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Secretome analysis of alkaliphilic bacterium *Bacillus lehensis* G1 in response to pH changes

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ABSTRACT

Bacillus lehensis G1 is an alkaliphilic bacterium that is capable of surviving in environments up to pH 11. Secretome related to bacterial acclimation in alkaline environment has been less studied compared to cytoplasmic and membrane proteome. The aim of this study was to gain better understanding of bacterial acclimation to alkaline media through analyzing extracellular proteins of *B. lehensis*. The pH range for *B. lehensis* growth was conducted, and two-dimensional electrophoresis and MALDI-TOF/TOF MS analysis were conducted to characterize changes in protein profiling in *B. lehensis* cultured at pH 8 and pH 11 when compared with those cultured at pH 10 (optimal growth pH). *B. lehensis* could grow well at pH ranging from 8 to 11 in which the bacteria showed to posses thinner flagella at pH 11. Proteomic analyses demonstrated that five proteins were up-regulated and 13 proteins were down-regulated at pH 8, whereas at pH 11, 14 proteins were up-regulated and 8 were down-regulated. Majority of the differentially expressed proteins were involved in the cell wall, main glycolytic pathways, the metabolism of amino acids and related molecules and some proteins of unknown function. A total of 40 differentially expressed protein spots corresponding to 33 proteins were identified; including GlcNAc-binding protein A, chitinase, endopeptidase lytE, flagellar hook-associated proteins and enolase. These proteins may play important roles in acclimation to alkaline media via reallocation of cell wall structure and changes to cell surface glycolytic enzymes, amino acid metabolism, flagellar hook-associated proteins and chaperones to sustain life under pH-stressed conditions.

1. Introduction

During the life cycle of bacteria, cells can be exposed to large amount of environmental stress that can impair their growth and survival. Such harsh conditions may include changes in the environmental temperature, pH, nutrient deficiency and others. Alkaliphiles are unique microorganisms that are undergoing increasing biotechnological exploitation due to their ability to grow under extreme conditions and the use for their enzymes, such as alkaline proteases and alkaline cellulases, in various industries (Fujinami and Fujisawa, 2010). Alkaliphilic *Bacillus lehensis* G1 is a Gram-positive soil bacterium that was isolated from a rubber plantation in Johor, Malaysia. The genome of *B. lehensis* G1 has been sequenced, and its alkalitolerance-related genes, including sodium-proton antiporters and proteins involved in respiration and cytoplasmic pH maintenance, have been identified (Noor et al., 2014). Although the study of *B. lehensis* G1 genome is crucial, studying its proteins is equally important as proteins carry out most of life functions and comprise the majority of cellular structures. Therefore, proteome studies are needed to understand the alkali-adaptive properties of this bacterium. *B. lehensis* G1 is well known for its ability to secrete proteins into the extracellular medium, including the starch degrading enzyme cyclodextrin glucanotransferase (CGTase) (Sian et al., 2005). Because this bacterium can survive in alkaline environments, studying the secretome will provide interesting insights into its cellular acclimation mechanisms.

The term secretome refers to a set of proteins that includes extracellular matrix proteins, vesicle proteins and proteins that are shed from the cell membrane (Makridakis and Vlahou, 2010). Two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS) has been used to study the secretome of many microorganisms (Calasso et al., 2013; Enany et al., 2013; Målen et al., 2007). Extracellular protein expression can be seen as the physiological response of cells to specific growth conditions such as salt stress (Rubiano-Labrador et al., 2015) and is also an indicator of how microorganisms interact with

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their environment (Armengaud et al., 2012). In growth media with a high pH, it is crucial for the cell to counteract intracellular alkalization by activating systems for protein repair or degradation, elevating the production of transporters and enzymes for proton capture and retention, increasing acid production via metabolic changes and inducing changes in cell surface layers to retain cytoplasmic protons (Padan et al., 2005). The pH of the environment can affect the balance of electric charges on the cell surface (Aono et al., 1995) and thus can induce swift hyperpolarization of the membrane at alkaline pH (Zilberstein et al., 1984).

Adaptation of the cellular proteome and membrane proteome in alkaline environments has been reported in several studies. Certain iron transport-associated membrane proteins and proteins involved in bioenergetic processes, such as the metabolism of carbohydrates, fatty acids, amino acids and nucleotides, were affected as the external pH increased (Wang et al., 2009). Survival of *Listeria monocytogenes* under lethal alkaline stress (pH 12) involves genes that are associated with virulence, the general stress response, cell division and changes in cell wall structure, as well as genes with unknown functions (Giotis et al., 2008). Under stress situations, the functional integrity of cellular proteins is maintained through protein quality control mechanisms involving molecular chaperones, protein-folding catalysts and ATP-dependent proteases (Hahne et al., 2010). Stress conditions such as pH, temperature and oxidative stress can induce similar genes that appear to activate cellular defenses (Barriuso-Iglesias et al., 2008), which play essential roles in adaptation and resisting the damaging effects of stressful environments (Li et al., 2012). However, to our current knowledge, little is known about the bacterial secretome during pH acclimation and the effects of pH on the cell and its secretome.

Here, the secretome changes in *B. lehensis* G1 were investigated under three different alkaline pH conditions (pH 8, 10 and 11). The presented findings describe the first proteomic analysis of the alkaliphilic *B. lehensis* G1 secretome, which provides novel insight into the acclimation that occur in this alkaliphilic bacterium, including reallocation of the cell wall structure and changes to cell surface glycolytic enzymes, amino acid metabolism, flagellar hook-associated proteins and chaperones, to sustain life under pH-stressed conditions.

2. Materials and methods

2.1. Bacterial strain and culture conditions

B. lehensis G1 was cultured in Horikoshi medium under different pH conditions using 100 mM sodium phosphate monobasic/sodium phosphate dibasic buffer (pH 7–8), 100 mM sodium carbonate/sodium bicarbonate buffer (pH 9–10) and 100 mM sodium carbonate /sodium hydroxide buffer (pH 11–12) (Nakajima et al., 2005). To prepare the inoculum, cells were incubated at 37 °C with shaking for 15 h. One percent of cells with an optical density of approximately 0.6 (550 nm) was inoculated into a 500 ml conical flask containing 100 ml of medium. Then, the flask was incubated at 37 °C with shaking for 15 h to determine the cell growth profile. Aliquots were withdrawn from the culture media at intervals followed by OD measurement at 550 nm. The maximum specific growth rate at log phase was determined by plotting OD values at log scale against time. For all tested growth conditions, bacterial cells were harvested at mid-log phase.

2.2. Cell morphology

The immobilized cells on the PVDF membrane were analyzed using Field Emission Scanning Electron Microscope (FESEM) (Yang et al., 2006). Fiber pieces were soaked in 2.5% (w/v) glutaraldehyde overnight at 4 °C to fix the bacteria. The treated fiber pieces were dehydrated in graded ethanol solutions as follows: 25% (10 min), 50% (10 min), 75% (10 min), 80% (10 min), 95% (10 min), 100% (10 min). Then, the dehydrated fiber samples were dried at 80 °C for 2 h. The fibers were broken into small pieces several millimeters in size and mounted on a FESEM sample stub on a carbon tape. The samples were coated with gold before examination using a ZEISS SUPRA 35VP (Oxford Instruments, Germany).

2.3. Electron microscopy for flagella observation

For transmission electron microscopy (TEM) sample preparation, formvar carbon film copper grids (400 mesh) were placed into aliquots of bacterial suspension and equilibrated for 5 min. The grids were rinsed gently with distilled water and excess fluid was wicked away with filter paper. The sample grids were negatively stained with 2% uranly acetate for 5 min. The bacteria samples were imaged with TEM (JEOL JEM2100F). The data in the TEM images were measured using ImageJ software.

2.4. Preparation and quantification of extracellular proteins

B. lehensis G1 extracellular proteins were collected at mid-log phase as previously described (Antelmann et al., 2000) with slight modification. Cells were removed from the growth medium via centrifugation at 9000 rpm/min and 4 °C for 15 min. Next, proteins in the supernatant were precipitated with 10% (w/v) pre-chilled trichloroacetic acid for 30 min and were collected via centrifugation at 9000 rpm/min for 15 min. The resulting protein pellet was collected and washed twice with pre-chilled acetone. The supernatant was removed, and the resulting protein pellet was air-dried for 5 min. Finally, the pellet was resolubilized in rehydration buffer (8 M urea, 40 mM dithiotreitol (DTT), 2% CHAPS, 0.5% (v/v) carrier ampholytes, 1 mM protease inhibitor cocktail, 0.002% bromophenol blue). The protein concentration of the extracellular protein sample was determined using a 2-D Quant Kit (GE Healthcare) according to the manufacturer's protocols.

2.5. Two-dimensional gel electrophoresis (2-DE) and gel analysis

1D isoelectric focusing (IEF) was carried out using an IEF 100 (Hoefer) and 2D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using a VS20 WAVE Maxi (Cleaver Scientific Ltd). The protocols were carried according to manufacturer recommendations. Aliquots of 400 µg protein samples were used to rehydrate gel strips (Immobiline DryStrip pH 4–7, Bio-Rad) for 12 h. After IEF, the strips were equilibrated with 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue in a two-step process. For the first step, 10 mg/ml DTT was added, and 25 mg/ml iodoacetamide (IAA) was used in the second step. After equilibration, IPG strips were placed on pre-casted 15% gels. SDS-PAGE was performed at 4 °C with 10–15 mA for 30 min, followed by separation at 110–120 V until the tracking dye reached the bottom of the gel. The gel was visualized by staining with Coomassie blue G-250 solution. Each independent experiment was repeated in triplicate.

The stained gels were scanned using a Densitometer GS-800 (Bio-Rad Laboratories). Spot detection was performed using Progenesis software (Nonlinear Dynamics, Newcastle). Spots were automatically co-detected on all images in the analysis, creating a single spot pattern of the analysis. Next, spot picking files were created for manual picking or using spot picking robot. All the visible spots on the 2-DE were picked and pooled from triplicate gels. Proteins were considered to be differentially expressed if the mean percentage spot normalized volume for an individual protein was at least 2.0-fold up- or down-regulated. Significance was determined using Student's *t*-test with a P value of 0.05, and data that did not meet the above criteria were eliminated from the differentially expressed analysis.

2.6. In-gel digestion, mass spectrometry (MS), and database searching

Selected protein spots were in-gel digested using a trypsin digestion