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Cellulolytic *Bacillus* species isolated from the Gut of the Desert Locust *Schistocerca gregaria*

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Abstract

The ability of many insects to thrive on wood, foliage, and detritus has stimulated investigation on the extent to which the gut microbial flora of such species are able to digest lignocellulosic material in their diet. In this study, the capability of the intestinal flora from the gut of *Schistocerca gregaria* to degrade cellulose model compound was investigated. Two cellulolytic *Bacillus* strains with Index cellulolytic activities (ICA) values of 1.146 ± 0.109 and 0.8442 ± 0.09203 were isolated and identified by 16S rDNA gene sequence homology and phylogeny tree building. The isolates were identified as *Bacillus* sp. *MED* and *Bacillus* sp. *CACO* and their respective 16S rDNA sequences deposited to GenBank under accession numbers JQ 837242.1 and JQ 837243.1.

***Keywords*:** Cellulases, Lignocellulose, Carboxymethylcellulose, Desert locust, Schistocercagregaria

Introduction

Lignocellulose, the major structural component of plants represent a major source of renewable organic material [1]–[4]. Lignocellulose consists of Lignin, hemicellulose, and cellulose, and is considered an optimal feedstock for industrial production of biofuels and other value-added products [3]–[5]. Bioconversion of lignocellulose is initiated by microbial cellulases from fungi and bacteria which are capable of degrading lignocellulosic material [1], [2]. Lignocellulose is, however, a recalcitrant polymer as it is insoluble and is present as hydrogen-bonded crystalline fibres [6]–[8]. Therefore Current industrial bioconversion of lignocellulose requires the application of high temperature and acidic or basic conditions to breakdown lignin, decrease the crystallinity, increase pore volume and solubilize cellulose and hemicellulose, a phenomenon referred to as pre-treatment [8], [9]. This process is, however, both expensive and inefficient [4], [8]. Attempts to improve the feasibility of the bioconversion of lignocellulose have increased interest in prospecting for novel cellulolytic enzymes capable of increasing the depolymerization efficiencies [4], [5], [7], [10].

A diverse spectrum of microorganisms with the capacity to biodegrade cellulosic substrates, mainly fungi and bacteria, have previously been isolated and identified over the years and the list continues to grow rapidly [3], [6], [7], [10]. The ability of many insects to thrive on wood, foliage, and detritus has stimulated investigation on the extent to which the gut microbial flora of such species are able to digest lignocellulosic material in their diet [11], [12]. Though several reviews on cellulolytic activity in insects are available [11]–[13] the role of intestinal microbes in the recycling of organic matter in the gut system has often been disregarded [14]. The desert locust *S. gregaria* is a notorious polyphagous insect species capable of consuming several plant species in its surroundings [15], [16]. The desert locust has been reported to have a rich gut bacterial flora, however, little is known of the relationship between the microbes and their host [16]–[19]. The gut microbiota of the desert locusts is, however,

thought to help protect the host from invasion by pathogenic microorganisms, a process known as colonization resistance (CR) [17], [18]. Previous reports also show that key components of the locust aggregation pheromone are produced by the gut bacteria [16]–[18]. Although low levels of cellulolytic activity have been detected in the gut of the desert locust *S.gregaria* and other related species [5], [12], [20], [21] there is limited information regarding cellulolytic bacteria present [19], [21]. In this study, the capability of the intestinal flora from the gut of the desert locust *S. gregaria* to degrade cellulosic compounds was investigated. To do so we used a combination of enrichment culture and 16S rRNA gene sequencing to isolate and identify cellulolytic bacteria from the gut of the desert locust S. gregaria using carboxymethyl cellulose as a model substrate. To our knowledge, this is the first case for isolation of such bacteria from desert locust and their potential impact in the biotechnological transformation of Lignocellulosic biomass.

Material and Methods

Isolation of the Bacteria

An adult desert locust, *S. gregaria* obtained from the School of Biological Sciences at the University of Nairobi, Kenya was surface sterilised in 70% ethanol, then rinsed with sterile distilled water and air-dried for 5 minutes at room temperature. The locust was then dissected with a pair of a sterile scalpel and the entire gut removed and suspended in 10 ml of 0.85% NaCl. An aliquot of 0.5 ml of the gut suspension was mixed with 4.5 ml of sterile Medium 1, a basal medium described by Chakraborty et al.[22] containing $0.2gL^{-1}$ yeast extract and $5gL^{-1}$carboxymethyl cellulose (CMC) as a sole carbon source and the pH set at 6.9. The culture was incubated at 37^0 C for 3 days in an airtight falcon tube (IWAKI, Japan). A second media, Medium 2, a modified DSMZ medium 65 containing $5gL^{-1}$Carboxymethyl cellulose (CMC), $0.1gL^{-1}$ Malt extract, $0.04gL^{-1}$ Yeast extract and pH set at 7.2 was also used for isolation Wenzel et al. [23]. The culture was then incubated for 4 weeks at 37ºC in an airtight

falcon tube (IWAKI, Japan). After respective incubation periods10µl of the resulting cultures were spread on nutrient agar plates and incubated for 24hrs at 37ºC. Pure colonies were obtained by several (five-six) subsequent plating on nutrient agar plates (OXOID, UK) (Supplementary Information **Fig S1**). The isolated bacterial strains were finally cultivated on nutrient broth (OXOID, UK). All steps were performed under aseptic conditions.

Screening for cellulose-degrading capacity

To test for cellulolytic activity of the bacterial isolates, a solid medium described by Wenzel et al. [23] designated Medium 3 [5gL^{-1} CMC, 4gL^{-1} Yeast extract, 10gL^{-1} Malt extract, and 12gL^{-1} Agar, pH 7.2 (modified, DSMZ medium 65 without $CaCO_3$ and glucose)] was used. The isolates were spread on Medium 3 plates and incubated for 5 days at 37ºC. Carboxymethyl cellulose degradation was tested by flooding the plates with 1mg/ml congo red dye (Sigma, USA) for 15 minutes followed by destaining with 1M NaCl. Bacterial strains that showed enzymatic activity towards CMC were used in subsequent studies.

Morphological and molecular identification of the bacterial isolates

The isolates were subcultured on Nutrient agar plates for 24 hours before a colony of each isolate was transferred on to sterile glass slides. Gram stain [24] and KOH test [25] were performed. KOH test uses 3% potassium hydroxide to determine gram reaction [25]. For molecular identification 16s rRNA gene was used. For this purpose, the procedure described by Wilson [26] was used to harvest total genomic DNA from the bacterial cells. Polymerase chain reaction (PCR) was performed in a TProfessional thermocycler (Biometra, Germany). The 16S rRNA gene primers previously described by Baumgarte et al. [27] were used: 16sF27, forward 5'…AGA GTT TGA TC(AC) TGG CTC AG…3' and 16sR 1492, reverse 5'…TAC GG(CT) TAC CTT GTT ACG ACT T…3'. The PCR reaction was performed in a total volume of 50µl containing 2mM $MgCl_2$, 200µM dNTPs, 1µM of each primer, 2.5U Taq polymerase and 100ng of the DNA template (Supplementary information TableS2). The PCR

was carried out according to Baumgarte, et al. [27] with some modifications (Supplementary information TableS3). The PCR products were then gel purified using Illustra GFX PCR DNA and Gel purification kit (GE Healthcare, UK), analysed on a 1% agarose gel to determine their sizes and quality before they were sent to ILRI (International Livestock Research Institute, Kenya) for sequencing. The deduced sequences were used for BLAST searches at NCBI using the default settings of BLASTn algorithm with the Database set at 16S ribosomal RNA sequences (Bacteria and Archaea) and optimized for highly similar sequences [28]. Fifteen top hits with identities above 99% and 100% coverage retrieved from the database, were aligned in MUSCLE [29] and a phylogenetic tree constructed based on the nucleotide sequences with the Bayesian phylogenetic method in MrBayes [30]. The tree was then visualised using fig tree software obtained at http://tree.bio.ed.ac.uk/.

Results and Discussion

For many years research efforts on cellulolytic microbial symbionts have mainly been focused on beetles and termites due to their high level of destruction to plant and wooden material [21], [23], [31]. In contrast, research into the mechanisms underlying the degradation of plant material, an important food source, in locust has widely been neglected [21]. This study was therefore motivated by the limited information available regarding cellulolytic symbiotic microbes in the gut of the desert locust *S. gregaria* and their potential applications in industrial biotransformation of cellulosic material. The organism was considered an appropriate target for screening cellulolytic bacteria because of its ability to utilise lignocellulose plant material resulting in massive destruction of cultivated fields and vegetation as previously reported [16]. The desert locusts used in the study were laboratory-bred adults whose diet comprised of freshly cut grass. The alimentary canal of the desert locust *Schistocerca* is anatomically similar to that of *Locuster migratoria* for which detailed descriptions are available and the pH of the gut increases posteriorly from pH 5.7 at the

mouth to pH 7.1 in the hindgut [5], [20]. The approach used by Wenzel et al. [23] to isolate bacteria from termites was extended to the desert locust *S. gregaria* where two distinctive media (Medium 1 and Medium2) of different pH (Supplementary information TableS1) were used to increase the chances of isolating bacteria along the entire gut. From the study, two bacterial isolates tagged CB1 and CB2 were obtained (Table 1) each from the two distinctive media used respectively. The isolates were both able to grow on CMC media at 37ºC, and from the cellulolytic test result (Fig. 1), clear zones around the bacterial colonies on CMC plates after staining with Congo red dye indicated cellulolytic activity. Index cellulase activity (ICA) was then calculated as **ICA = [(Diameter clear zone – Diameter bacterial colony)/Diameter bacterial colony]** according to Mangunwardoyo et al. [32] (Supplementary information TableS4). ICA values of 1.146 ± 0.109 and 0.8442 ± 0.09203 were obtained for CB1 and CB2 respectively (Table 1) indicating the ability to degrade cellulose. The ICA values (ICA) for CB1 was slightly higher that of CB 2, however, the difference was not statistically significant (p-value >0.05).

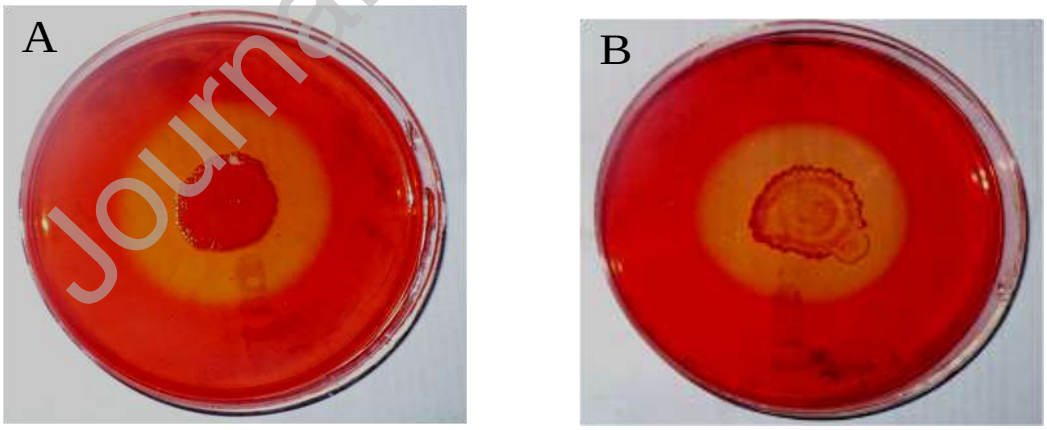

FIGURE 1: Photographs of agar plates containing CMC spotted with bacterial isolates. Clear zones around the isolates after flooding the plates with 1mg/ml Congo red are indications of cellulolytic activity. (A) CB 1**; *Bacillus* sp. MED** from Medium 1 (B) CB 2**; *Bacillus* sp. CACO** from Medium 2.

Characterisation of the isolates relied on morphological characteristics and molecular tools. The morphological features of the isolates were determined by gram stain [24] and KOH tests [25]. KOH test is normally used as a confirmatory test for gram stain. It uses 3% potassium hydroxide to determine the gram reaction. Gram-negative bacteria become viscous and string out, while gram-positive bacteria are not affected [25]. The two tests confirmed the two isolates were gram-positive rods (Table 1).

Table 1: The morphology characteristics of isolated bacteria and cellulolytic activities

	Isolates	
Tests	**CB 1 (*Bacillus* sp. MED)**	**CB 2 (*Bacillus* sp. CACO)**
Gram stain	+ve	+ve
KOH test	No effect	No effect
Catalase	+ve	+ve
Colony appearance on Nutrient agar	Creamy, Circular, raised	Creamy, irregular, flat
Shape	rod	rod
ICA number	1.1463 ±0.109	0.8442 ±0.09203

The isolates were further identified by 16S rRNA gene sequence homology at NCBI using the BLAST search algorithm. Blast search of the 16S partial sequences revealed that both isolates (CB1 and CB2) were closely related to *Bacillus safensis* with sequence identities >99% with *Bacillus safensis* strain NBRC 100820 (accession no. NR113945) and Bacillus safensis FO-36b (accession no. NR041794). The 16S rRNA gene sequences for the two isolates were deposited to GenBank at NCBI where the isolates were tagged as *Bacillus* sp. MED 1and *Bacillus* sp. CACO with accession numbers JQ 837242.1 and JQ 837243.1 for CB1 and CB2 respectively. A Phylogenetic tree (Fig 2) based on the homologous sequences obtained from blast searches [28] was constructed, where the isolates clustered with

members of *B. safensis* clade as expected with a posterior probability value of 94% (< 97%) signifying that these isolates are likely to be new members of this group. From the tree topology, the two isolates also appeared to be closely related.

Hydrolytic activity towards cellulose powder has previously been detected in the gut fluids of a few species of locusts [20], [21] and related grasshoppers (Willis, et al., 2010). There is however limited information correlating the observed hydrolytic activity of cellulose microbial degradation in the gut of desert locusts [19]–[21]. In their study, Evans & Payne [20] eliminated bacterial degradation of cellulose by addition of toluene to their reaction mixtures, where they noted, addition of toluene to the reaction mixture containing fresh gut content caused a 40% decrease in cellulose hydrolysis. They, therefore, suggested that cellulolytic bacteria played a part in cellulose digestion in addition to endogenous cellulase enzymes, however, they were unsuccessful in the detection of protozoa, which could have been a source of cellulases on examination of the gut content under a microscope [20]. More recently Su et al. reported cellulolytic activity and the community structure of symbiotic bacteria in the gut of locusts from five different families including Acrididae, Arcrypteridae, Oedipodidae, Catantopidae, and pyrgomorphidae [21]. Their study showed differences in the intestinal bacterial community structure of different locust species and that majority of these bacterial symbionts had cellulose degradation capacity [21].

Studies on the microbial flora of *S. gregaria* indicate that the gut contains a relatively simple but abundant microbiota which originates from the insect diet [16]–[19]. The organisms identified to date include *Escherichia coli*, *Enterobacterliquefaciens*, *Klebsiellapneumoniae*, *Enterobacter cloacae* and a number of gram-positive *streptococci* [18]. The gut microbes of the desert locust are thought to help protect the host from invasion by pathogenic microorganisms, a phenomenon is known as colonization resistance. It has also been reported that bacterially derived phenolics (Guaiacol& Phenol) provide key components of the locust

aggregation pheromone [16]–[18] and the digestion and processing of plant material in the gut appears to be a pre-requisite for production of guaiacol by bacteria. The most obvious precursor has been reported to be the plant-derived vanillic acid from lignin[17]. The failure to isolate cellulolytic bacteria from the gut of desert locust, *S. gregaria* to date is therefore not compelling evidence against the involvement of cellulolytic bacteria in the digestion of dietary cellulose. This study, therefore, provides the likelihood of bacterial involvement in the digestion of cellulosic material and associated plant polymers in *S. gregaria* as previously proposed by Su et al. for other locust species [21]. This suggests that locust gut flora offers a potential source of cellulolytic bacteria, that could be useful in the biotransformation of cellulosic material to biofuels and other chemicals using inexpensive substrates.

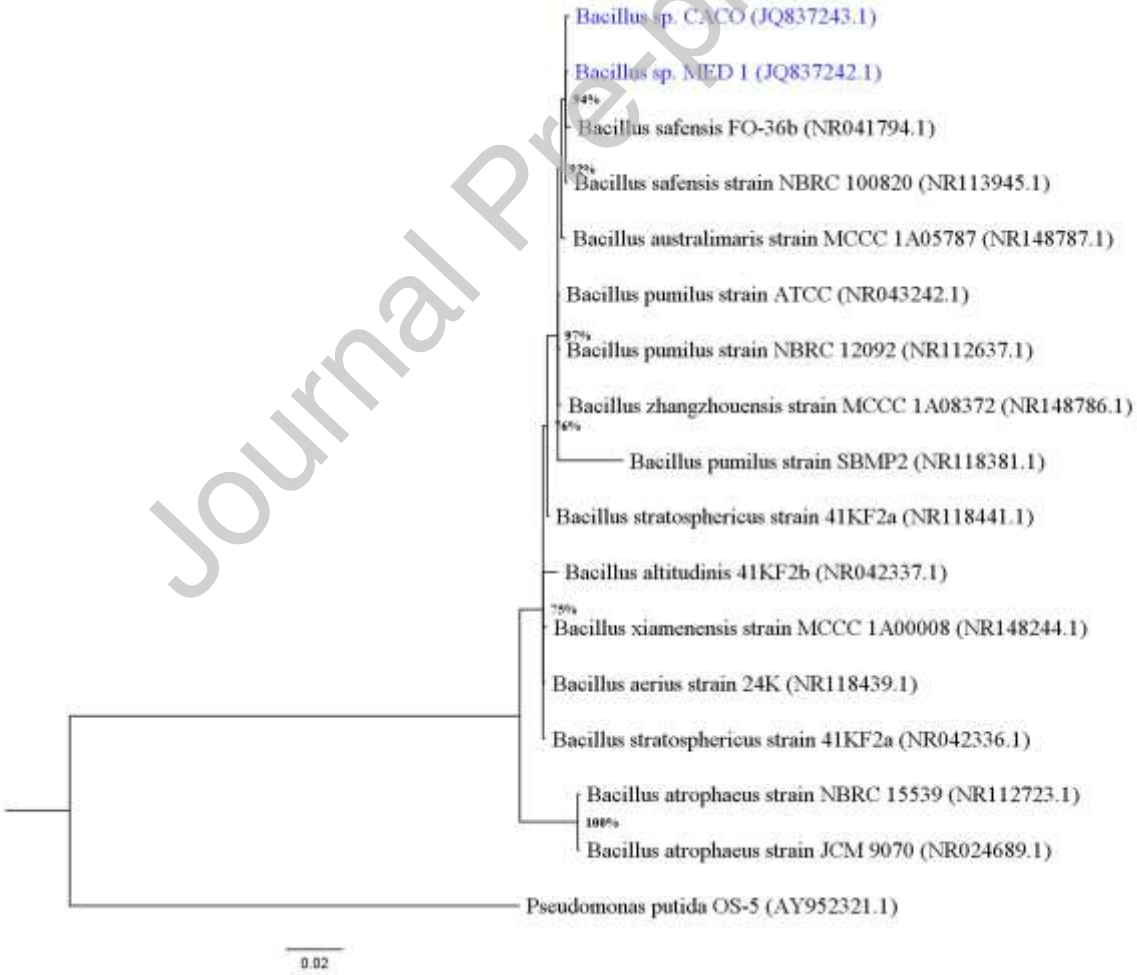

FIGURE 2: Phylogenetic tree based on 16s rDNA gene sequences. The tree was constructed in MrBayes, a program for the Bayesian inference of phylogeny that is based on the Markov

Chain Monte Carlo (MCMC) method. Numbers at the nodes indicate the percentage of posterior probabilities indicating topological robustness of the Phylogenetic tree. *Pseudomonas putida* strain OS-5 was used as an outgroup to root the tree.

Conclusion

Results from this study suggest that the desert locust (*S. gregaria*) gut offers a potential source of microbial cellulases, which may be useful in the biotransformation of cellulosic material to biofuels and other chemicals using inexpensive substrates.

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Conflict of interest

No conflict of interest to declare.

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