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Review

Genetic engineering of *Geobacillus* spp.

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ABSTRACT

Members of the genus *Geobacillus* are thermophiles that are of great biotechnological importance, since they are sources of many thermostable enzymes. Because of their metabolic versatility, geobacilli can be used as whole-cell catalysts in processes such as bioconversion and bioremediation. The effective employment of *Geobacillus* spp. requires the development of reliable methods for genetic engineering of these bacteria. Currently, genetic manipulation tools and protocols are under rapid development. However, there are several convenient cloning vectors, some of which replicate autonomously, while others are suitable for the genetic modification of chromosomal genes. Gene expression systems are also intensively studied. Combining these tools together with proper techniques for DNA transfer, some *Geobacillus* strains were shown to be valuable producers of recombinant proteins and industrially important biochemicals, such as ethanol or isobutanol. This review encompasses the progress made in the genetic engineering of *Geobacillus* spp. and surveys the vectors and transformation methods that are available for this genus.

Contents

1. Introduction

The genus *Geobacillus* was first described in 2001 by Nazina et al. (2001). It comprises thermophilic bacteria that were originally attributed to the genus *Bacillus*. Geobacilli are Gram-positive, endospore-forming, aerobic or facultative anaerobic thermophiles, growing optimally at temperatures between 50 °C and 60 °C. However, growth temperature can vary within a range of 37 °C to 80 °C in some species or strains. Members of this genus are isolated from a wide range of environmental samples: from subterranean oilfields to the soil of moderate or even permafrost temperatures. They do not usually have special growth requirements and are able to utilize various carbon sources (Coorevits et al., 2012; Nazina et al., 2001; Omokoko et al., 2008; Zeigler, 2014). Taking into account all of these properties, *Geobacillus* spp. could be beneficial in various biotechnological applications.

Bacteria of the genus *Geobacillus* draw attention as a source of many biotechnologically relevant thermostable enzymes, such as proteases,

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amylases, lipases (McMullan et al., 2004), DNA polymerase (Mead et al., 1991) and reverse transcriptase (Vellore et al., 2004). Most of these enzymes are produced in a mesophilic host, which has a well-established expression system, usually in *Escherichia coli*. However, heterologous expression of proteins in a phylogenetically distant host, which grows under remarkably different temperatures, has some drawbacks. Lower temperatures cause the incorrect folding of several thermophilic proteins (Suzuki et al., 2013a). Furthermore, differences in codon usage and the lack of required co-factors can result in reduced expression levels (Turner et al., 2007). To avoid these circumstances, it is of great interest to establish an effective protein production system in *Geobacillus* spp. cells.

Due to its wide growth temperature range, geobacilli, when cultivated at the proper temperature, can serve as a host for the expression of proteins derived not only from thermophiles but also from mesophiles (Blanchard et al., 2014; Couñago and Shamoo, 2005). Several applications of *Geobacillus* spp. in the production of thermostable variants of mesophilic proteins were demonstrated (Couñago et al., 2006; Liao and Kanikula, 1990; Suzuki et al., 2014).

In addition, *Geobacillus* spp. have tremendous possibility to be used as whole cell-biocatalysts. They are able to produce a large variety of enzymes required for the production of valuable bio-products (Xiao et al., 2012) or for the biodegradation of pollutants (Banat and Marchant, 2011; Feng et al., 2007; Shintani et al., 2014; Zheng et al., 2014). Using an appropriate genetic toolkit, the quantities and range of synthesized enzymes and biochemicals could be further expanded.

The application of thermophiles for industrial purposes has been well described in several reviews (Lin and Xu, 2013; Taylor et al., 2011; Turner et al., 2007). The advantages of high-temperature (≥50 °C) bioprocesses include: (1) minimization of microbial contamination, (2) reduction of energy input for cooling, and (3) facilitation of the downstream recovery of volatile products (like ethanol). Other thermophiles, including the Gram-negative bacteria *Thermus thermophillus*, *Thermotoga maritima*, and archaeon *Thermococcus kodakariensis*, could also be used in bioprocesses at elevated temperatures (Taylor et al., 2011). However, *Geobacillus* spp. have some advantages: (1) geobacilli are not fastidious and they can be cultivated on various substrates, (2) they form endospores which allow them to survive unfavorable conditions and facilitate their storage and transportation (Blanchard et al., 2014), and (3) the high growth rate allows high cell densities to be reached and consequently the high production of the required products (Suzuki et al., 2013a).

Thus, the biotechnological potential of *Geobacillus* spp. gives an incentive to develop molecular biology techniques for analysis and genetic modification of these thermophiles. A genetic transformation system consists of three elements: a suitable host, a methodology for introducing exogenous DNA to this host and a molecular vehicle for recombinant gene (or other DNA insert) transfer (Inoue and Sako, 2013). Comparing the host-vector systems that are available today, geobacilli are still lagging behind other bacteria, such as *E. coli* or *Bacillus subtilis*. However, continuous progress is being made towards improving the tools for the genetic manipulation of these thermophiles.

The establishment of reliable transformation techniques for new bacterial species is a challenging process. Geobacilli are phylogenetically relevant to relatively well-studied members of the genus *Bacillus*. This implies the similarity of these two genera and facilitates the construction of a genetic transformation system for *Geobacillus* spp., as many methods developed for *Bacillus* spp. could be modified and adapted for *Geobacillus* spp. Moreover, the increasing number of publicly available complete genome sequences of geobacilli (Studholme, 2015) provides more information for a better understanding of their biology and the development of host-vector systems.

This review provides an overview of the genetic tools and methodologies available for geobacilli and describes the current tendencies for their further enhancement and application.

2. Hosts: most commonly used *Geobacillus* strains for transformation

A convenient host strain should be easily cultivable and readily transformable. The choice of *Geobacillus* spp. as a host is restricted, since genetic engineering methods are not well established for these thermophiles. Nevertheless, several transformation systems for some *Geobacillus* strains have been described (Table 1). The most commonly used strains are *Geobacillus stearothermophilus* NUB36, *Geobacillus thermoglucosidasius* NCIMB 11955 and *Geobacillus kaustophilus* HTA426.

G. stearothermophilus NUB36 and its derivatives (NUB3621 and NUB3621R) are among the most studied *Geobacillus* strains. Despite the scientific significance of strain NUB36, its systematic position remains uncertain. After the isolation of NUB36 from soil, it was assigned to *Bacillus stearothermophilus* (Chen et al., 1986). Genetic analysis of this strain showed that it is too distinct phylogenetically to belong to the species *G. stearothermophilus* (Studholme et al., 1999) and should be reclassified as *Geobacillus caldoxylosilyticus* or assigned to a novel species of *Geobacillus* (Studholme, 2015; Zeigler, 2005). However, this strain still holds the original name allocated upon discovery.

The growth temperature of NUB36 strains can vary from 39 °C to 75 °C, but optimal growth is reached at ~65 °C (Chen et al., 1986; Wu and Welker, 1991). *G. stearothermophilus* NUB36 was intensively investigated by Welker and colleagues (Vallier and Welker, 1990; Wu and Welker, 1989), who isolated a number of mutant variants of this strain. The strain NUB3621 is a mutant that is resistant to rifampin and lacking a restriction modification (R-M) system (Chen et al., 1986). An efficient protoplast transformation protocol is available for this strain (Wu and Welker, 1989). Another strain, NUB3621-R, is a derivative of NUB3621 and is capable of producing an even higher number of transformants (using protoplast transformation), compared to its parental strain. Unfortunately, strain NUB3621-R and mutations that led to such phenotype have never been described in the literature (Personal communication with prof. D. R. Zeigler). Recently, Blanchard et al. (2014) presented a draft genome sequence of strain NUB3621, which makes it an even more attractive host for genetic engineering.

G. thermoglucosidasius strain NCIMB 11955 (type strain of the species, also known as DSM 2542^T) is primarily of interest because of its potential to be used in the biofuel industry (Cripps et al., 2009). Therefore, techniques for genetic engineering of this strain are rapidly developing. This strain (and its derivatives) can be efficiently transformed by a protocol described by Taylor et al. (2008). Moreover, an integrative vector system is available for genome manipulations (Cripps et al., 2009) and an expression system is currently under intensive development (Bartosiak-Jentys et al., 2013; Lin et al., 2014).

G. kaustophilus HTA426 is another promising thermophilic host. This strain can be cultivated at temperatures between 42 °C and 74 °C under aerobic conditions and is known to tolerate NaCl concentrations up to 3% (Suzuki and Yoshida, 2012). It grows as rapidly as *E. coli* and reaches high cell densities. Consequently, high yields of the desired product can be expected (Suzuki et al., 2013a). The published genome sequence of *G. kaustophilus* HTA426 (Takami et al., 2004) provides opportunities for its thorough research and subsequent practical applications. Suzuki et al. (2012, 2013b, 2014) made significant progress within the field by establishing a host–vector system for *G. kaustophilus*. They developed a genetic transformation protocol using conjugative transfer (Suzuki and Yoshida, 2012), constructed a highly transformable strain MK244 (auxotrophic for uracil and deficient in some R-M system genes) and showed the possibility of using *G. kaustophilus* for the construction of gene libraries and the identification of thermophile enzyme genes by in vivo functional screening (Suzuki et al., 2013b). Furthermore, the same group created a strain MK480, which is deficient for five DNA repair genes, and is therefore useful for the generation of mutant proteins with enhanced properties (Suzuki et al., 2014, 2015).