bioprocess design (Cripps et al., 2009).

p0295 Bioethanol (C_2H_5OH) is now a well-established biofuel that is being commercially produced in first-generation (from cane sucrose or corn/wheat-derived glucose) processes, with second-generation processes (from lignocellulosic feedstocks) coming on stream. As well as its value as a high octane fuel, it has extensive secondary applications (e.g., ethylene, higher alcohols); ethanol itself is biodegradable and has low toxicity, causing little environmental pollution (Hansen, Zhang, & Lyne, 2005). In the United States, first-generation ethanol production has now saturated the demand for inclusion at 10% of US gasoline fuel supply (Bajpai, 2013).

p0300 Ethanol is a natural, but not the main fermentation, product of facultatively anaerobic *Geobacillus* spp. After knocking out the L-lactate dehydrogenase pathway, it was expected that the fermentation products would be determined by the residual PFL pathway. However, higher yields of ethanol were obtained than expected from the PFL pathway and it was recognized that, as in *B. subtilis*, pyruvate dehydrogenase (Pdh) was still active under [Q20] anaerobic conditions. By ~~knockout out~~ the PFL pathway and upregulating expression of Pdh a novel, redox balanced homoethanol pathway was demonstrated, which forms the basis of an industrial process (Cripps et al., 2009). Ethanol yields from glucose of >90% of the theoretical value have been achieved in the triple mutant (Δ*ldh*, Δ*pflB*, and pdh_{up}) process strain *G. thermoglucosidasius* TM242 (Cripps et al., 2009) with productivities as high as 2.85 g/L h on glucose and 3.2 g/L h on cellobiose (Cripps et al., 2009).

p0305 An alternative avenue for increasing flux to ethanol is via pyruvate decarboxylase (Pdc, EC 4.1.1.1), the fermentation route use by *Saccharomyces cerevisiae* and the bacteria *Zymomonas mobilis* and *Zymobacter palmae*. Pdc catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde, which is then converted to ethanol by alcohol dehydrogenase (Adh, EC 1.1.1.1). Although Pdcs from plants and yeast have been widely studied, only a few bacterial examples have been identified and characterized, notably from *Z. mobilis*, *Z. palmae*, *Acetobacter pasteurianus*, and *Sarcina ventriculi* (Raj, Talarico, Ingram, & Maupin-Furlow, 2002). So far, a Pdc of thermophilic origin has not been discovered, and heterologous expression of both *Z. mobilis* and *Z. palmae* PDC in *G. thermoglucosidasius* does not result in functional enzyme activity at temperatures >55 °C (Thompson,

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Studholme, Green, & Leak, 2008). However, both enzymes were reported to show good in vitro thermostability at these temperatures, and previous studies indicate the native (prefolded) *Z. mobilis* Pdc being stable up to 60 °C (Pohl, Mesch, Rodenbrock, & Kula, 1995; Thompson et al., 2008). It has, therefore, been argued that this reflects the temperature sensitivity of Pdc assembly, rather than enzyme activity per se, based on the requirement of cofactor binding during Pdc folding (Pohl, Grötzinger, Wollmer, & Kula, 1994). Recently, a Pdc from *Gluconobacter oxydans*, which remains thermostable in vitro at 45 °C, was expressed in *G. thermoglucosidasius* TM89, a Δ*ldh* variant of the NCIMB 11955 strain and grown fermentatively at 52 °C, resulting in yields as high as 0.35 ± 0.04 g/g ethanol per gram of glucose consumed.

p0310 Although ethanol is currently the prime renewable fuel, it has been argued that it may not provide an optimal economic solution across the feedstock-to-consumer value chain (Ryan, Munz, & Bevers, 2011). As a blend fuel, isobutanol has several advantages over ethanol, including greater energy content, lower oxygen content, lower Reid Vapor Pressure, and a low water solubility (Ryan et al., 2011). To this end, Lin and colleagues prospected and characterized enzymes required for isobutanol biosynthesis at elevated temperatures, and demonstrated isobutanol titres of 3.3 g/L of isobutanol from glucose at 50 °C, although titres from cellobiose were lower at 0.6 g/L of isobutanol (Lin et al., 2014). This was achieved by overexpression of genes encoding the native *G. thermoglucosidasius* DSM2542^T acetohydroxy acid isomeroreductase, a *L. lactis* α-ketoisovalerate decarboxylase, and a *B. subtilis* acetolactate synthase gene (Atsumi, Hanai, & Liao, 2008; Lin et al., 2014).

s0155 7.2 Enzymes for Biocatalysis

s0160 *7.2.1 Proteases*

p0315 Proteases have a long history in industrial processes such as leather processing, addition to detergents, peptide synthesis, applications in the food industry, and various other biotechnological applications (Haki & Rakshit, 2003). Thermostable proteases offer compatability with processes that function more optimally at higher temperatures (e.g., through reduced viscosity), can have high catalytic efficiencies, and offer resistance from mesophilic microbial contamination. Thermostable proteases have been isolated and characterized from a number of *Geobacillus* isolates, and genomic analysis demonstrates widespread putative proteolytic capabilities across the genus (Chen, Stabnikova, Tay, Wang, & Tay, 2004; Hawumba, Theron, & Brözel,

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2002; Iqbal et al., 2015; Zhu, Cha, Cheng, Peng, & Shen, 2007). This includes the commercially produced *G. stearothermophilus* thermostable metalloendopeptidase Thermolysin, which is used for the commercial synthesis of N-(benzyloxycarbonyl)- L-aspartyl-L-phenylalanine methyl ester, the precursor for the artificial sweetener aspartame (Sigma).

p0320 A subset of proteases finding increasing clinical applications and use as experimental reagents are thermostable collagenolytic proteases (Watanabe, 2004). Collagen-degrading *Geobacillus collagenovorans* MO-1 has since been isolated, and a range of collagenolytic enzymes have been characterized (Itoi, Horinaka, Tsujimoto, Matsui, & Watanabe, 2006; Miyake et al., 2005).

s0165 7.2.2 Carboxylesterases and Lipases

p0325 Although both carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) catalyze the hydrolysis of carboxyl esters, and are both found in macroorganismsand microorganisms, they exhibit some fundamental differences in their enzyme kinetics and substrate specificities (Jaeger, Dijkstra, & Reetz, 1999).

p0330 Lipases have a preference for water-insoluble substrates, and hydrolyze long chain triglycerides (Bornscheuer, Bessler, Srinivas, & Krishna, 2002). Lipases are generally more enantioselective than carboxylesterases, show greater resistance to organic solvents, and contain a hydrophobic domain covering the active site that causes a remarkable increase in activity on interaction with a hydrophobic substrate (Patel, 2006). Lipases are increasingly used in the cosmetics industry for the synthesis of emolient esters, which improve the appearance and glide of personal care products (reviewed by Ansorge-Schumacher and Thum (2013)). In the food industry, lipases are heavily involved in cheese making, and the synthesis of sugar esters (reviewed by Khanniri et al. (2015)). The excellent stereoselectivity and enantioselectivity of lipases is exploited by the fine-chemicals industry for the production of optically active building blocks for applications in synthetic chemistry (reviewed by Pandey et al. (1999)).

p0335 To date, thermostable lipases have been isolated and characterized from *G. thermodenitrificans* IBRL-nra (Balan, Ibrahim, Abdul Rahim, & Ahmad Rashid, 2012; Balan, Magalinggam, Ibrahim, Rahim et al., 2010), *G. thermodenitrificans* AZ1 (Abdel-Fattah, Soliman, Yousef, & El-Helow, 2012), *G. stearothermophilus* JC (Jiang, Zhou, & Chen, 2010), *G. stearothermophilus* L1 (Kim, Park, Lee, & Oh, 1998), *G. stearothermophilus* DSMZ 1550 (Sheng, Jun, Hong, & Lehe), *G. stearothermophilus* strain 5 and 6 (Berekaa, Zaghloul,

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Abdel-Fattah, Saeed, & Sifour, 2009; Sifour, Zaghloul, Saeed, Berekaa, & Abdel-fattah, 2010), *G. thermoleovorans* CCR11 (Quintana-Castro, Díaz, Valerio-Alfaro, García, & Oliart-Ros, 2009), *G. thermoleovorans* Toshki (Abdel-Fattah & Gaballa, 2008), *Geobacillus zalihae* (Nurbaya, Bakar, Raja, Chor, & Basri, 2014), and many other thermophiles attributed to the *Geobacillus* genus (Hamid et al., 2003; Leow, Rahman, Basri, & Salleh, 2007; Li & Zhang, 2005; Mahadevan & Neelagund, 2014; Wang, Srivastava, Shen, & Wang, 1995; Zhu et al., 2014).

p0340 In contrast to lipases, carboxylesterases catalyze the cleavage of ester bonds of shorter acyl chain length (less than C8), with a preference for water-soluble substrates. Carboxylesterases generally require no cofactor and exhibit high regiospecificity and stereospecificity due to the presence of a unique small acyl binding pocket, which optimally fits to the acyl moiety of their substrates (Panda & Gowrishankar, 2005). To date, thermostable carboxylesterases have been isolated and characterized from *G. stearothermophilus* ATCC12980 and ATCC7954 (Ewis, Abdelal, & Lu, 2004), *G. thermoleovorans* YN (Soliman, Abdel-Fattah, Mostafa, & Gaballa, 2014), *G. thermodenitrificans* T2 (Yang et al., 2013), *G. thermodenitrificans* CMB-A2 (Charbonneau, Meddeb-Mouelhi, & Beauregard, 2010), *G. kaustophilus* HTA426 (Montoro-García et al., 2009), *G. caldoxylosilyticus* TK4 (Yildirim, Colak, Col, & Canakci, 2009), *G. thermoglucosidasius* EAEC, and many other thermophilic strains attributed to the *Geobacillus* genus (Ayna, 2013; Özbek, Kolcuoğlu, Konak, Colak, & Öz, 2014; Tekedar & Şanlı-Mohamed, 2011; Zhu et al., 2012).

p0345 Notably, studies of a *G. kaustophilus* HTA426 carboxylesterase, and related carboxylesterases, revealed stark topological differences from preexisting families of lipolytic enzymes, and has led to the argument that the *Geobacillus* carboxylesterases represent a new bacterial carboxylesterase family (Montoro-García et al., 2009).

p0350 One carboxylesterase subfamily of recent interest in the second-generation biofuel industry comprises the acetylxylan esterases (EC 3.1.1.72), which hydrolyze the ester linkages of acetyl groups at positions 2 and/or 3 of the xylose moieties in xylan. Studies have shown that the introduction of acetylxylan esterases in hemicellulase mixtures enhances the accessibility of xylanases to the xylan backbone (Zhang, Siika-aho, Tenkanen, & Viikari, 2011). Based on comparisons with known acetylxylan esterase families in the CAZy Database, Axe2 isolated from *G. stearothermophilus* T6, is thought to represent a new family of carbohydrate esterases (Alalouf et al., 2011). This is supported by a detailed three-dimensional X-ray crystal

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structure of Axe2, which revealed that the homooctamer has a unique quaternary structure built of two staggered tetrameric rings (Lansky et al., 2014).

p0355 Due to the unusual properties of these carboxylesterases and lipases, such as their resistance to organic solvents and enantiospecificity and stereospecificity, these enzymes may prove to be valuable for industrial applications in biocatalysis.

s0170 *7.2.3 L-arabinose Isomerase*

p0360 Due to its low calorific content, yet being 92% as sweet as sucrose, Tagatose, an isomer of D-galactose could be used as a novel sweetener in food products, as well as aiding in the amelioration of type-2 diabetes and hyperglycemia (Seo, 2013). [Q21] It has been heralded as a low-calorie sweetener in a wide variety of foods, beverages, health foods, and dietary supplements, as well as nonchronic drugs, tooth paste, and mouth wash (Oh, 2007). A growing body of research has highlighted additional numerous health benefits, including improvement of pregnancy and foetal development (Levin, 2001), antiplaque properties (Wong, 2000), antihalitosis properties (Laerke, Jensen, & Højsgaard, 2000), and prebiotic properties (Bertelsen, Jensen, & Buemann, 1999), as well as acting as a potent cytoprotective agent against chemically induced cell injury during organ transplants (Paterna, Boess, Stäubli, & Boelsterli, 1998).

p0365 To this end, there has been concerted biotechnological effort to develop Tagatose for mass production through chemical and enzymatic isomerization. This has focussed on enzymatic conversion using L-arabinose isomerases (L-AI; EC 5.3.1.4), since chemical production of D-tagatose from D-galactose has some disadvantages, such as complex purification steps, chemical waste formation, and by-product formation (Fan et al., 2014).

p0370 L-arabinose isomerases have been isolated and characterized from several *Geobacillus* strains, including *G. stearothermophilus* KCCM 12265 (Kim, Kim, Oh, & Oh, 2006), *G. stearothermophilus* US100 (Rhimi & Bejar, 2006), *G. stearothermophilus* IAM 11001 (Cheng, Mu, & Jiang, 2010), *G. stearothermophilus* DSM 22 (Lee et al., 2005), *G. thermodenitrificans* (Kim & Oh, 2005), and *G. thermoglucosidasius* KCTC 1828 (Seo, 2013). Higher optimum reaction temperatures (50–70 °C) change the aldose–ketose equilibrium adding weight to the exploitation of *Geobacillus* in this regard. In fact, the highest reported production level is 230 g/L D-tagatose from 500 g/L D-galactose using immobilized *G. stearothermophilus* L-arabinose isomerase in the continuous recycling mode of a packed-bed bioreactor, boasting a tagatose productivity of 230 g /L day (Kim, Ryu, Kim, & Oh, 2003).

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Another characterized L-arabinose isomerase to note is that from *G. stearothermophilus* US100, which does not require any metal ion as cofactor for enzymatic activity below 65 °C, although Mn^{2+} and Co^{2+} are required to enhance activity at its optimum temperature of 80 °C (Rhimi & Bejar, 2006). Other methods for producing D-tagatose, such as from D-fructose via a D-psicose 3-epimerase intermediate step, have been suggested to have little potential for commercial application (Wanarska & Kur, 2012). As such, isomerization of D-galactose by *Geobacillus* L-arabinose isomerases remains an economically attractive method for commercial D-tagatose production.

s0175
7.2.4 Pyrimidine Nucleoside Phosphorylases

p0375 Another family of enzymes of biotechnological interest comprises pyrimidine nucleoside phosphorylases (PyNP; EC 2.4.2.2) that, in the presence of phosphate ions, catalyze the reversible phosphorolytic cleavage of the glycosidic bond of pyrimidine nucleosides or closely related derivatives. In fact, PyNP does not discriminate between uridine and thymidine and accepts both compounds as natural substrates, making PyNP a versatile biocatalyst suitable for the enzymatic synthesis of modified nucleosides. These nucleoside analogs are widely used in the treatment of diverse human tumours (Vander Heiden, 2011), as well as pharmaceutical agents for the treatment of viral infections (De Clercq, 2011). PyNP have been isolated and characterized from several *Geobacillus* strains, including *G. stearothermophilus* JTS 859 (Hori, Watanabe, Yamazaki, & Mikami, 1990), *G. stearothermophilus* TH 6-2 (Hamamoto, Noguchi, & Midorikawa, 1996), *G. stearothermophilus* St-10 (Saunders, Wilson, & Saunders, 1969), and *G. thermoglucosidasius* NCIMB 11955 (Szeker et al., 2012).

p0380 Notably, immobilized nucleoside phosphorylases from *G. stearothermophilus* B-2194 have been demonstrated for the production of the chemotherapeutic drugs, fludarabine and cladribine as well as other nucleoside analogs, to yields of >85% (Taran, Verevkina, Feofanov, & Miroshnikov, 2009). These reports illustrate the possibilities of producing organic compounds of high market value and societal demand using enzymes from *Geobacillus* spp.

s0180
7.3 Biomimetic Nanoscale Structures

p0385 The crystalline cell-surface layers (S-layers) of some Gram-positive bacteria are composed of a single protein or glycoprotein species, binding to the peptidoglycan surface layer. Based on their nonbinding functional domains,

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these S-layer proteins exhibit square, oblique, or hexagonal lattice symmetry, suggesting a potential for exploitation as carriers for enzyme immobilization. Furthermore, the pores passing through these monomolecular arrays show identical size and morphology in the 2- to 8-nm range (Ilk, Egelseer, & Sleytr, 2011), opening up potentially broad applications functioning as biomimetic membranes (reviewed by Shen, Saboe, Sines, Erbakan, and Kumar (2014)).

p0390 [Q22] Currently, the most intense research is on the structural S-layer protein SgsE from *G. stearothermophilus* NRS 2004/3a, which self-assembles into an oblique cell-surface array (Steiner et al., 2006). Functional SgsE-based biocatalysts, engineered by fusion of a glucose-1-phosphate thymidylyltransferase to the cell wall-binding regions of recombinant SgsE, were shown to self-assemble in solution and on the surface of liposomes (Schäffer et al., 2007). In fact, liposomes covered by the SgsE/RmlA biocatalyst were also recyclable, retaining 61% of detectable enzymatic activity after one cycle (Schäffer et al., 2007).

p0395 [Q23] Self-assembly of a bifluorescent SgsE tandem fusion protein (~~ECFP~~—SgsE—~~YFP~~) has been demonstrated on solid supports, such as silicon dioxide substrates, with similar lattice symmetry to that observed with wild-type SgsE (Kainz, Steiner, Sleytr, Pum, & Toca-Herrera, 2010). Both N- and C-terminally incorporated fluorescent tags retained their specific fluorescent properties, as determined by steady-state fluorimetry, flow cytometry, and [Q24] confocal microscopy (Kainz et al., 2010). Together with studies on the SbpA S-layer protein of *Lysinibacillus sphaericus*, these advances open up a promising field in surface 2D nanofunctionalization, exploiting S-layer matrices for the controlled immobilization of antibodies, ligands and enzymes as biosensors, affinity membranes and affinity microparticles as well as for solid phase assays.

s0185 7.4 Whole-Cell Applications

s0190 *7.4.1 Biorefinement of Linen Fibres*

p0400 In addition to the use of engineered strains for fuel and chemical production from lignocellulosic substrates, where the hydrolysis of hemicellulose requires multiple synergistic enzyme activities, cells expressing a limited subset of enzyme activities can also find niche applications. One such application is the use of *Geobacillus* spp. for extraction of fine long linen fibres for use in textiles (linen) and other technical applications, such as specialty papers, composites, and insulating material (Valladares Juárez, Rost, Heitmann, Heger, & Müller, 2011). This is a promising environmentally friendly and

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reliable alternative to traditionally retting methods used for fibre extraction, such as dew retting and water retting. The current process is mediated by *G. thermoglucosidasius* PB94A (Valladares Juárez et al., 2009), which was isolated on hemp pectin and secretes pectin lyases to facilitate degradation of not only hemp pectin, but sugar beet, apple pectin, polygalacturonic acid, and citrus (Valladares Juárez et al., 2011). Like most *Geobacillus* spp., the strain does not secrete cellulases, avoiding any damage to the fibres, and fibre quality remains equally high after seven reuses of the pectinolytic bacterial culture. Operation of a purpose-built 200 L pilot-plant for one year has been reported, with future plans to commercialize the enzymatic refining of raw decorticated fibres using *G. thermoglucosidasius* PB94A cell culture (Juárez, Rost, Heitmann, Heger, & Müller, 2013).

s0195
7.4.2 Bioremediation of Environmental Pollutants

p0405 Due to increasing levels of environmental pollution, there has been a concerted effort to explore the use of microorganisms to remove and neutralize organic pollutants as an effective and economical means of environmental remediation (reviewed by Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, and Naidu (2011)). In fact, bioremediation continues to be the preferred method for household waste recycling and heavy metal, toxic chemical, and radioactive pollutant removal (Bonaventura & Johnson, 1997). Often, synthetic environmental pollutants degrade too slowly in the environment, requiring more intensive and directed methods for treatment at source including the use of *Geobacillus* spp.

p0410 Phenol and phenolic compounds represent one of the largest groups of environmental pollutants due to their broad applications as antibacterial and antifungal agents. The ability of *Geobacillus* strains to metabolize aromatic compounds has been described above and Feitkenhauer, Muller, and Markl (2003) have studied the kinetics of phenol degradation in continuous culture at 65 °C using *G. thermoleovorans*. However, most of the studies to date have focussed on fundamental biochemistry. Similarly, studies of alkane degradation have largely focussed on fundamental enzymology although the long chain alkane monooxygenase LadA clearly has potential in environmental cleanup (Feng et al., 2007; Li et al., 2008; Wang et al., 2006) and has recently been included (Brinkman et al., 2012) as an interchangeable "biobrick" part in an alkane utilizing *E. coli* strain.

p0415 Synthetic organophosphonates are another example of industrial compounds that pose a threat to the environment. In 2011, worldwide sales of the organophosphonate herbicide glyphosate were worth around

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$6.5 billion, more than the combined value of all other herbicides (Sansom, 2012).

p0420 The glyphosate-degrading *G. caldoxylosilyticus* strain T20, isolated from a central heating system, represents the first report of organophosphonate degradation by a thermophilic bacterium (Obojska, Ternan, Lejczak, Kafarski, & McMullan, 2002). The strain is capable of utilizing a number of organophosphonates as the sole phosphorus source (Obojska et al., 2002). Intriguingly, the isolation of this strain from a source unlikely to have been exposed to the herbicide glyphosate, as well as isolations of glyphosate-degrading bacteria prior to widespread commercial introduction of the herbicide, indicate that this pathway may have evolved for a different, natural substrate (Obojska et al., 2002; Pipke & Amrhein, 1988).

p0425 Acrylamide, the monomer widely used in the synthesis of polyacrylamides, is neurotoxic, genotoxic, and recently suspected to be carcinogenic (Program, 2011). The release of acrylamide during its production process and downstream applications has contaminated soil and water. For example, residual acrylamide concentrations in acrylamide flocculants approved for use in water treatment plants have been measured to range from 0.5 to 600 ppm (Program, 2011). Although there has been limited work on acrylamide-degrading *Geobacillus* isolates, the isolation and characterization of *G. thermoglucosidasius* AUT-01, a strain capable of degrading acrylamide at concentrations of 7 mM, has shown promise (Cha & Chambliss, 2013). Although the strain grew poorly on higher concentrations of acrylamide and was unable to degrade acrylic acid, the product of acrylamide degradation, it was regarded as a possible candidate for the treatment of acrylamide in foods (Cha & Chambliss, 2013). *Geobacillus thermoglucosidasius* AUT-01 cell-free extracts were subsequently used in acrylamide degradation studies on coffee, the primary source of acrylamide consumed in the diet (Cha, 2013). Although acrylamide was not totally degraded at higher concentrations of coffee in water, complete removal of acrylamide was achieved at concentrations of 100 mg of coffee/10 mL ddH_20 (Cha, 2013). However, the issue of acrylic acid degradation to smaller and less-toxic molecules must be addressed before acrylamide bioremediation can be feasible as a future bioremediation technology.

s0200 7.5 Biocontrol

p0430 One divergent biotechnological application of *Geobacillus* spp. is in the manipulation of the crop rhizosphere for the biocontrol of plant pathogens, such as *Fusarium* wilt (Weller, 1988). *Fusarium* wilt, which is the most

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widespread disease of tomato, banana, and other economically important crops is caused by the fungal pathogens, *Fusarium oxysporum* or *Fusarium solani* (Ploetz, 2006). To combat this disease, rhizospheric and endophytic microorganisms that survive and compete favourably with the *Fusarium* wilt pathogen have been isolated, most of which are *Bacillus* and *Pseudomonas* species (Siddiqui, 2006, pp. 112–142). However, one study demonstrates the use of *G. thermoglucosidasius* strain PMB207 as a biocontrol agent in the commercial production of lily bulbs, with the capacity to be used alone or in combination with the fungicide Sporgon at low concentration (<100 μg/mL) (Chung, Wu, Hsu, Huang, & Huang, 2011). Another study demonstrated the potential use of *G. caldoxylosilyticus* IRD in controlling or protecting maize plants against high salt stress (Abdelkader & Esawy, 2011). The field of biocontrol is currently still in its infancy, but may be exploitable for commercial use once regulatory hurdles imposed by the International Organization for Biological Control and REBECA (Regulation of Biological Control) are overcome (Bale, Van Lenteren, & Bigler, 2008).

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Non-Print Items

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