African Journal of Bacteriology Research

Volume 7 Number 3, April 2015

ISSN 2006-9871

Academic Journals

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Bacteriology Research

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academicJournals

Vol. 7(3), pp. 19-23, April, 2015
DOI: 10.5897/JBR2014.0137
Article Number: EA21EF551708
ISSN 2006-9871

http://www.academicjournals.org/JBR

Journal of Bacteriology Research

Full Length Research Paper

Acinetobacter spp. in the patients and environment of University Hospital of Yopougon, Côte d'Ivoire, from 2007 to 2011

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Received 11 September, 2014; Accepted 13 March, 2015

The objective of this work was to study the epidemiology and antibiotic resistance of strains of *Acinetobacter* spp. in the University Hospital of Yopougon Abidjan. This work studied the *Acinetobacter* strains isolated from humans and environment of the hospital; they were preserved in the culture collection of the Laboratory of Bacteriology from January 2007 to December 2011. Isolation and identification were made by conventional bacteriological methods, and antibiotic susceptibility was studied by the method of agar diffusion. Interpretation was made according to the standards of the CA-SFM. 110 strains of *Acinetobacter* spp. have been studied (61% of human strains and 39% of strains isolated from the hospital environment). *Acinetobacter baumannii* was the most isolated in 66% of cases. 52. 8% of strains were resistant to ceftazidime; 5.6% to imipenem; 21.2% to gentamicin and 35.2% to ciprofloxacin. 12.5% of human strains of *A. baumannii* were multi-resistant bacteria. *Acinetobacters* spp. are present in the hospital environment and patients with a predominance of *A. baumannii* species. The presence of imipenem-resistant strains is a major public health problem because their disclosure could lead to therapeutic impasse in hospital.

Key words: *Acinetobacter* spp., epidemiology, antibiotic resistance.

INTRODUCTION

Acinetobacter spp. are emerging opportunistic pathogenic bacteria that play an important role in hospitals worldwide (Munoz-Price and Weinstein, 2008; Schreckenberger et al., 2007). Indeed, in recent years, these bacteria have become a concern in hospital services in different countries.

Thus, they are responsible for nosocomial infections manifested by septicemia, urinary tract infections, secondary meningitis and especially lung infections in patients under mechanical ventilation in intensive care units (Balkhy et al., 2014; Meite et al., 2011). Moreover, in recent years, *Acinetobacter* infections of the central nervous system, skin, soft tissue and bone have been observed (Bergogne-Berezin and Towner, 1996; French et al. 1980; Jaffar et al., 2007).

These infections are difficult to treat, due to the

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increasing resistance of *Acinetobacter* strains growing on different families of antibiotics. Several studies have shown an appearance and an increase in the resistance of *Acinetobacter* spp. to carbapenem, choice antibiotics for the treatment of infections caused by these bacteria (Balkhy et al., 2014; Boni-Cisse et al., 2011; Chaisathaphol and Chayakulkeeree, 2014; Halstead et al., 2007; Unal and Garcia-Rodriguez, 2007).

In addition, antibiotic resistant strains of *Acinetobacter* spp. have a great ability to survive for several days in hospital environment, inert material or dust, thereby increasing the likelihood of transmission of the inter-human bacteria via a human or tank material (Boni-Cisse et al., 2011).

In Côte d'Ivoire, although *Acinetobacter* is responsible for nosocomial infections (Boni-Cisse et al., 2011; Meite et al., 2010, 2011), few data are available on circulating bacteria including species, the resistance profile of strains to antibiotics and the main hospital tank bacteria.

The objective of this work is to study *Acinetobacter baumanii* complex circulating in patients and hospital environment as well as their antibiotic resistance profile.

MATERIALS AND METHODS

The authors studied 110 strains of *Acinetobacter* spp. kept in the culture collection of the Laboratory of Bacteriology Virology of CHU Yopougon, from January 2007 to December 2011. These strains of *Acinetobacter* spp. were isolated from Biologic patients and hospital environment (sink, handle of door, sick bed and respirators).

Isolation of strains

Acinetobacter strains were revived in Brain Heart broth for 3 h at 37°C and then re-plated on nutrient agar, sheet blood agar and non selective lactose agar; they were incubated at 37°C for 18 to 48 h. The colonies were identified by standard bacteriology tests (culture on minimal agar) as the genus *Acinetobacter* ssp. which are Gram negative bacilli, non-motile; strictly aerobic, oxidase-negative and glucose non fermentative. Reference strains, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used for the validation of rapid biochemical tests used.

Biotypique study

For identification of *Acinetobacter calcoaceticus-A. baumanii* complex, the following characters were investigated: the ability to grow at 41 and 44°C, using citrate as sole carbon source, the production of an ornithine decarboxylase (ODC) and the production of arginine dihydrolase (ADH). Reference strains ATCC 13883 *Klebsiella pneumoniae*, *Shigella sonnei* ATCC 25931 and ATCC 27853 *Pseudomonas aeruginosa* were used to validate the research of ODC and ADH.

Studies of antibiotic susceptibility

The search for antibiotic susceptibility was performed by the method of agar diffusion. The following antibiotics disk of biorad were tested: ticarcillin/clavulanate (75/10 µg); ceftazidime (30 µg); imipenem (10 µg); aztreonam (30 µg); gentamicin (10 IU); amikacin (30 µg); netilmicin (30 µg); tobramycin (10 µg) and ciprofloxacin (5 µg). Interpretation of results was done according to the standards committee of the susceptibility of the French society of Microbiology (CA-SFM) recommendations 2012.

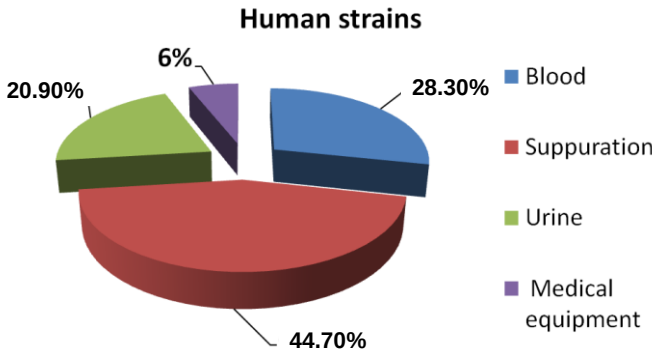

Figure 1. Distribution of human strains according to organic products.

RESULTS

A total of 110 strains of *Acinetobacter* were analyzed, of which 61% were strains of human origin and 49% of environmental strains. 44.8% of the human strains were from suppurations, 28.3% from blood, 20.9% from urine and 6% from medical equipment (Figure 1). In the environment surface, 38.1% were isolated from sink, 23.8% from the patient bed, 21.4% from the handle of door and 12% from respirators. 100% of the strains gave positive cultures on isolation media used. About biotypique study, none of the strains yielded positive test for glucose fermentation.

ADH test was negative for all the strains and hemolysis test in the sheet blood agar. 94% of cultures at 41°C were positive (Table 1). For the identified species, 94% were *A baumanii-calcoaceticus* complex and 6% of *Acinetobacter johsonii*. 62% *A. baumanii–calcoaceticus complex* were of human origin.

The antibiogram for beta-lactam revealed that, 39.4, 52.8 and 05.6% of tested strains were resistant to the combination of respectively Ticarcillin-clavulanic acid, and preview Ceftazidime, Imipenem. Regarding amino-glycosides, 21.2 and 10.3% of the strains tested were resistant to Gentamicin and Amikacin. 35.2% of tested strains were resistant to ciprofloxacin regarding quinolones. The high proportion of antibiotic resistance was found in strains of human origin and in *A. baumanii* complex species.

Thus, 100% of resistant strains were imipenem essentially *A. baumanii* species. 05.2% of tested strains were resistant to three antibiotics families tested. This was the case of human stem *A. baumanii.* Resistance to two different antibiotics family was not observed with

Table 1. Distribution of frequency of positive biochemical tests.

Test	Number of positive (N = 110)	Percentage (%)
Positive culture at 44°C	73	66
Positive culture at 38°C	104	94
Positive culture on Simmons citrate	110	100
Fermentation of glucose	00	00
Oxidation of lactose	00	00
Production of ODC	30	28
Production of ADH	00	00
Hemolysis on GSF	00	00

ODH: Ornithine decarboxylase, ADH: arginine dihydrolase, GSF: In fresh blood agar

environmental strains, except the three strains resistant to imipenem.

DISCUSSION

In this study, in humans, *Acinetobacter* was found in suppuration from blood and urine. This is consistent with some data from the literature (Van Looveren et al., 2004). Predominantly, the suppuration is linked to the fact that *Acinetobacter* is from commensal skin bacterium (Nordmann, 2004).

The environments handled by the nursing staff and visitors (sinks, beds, door handle) were colonized with *Acinetobacter.* This presents a risk of nosocomial infection (Oie et al., 2002). This is also the problem of hygiene in our hospitals.

A. baumanii-calcoaceticus complex is a predominance species in this study. These results are in agreement with those of literature. Indeed, several studies in different countries have shown a predominance of *A. baumanii* species. It is found in two thirds of infections caused by *Acinetobacter* spp. In a study conducted in Kosovo (Raka et al., 2009), *A. baumanni* represented 81.2% of *Acinetobacter* spp. In 1993, Seifert et al. (1993) identified 73% of *Acinetobacter* strains from clinical isolates as *A. baumannii*.

Acinetobacter lwoffi, *Acinetobacter hemolyticus* and *Acinetobacter junni* species were not isolated in this study. These bacteria are rarely clinically isolated; the isolation of *A. lwoffi* suggests a port rather than an infection. For resistance to beta-lactam antibiotics, the rate of resistance to Ceftazidime and Cefsulodin was respectively, 52.8 and 89.6%. This resistance was greater with *A. baumanii* complex strains of human origin. The rate of resistance to Ceftazidime was higher than that observed in our previous studies (Meite et al., 2010, 2011). This is in favor of a steady increase in antibiotic resistance of *Acinetobacter* in our health facility. These results corroborate those of Balkhy et al. (2014) in Saudi Arabia and Rahbar et al. (2010) Iran, who found 84.1 and 99% rates of resistance to ceftazidime. Regarding resistance to carbapenems, it was 5.6%, including 75% of the strains of environmental origin. *A baumannii* complex resistance to carbapenems has appeared in many parts of the world and is clearly increasing (Balkhy et al., 2014; Chaisathaphol and Chayakulkeeree, 2014; Gootz and Marra, 2008; Perez et al., 2008).

The main mechanism of resistance is the acquisition of carbapenemases Class B and D (Gootz and Marra, 2008). Low levels of resistance (3 and 4.5%) of *A. baumannii* to imipenem were reported in Saudi Arabia by Jaffar et al. (2007) and in Iran by Rahbar et al. (2010). However, more recent studies in these countries have shown a marked increase in resistance to carbapenems by *Acinetobacter* spp., especially in Saudi Arabia where it is grown to over 80% (Sameera et al., 2010). Asencio et al. (2010)'s study carried out in Spain showed a resistance rate of 83% by *A. baumanii* to Imipenem, while that of Cisneros et al. (2005) was 43% resistance rate. These high rates of resistance to Imipenem are found in hospitals. The variations in time of the resistance of *A. baumanii* to Imipenem are related to increased use of the molecule and use not mastered by some prescribers, sometimes. Monotherapy is not recommended and should be used in combination synergistically, taking into account the bioavailability of each molecule, the site of action, the causative organism and risk factors related to patients. Strains tested were sensitive enough to aminoglycosides. Resistance was 21.2% for Gentamicin and 10.3% for Amikacin. These results are quite close to that of Asencio et al. (2012), in which the rates were very low (12% for Gentamicin and 2% for Amikacin). These low levels of resistance are due to the fact that aminoglycosides are rarely used in treating infections in our country, generally. Oral forms are rare and injectable forms available are used only in association with other molecules and in hospitals.

In this study, the rate of resistance to ciprofloxacin was 49%. Our results corroborate those of Ben Haj et al. (2010) in Tunisia who regained a resistance rate of 50%. These results, however, are contrary to those found in Iran by Rahbar et al. (2010) which was 90.9%. Described for the first time in Taiwan in 1998 and defined as being

resistance to more than three classes of antibiotics, the incidence of strains of multi-resistant *A. baumannii* (MDR) continues to grow in recent years (Appleman et al., 2000). In a study realized in the United States by Dent et al. (2010), it involved almost 72% of *A. baumannii* studied. In our present study, it was 5.2% for all strains and reached 12.5% for strains isolated from humans.

A. baumannii with P. aeruginosa are frequently pan-resistant bacteria of antibiotic. In effect, these bacteria may be resistant to all antibiotics, including the aminoglycosides, cephalosporins, carbapenems, carboxypénicillines and fluoroquinolones. Dent et al. (2010) in USA found about 46% in their series. This profile was not found in our study. However, the phenotypes of resistance to at least two families of antibiotics have been observed. This resistance was generally observed as regards the lactams and amino-glycosides. Strains involved were the cases of *A. baumanii* especially of human origin. The proportion of resistance to these two antibiotic families is linked to therapeutic habits of our health facilities. The combination of these two antibiotic families is the first therapeutic choice in many empirical studies in Côte d'Ivoire.

Conclusion

Acinetobacter remains an environmental bacterium whose species *A. baumanii* complex is the most involved in human infections in hospitals. It presents a profile of increasingly resistant to conventional antibiotics in our health facility and its main concern remains the appearance of resistant strains to imipenem. This multidrug-resistant *Acinetobacter* to imipenem requires the implementation of policy microbiological monitoring and control to limit dissemination.

Conflict of interests

The authors did not declare any conflict of interest.

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Vol. 7(3), pp. 24-28, April, 2015
DOI: 10.5897/JBR2015.0152
Article Number: 278D96951714
ISSN 2006-9871

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Journal of Bacteriology Research

Full Length Research Paper

Potential biodegradation of low density polyethylene (LDPE) by *Acinetobacter baumannii*

R. Pramila and K. Vijaya Ramesh*

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Received 26 January, 2015; Accepted 17 March, 2015

Acinetobacter baumannii **was isolated from municipal landfill area, Pallikaranai, Chennai, Tamilnadu. The degradation ability of the bacteria was determined by performing Fourier Transform Infrared Spectroscopy (FTIR). The by-products of polyethylene degradation were monitored by gas chromatography-mass spectrometer (GC-MS) analysis. The toxicity of degradation by-products of low density polyethylene (LDPE) was tested on the plant *Vigna radiata* by determining the morphological parameters such as root length, shoot length and chlorophyll content. After 30 days of degradation process, the FTIR results revealed an increase in carbonyl index and formation of peaks and occurrence of stretches. Alkane compounds were analyzed in GC-MS analysis. Determination of toxicity level of intermediate degraded products showed no changes in morphological characters.**

Key words: Biodegradation, Low density polyethylene (LDPE), *Acinetobacter baumannii*, Fourier transform infrared spectroscopy (FTIR), gas chromatography-mass spectrometer (GC-MS), *Vigna radiata.*

INTRODUCTION

Polyethylene plays an important role in our everyday life. It is a synthetic polymer, made of long chain of monomers of ethylene. Its density ranges from 0.915-0.9359 gcm^3. Polyethylene is classified into different types such as low density polyethylene (LDPE), high density polyethylene (HDPE), linear low density polyethylene (LLDPE), etc. Among these, LDPE has been used for various purposes such as packaging, making carry bags, disposable cups etc. In contrast, when considering disadvantages of polyethylene it poses one of the worst environmental problems. Polyethylene products tend to accumulate in the land areas and remain inert for several decades. This reduces the fertility of the soil, water percolating capacity into the plants and it also threatens animal life. On burning, it produces toxic chemicals polluting the environment, leading to diseases affecting the lungs and skin.

Numerous activities are carried out to reduce the usage of polyethylene and plastic, however less attention is focused on the degradation of polyethylene. Recent research focuses on biodegradation of polyethylene. Biodegradation is the process by which organic substances are broken down by living organisms like bacteria and fungi. During biodegradation process of polymers, two categories of enzymes are actively involved; extracellular and intracellular depolymerases. During degradation, exoenzymes from microorganisms break down complex polymers into smaller molecules, for example oligomers, dimers, and monomers that are small enough to pass the semi-permeable outer bacterial

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membranes, and then utilized as carbon and energy sources and release end products such as CO_2 and H_2O.

Biodegradation of LDPE was studied earlier (Albertsson et al., 1987; Shah, 2007; Suresh et al., 2011; Negi et al., 2011) however the results of these reports were based on pre-treating the LDPE with UV irradiation, thermally oxidized fragments and pro-oxidant additives containing LDPE and starch blended polyethylene.

Gilan et al. (2004) and Hadad et al. (2005) have reported the degradation of LDPE by pretreatment with UV-irradiation and subsequent incubation with *Rhodococcus ruber* and thermophilic bacteria *Brevibacillus parabrevis.*

Sudhakar et al. (2008) and Harshavardhan and Jha (2013) have isolated marine bacteria and utilized them for degradation study of thermally pretreated and starch blended LDPE. Mahalashmi et al. (2012) and Kyaw et al. (2012) have reported the degradation of untreated LDPE by *Pseudomonas* species.

A bacterial culture was isolated from a municipal land fill area and identified as *Acinetobacter baumannii* during previous study (Pramila et al., 2012). The preliminary degradation ability of *A. baumannii* was studied by measuring CO_2 evolution, calculation of generation time, protein estimation, and Bacterial adhesion to hydro-carbon (BATH) test. The significance of chosen municipal dump soil for isolation of bacteria was associated with the fact, that the cultures already had stressful conditions and could develop tolerance towards such environmental conditions.

The current study was focused on determina-tion of physical changes by tensile strength and chemical changes in LDPE by FTIR analysis to measure carbonyl Index (CI). Measuring the carbonyl index (CI) is neces-sary to elucidate the mechanism of biodegradation process where the initial step involves oxidation of the polymer chain and leads to the formation of carbonyl groups, since these groups undergo β-oxidation and are totally degraded via citric acid cycle resulting in formation of CO_2 and H_2O (Albertsson et al., 1987). Additionally, the current study also aimed to study the formation of intermediate by-products by GC-MS analysis and to test the toxicity level of the degraded by-products on plants by *A. baumannii.*

MATERIALS AND METHODS

Preparation of LDPE powder

LDPE sheets were cut into bits and immersed in xylene. It was boiled for 15 min as xylene dissolves the LDPE film and the residue was crushed while it was warm by using band gloves. The LDPE powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate to remove ethanol. The powder was dried in hot air oven at 60°C over night.

Isolation of microorganism

Bacterial culture was isolated by spread plate method in sterilized synthetic medium (SM) at 37ºC for 24 h. SM contains the following constitutions in 1000 ml distilled water (K_2HPO_4, 1 g; KH_2PO4, 0.2 g; NaCl, 1 g; $CaCl_2.2H_2O$, 0.002 g; $(NH_4)_2SO$, 1 g; $MgSO_4.7H_2O$, 0.5 g; $CuSO_4.5H_2O$, 0.001 g; $ZnSO_4.7H_2O$,0.001 g; $MnSO_4.H2O$, 0.001 g and $FeSO_4.7H_2O$, 0.01 g, amended with 500 mg LDPE powder. Synthetic mineral medium had LDPE as the sole carbon source.

Degradation study

The degradation study was carried out in synthetic medium broth. LDPE films were cut into 2×2 cm. The films were disinfected with 95% ethanol and washed with sterile distilled water. One full inoculation loop of isolated culture were inoculated in 5 ml SM broth and incubated at 37°C for 24 h. After 24 h, the broth was compared with McFarland scale ($CFU\times10^9$/ml) and poured into 45 ml of SM broth in a 100 ml conical flask. Four pieces of equally weighing LDPE films were placed in SM broth. The flasks were incubated at 37°C for 30 days with shaking at 100 rpm. SM broth with LDPE films without culture was maintained as control.

Tensile strength

For tensile strength measurement, test strips were retrieved after 30 days of incubation, washed with 2% sodium dodecyl sulphate (SDS) followed by distilled water and dried in oven overnight at 50°C. The strips were subjected to tensile strength tests as per ASTM A.370 (2012).

FTIR study

After 30 days of incubation, the LDPE sheets were taken and washed with 2% SDS followed by sterile distilled water. The LDPE sheets were dried in oven overnight at 50°C. The films were subjected to FTIR analysis to calculate carbonyl index, presence or absence of functional groups, stretches. The carbonyl index is a measure of the concentration of carbonyl group (acids, aldehydes, ketones) (Albertsson et al., 1987).

$$\text{Carbonyl index (CI)} = \frac{\text{Absorbance at 1715 cm}^{-1}\text{(Peak wavelength)}}{\text{Absorbance at 1465 cm}^{-1}\text{(Peak wavelength)}}$$

GC-MS study

After 30 days of incubation, 10 ml broth was centrifuged at 1000 rpm for 10 min. Supernatant was extracted with 10 ml dichloromethane using a separating funnel. Simultaneously, LDPE films were extracted with 5 ml dichloromethane. Both the extracts were determined by GC-MS (JOEL GCMATE II GC-MASS SPECTROMETER IIT CHENNAI) using HP5 column, helium gas, temperature from 70 to 200°C, injection liquid 1 µl. By retention time the compounds were identified by NIST library.

Toxicity study

Culture broth was analyzed for its toxicity after 30 days, towards plant *V. radiata*. 10 g of garden soil was placed in a pot. Seeds were sown and the soil was wetted regularly with 5 ml of the culture broth. The pots were kept in room temperature with normal condition. After 7 days, the seedlings were harvested and morphological parameters such as root length, shoot length and chlorophyll content of the plant were estimated by Arnon (1949) method. SM with LDPE without culture and SM alone served as controls.

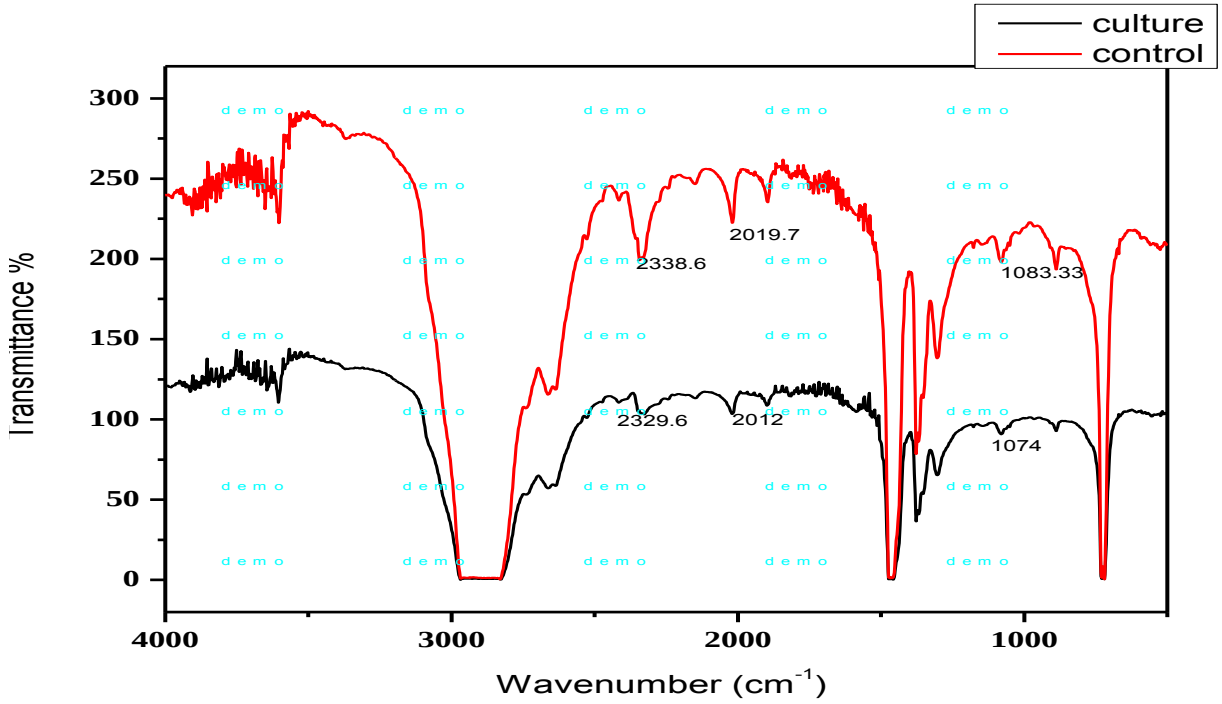


Figure 1. FTIR study of LDPE treated and untreated with *Acinetobacter baumannii* after 30 days of incubation.

RESULTS

FTIR study

Increase in carbonyl index (CI) of LDPE treated with *A. baumannii* after 30 days of incubation indicates the formation of carbonyl groups (Figure 1).

GC-MS study

Figures 2 and 3 indicate the formation of new peaks and compounds in 7.464- as 2-butene, 2-methyl, 8.250- Acetone and 17.288- ethene.

Toxicity test

Table 1 shows the toxicity results of LDPE biodegraded by-products after 30 days of incubation with *A. baumannii*. There are no changes in germination percentage as well as root length and shoot length when compared to control.

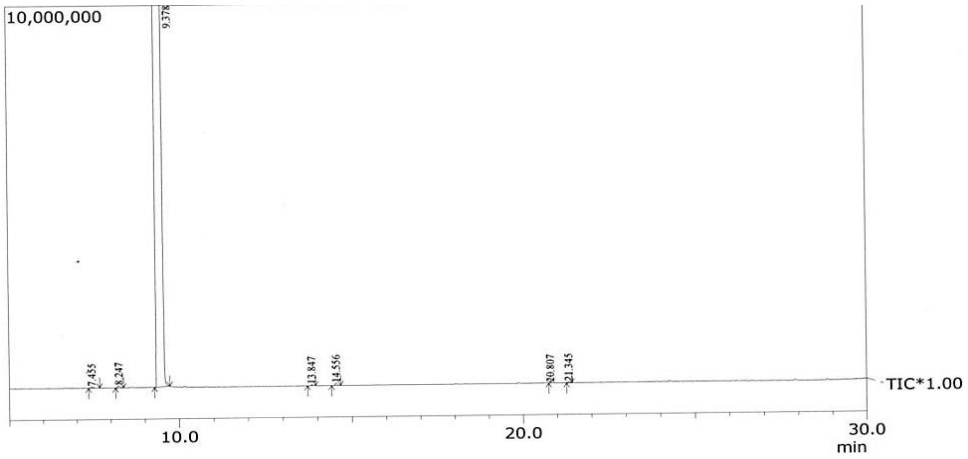
DISCUSSION

Biodegradation of polyethylene has been known for several years. In the previous study, the LDPE degradation ability of *A. baumannii* was reported (Pramila et al., 2012). The current study focused on monitoring the chemical changes of LDPE by FTIR analysis by measuring the carbonyl index (CI). The obtained results indicate the CI was increased by 0.1% after 30 days of incubation without pretreating the LDPE film.

Previous reports on polyethylene degradation utilized UV-irradated LDPE films and showed increase in CI after 30 days of incubation (Gilan et al., 2004; Hadad et al., 2005). Albertsson et al. (1987) has reported the 0.3% increase in CI after 10 years of incubation in soil burial method by pretreating with UV. Sudhakar et al. (2008) and Harshavardhan and Jha (2013) revealed the result of 0.15% increase in CI by incubating with marine bacteria for 30 days.

Suresh et al. (2011) and Negi et al. (2011) have reported the FTIR results by monitoring the changes in peaks such as formation or disappearance of peaks of LDPE film containing pro-oxidant additives by incubation with *Bacillus cereus* and soil burial method for 3 months. Mahalakshmi et al. (2012), studied the degradation of unblended or untreated LDPE using *Pseudomonas* spp. after two months of incubation and reported slight changes in peak wave numbers.

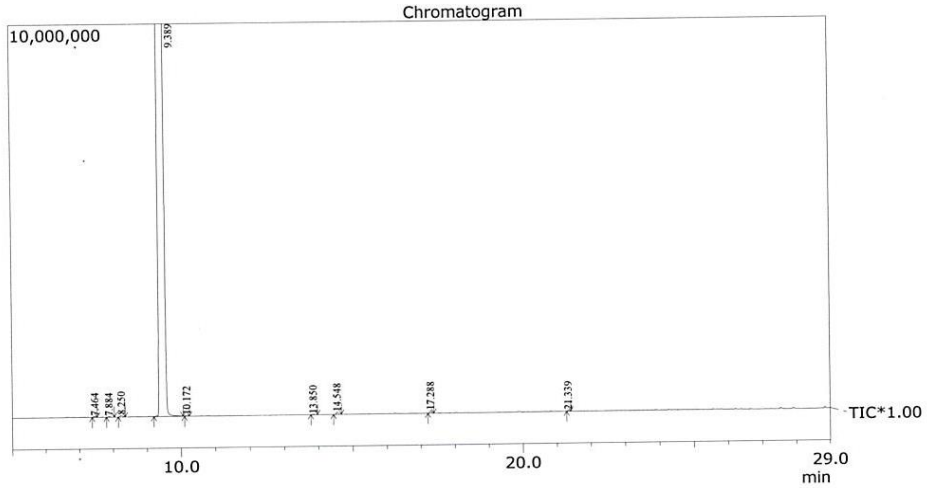
Kyaw et al. (2012) reported the result of 16-80% decrease in CI after 120 days of incubation in mineral based medium by *Pseudomonas* spp. The decrease was presumably due to the prolonged incubation time where the culture entered the Norrish II type mechanism (Albertsson et al., 1987). No changes were observed in tensile strength.



Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	7.455	150367	0.02	CYCLOPROPANE, 1,1-DIMETHYL-
2	8.247	283870	0.04	2-PROPANONE
3	9.378	734843731	99.88	METHANE, DICHLORO-
4	13.847	117086	0.02	ACETIC ACID ETHYL ESTER
5	14.556	260309	0.04	METHANE, TRICHLORO-
6	20.807	5404	0.00	2-(3'-PHENYLSULFONYLBUT-3'-ENYL)-
7	21.345	97452	0.01	HEXANAL
		735758219	100.00	

Figure 2. Compounds detected after GC-MS study of untreated LDPE after 30 days.



Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	7.464	94385	0.01	2-BUTENE, 2-METHYL-
2	7.884	45743	0.01	ETHENE, 1,2-DICHLORO-, (Z)- $$ 1,2-DIC
3	8.250	207912	0.03	Acetone
4	9.389	646176618	99.84	METHANE, DICHLORO
5	10.172	100101	0.02	ETHENE, 1,1-DICHLORO-
6	13.850	77234	0.01	ACETIC ACID ETHYL ESTER
7	14.548	241284	0.04	METHANE, TRICHLORO-
8	17.288	200178	0.03	ETHENE, TRICHLORO-
9	21.339	91222	0.01	Hexanal
		647234677	100.00	

Figure 3. Compounds detected after GC-MS analysis of LDPE after 30 days of incubation with *Acinetobacter baumannii.* Figures 2 and 3. Indicates the formation of new peaks and compounds in 7.464- as 2-butene, 2-Methyl, 8.250- acetone, 17.288- ethene.

Table 1. Toxicity results of LDPE biodegraded by-products after 30 days of incubation with *Acinetobacter baumannii.*

Culture	Germination percentage %	Root length (cm)	Shoot length(cm)	Chlorophyll content (mg/g)
Control	80	2.05±0.59	10.07±1.54	0.152
Culture Broth	80	2.52±0.45	11.47±2.16	0.177

Mean ± S.D n=3. There are no changes in germination percentage as well as root length and shoot length when compared to control.

GC-MS results presented in the framework of this study reveals the presence of compounds such as 2-butene, 2-methyl-, acetone, ethene. Presence of acetone indicates the formation of carbonyl groups. Kyaw et al. (2012) has reported GC-MS result of formation of alkanes, aromatic compounds and fatty acid such as hexadecanoic acid and octanoic acid after 120 days incubation.
The byproducts did not reveal any toxicity towards the tested plant characteristics.

Conclusion

Accumulation of polyethylene is becoming a serious environmental issue. Biodegradation of polyethylene process can be viewed as one of the strategic studies to overcome this problem. The current study focused on degradation of LDPE by *A. baumannii*. This isolate grows by utilizing LDPE as a sole carbon source. The bacteria are able to degrade LDPE without any additives and pretreatment in short time duration. This is the first report on degradation of non-pretreated LDPE by *A. baumannii*.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENT

The authors are grateful to the University Grants Commission (F.no. 42-480/2013 SR dated March 2013) for providing financial assistance for the completion of this work.

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academicJournals

Vol. 7(3), pp. 29-34, April, 2015
DOI: 10.5897/JBR2015.0155
Article Number: D622AAA51725
ISSN 2006-9871

http://www.academicjournals.org/JBR

Journal of Bacteriology Research

Full Length Research Paper

Effect of high temperature on viability of *Lactobacillus casei* and analysis of secreted and GroEL proteins profiles

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Received 29 January, 2015; Accepted 13 March, 2015

The bacterial heat shock response is characterized by the elevated expression of a number of chaperone complexes including the GroEL and the rate change of synthesis of certain proteins (total and secreted). In this work, after incubation at 45°C, total and secreted proteins profiles of stressed bacteria were found to be altered when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the level expression of GroEL was evaluated with Western blot. Our results show a marked increase in both GroEL expression and protein synthesis at 45°C. These modifications were manifested by the appearance and/or disappearance of bands as well as in the level of expression of certain proteins.

Key words: *Lactobacillus casei*, heat shock, GroEL, secreted and total proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting.

INTRODUCTION

The viability of probiotic in foods depends on various factors during processing and storage. Heat is used in the process of a lot of foods, so the survival of probiotics in thermal processing is the main obstacles to food manufacturers (Mansouripour et al., 2013). Thus, paying special attention to the effect of heat shock on the survival of probiotics is necessary. For an organism to grow at high temperatures, especially as high as those of the hyperthermophiles discussed here, all cellular components, including proteins, nucleic acids and lipids, must be heat protected. Desmond et al. (2004) showed that the mechanisms involved in thermoprotection of *Lactobacillus paracasei NFBC338* are probably controlled at the protein synthesis level. It has been previously reported that stress tolerance in *Lactobacillus delbrueckii ssp. bulgaricus* induced by a moderate heat shock was dependent on protein synthesis (Gouesbet et al., 2002) and Whitaker and Batt (1991) demonstrated the enhanced synthesis of a number of heat shock proteins, including GroEL and DnaK in a heat-adapted culture of *Lactococcus lactis* ssp*. lactis*. In addition, destabilization of macromolecules as ribosomes and RNA, and alterations of membrane fluidity were also described (Earnshaw et al., 1995; Teixera et al., 1997; Hansen et al., 2001). The heat-shock response has been studied notably in *Escherichia coli* (Gram negative) and *Bacillus*

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subtilis (Gram positive). Physiological studies demonstrated that lactobacilli elicit heat-shock responses similar to that of other Gram positive bacteria: *L. lactis* (Whitaker and Batt, 1991; Auffray et al., 1992; Kilstrup et al., 1997), *Leuconostoc mesenteroides* (Salotra et al., 1995), *Enterococcus faecalis* (Flahaut et al., 1996), *Oenococcus oeni* (Guzzo et al., 1997) and *L. bulgaricus* (Gouesbet et al., 2002). In order to understand the mechanisms of stress tolerance of lactobacilli, numerous studies have examined the physiological and genetic adaptations of these organisms during growth and survival in diverse environmental stresses (Corcoran et al., 2008; Spano and Massa, 2006; Van de Guchte et al., 2002) and postgenomic approaches have accelerated the understanding of the global (genome-wide) stress responses in lactobacilli to acid, lactate, oxidative, bile and heat stresses (Broadbent et al., 1998; Stevens, 2008; Serrano et al., 2007; Bron et al., 2006; Pieterse et al., 2005; De Angelis et al., 2004). These studies have shown that lactobacilli respond rapidly to their environment by modulating expression levels of genes involved in different cellular processes, including stress response pathways, cell division, transport and cell envelope composition. However, the proteins synthesized by the stressed cells during the stationary phase were necessary for maintaining of the viability during prolonged stress (Kolter et al., 1993). These proteins enable the cell to neutralize stress to adapt or repair damages caused by stress (Hecker et al., 1996). Examination of lactobacilli heat shock responses using bi-dimensional electrophoresis revealed variable numbers of induced proteins: 34 in *E. faecalis* (Flahaut et al., 1996), 17 in *L. lactis* (Kilstrup et al., 1997) and 40 in *Streptococcus mutans* (Svensater et al., 2000), among them, only six proteins were specifically up regulated by heat (Svensater et al., 2000). In *L. lactis*, the 12 proteins were induced by NaCl and detected on bi-dimensional electrophoresis, all belong to the heat shock stimulon (Kilstrup et al., 1997). This striking overlap between heat-shock and osmotic-stress responses may also exist in *S. mutans* as 21 heat-inducible proteins also belong to the osmotic stress response. Among the heat shock proteins, well conserved chaperones (DnaK, DnaJ, GrpE, GroES and GroEL) and proteases (Clp, HtrA and FtsH) have often been identified.

Some of the most intensively investigated heat shock proteins include the molecular chaperones GroEL and GroES, which are highly conserved in *E. coli* and eukaryotic cells (Gupta, 1995). The GroEL and GroES chaperones (also known as Hsp60 and Hsp10 chaperones) have been recognized as heat shock proteins in many bacteria, including *E. coli*, *B. subtilis* (Hecker et al., 1996), *Agrobacterium tumefaciens* (Segal et al., 1996), *Streptomyces lividans* (De Leon et al., 1997), *L. lactis* (Kilstrup et al., 1997), *Lactobacillus helveticus* (Broadbent et al., 1998), *Pseudomonas aeruginosa* (Fujita et al., 1998) and *Lactobacillus johnsonii VPI 11088* (Walker et al., 1999).

In the present work, we focused on the effect of heat stress on GroEL expression of two strains of *Lactobacillus* and their influence on secreted and total proteins profiles of stressed cells. The cells were analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

MATERIALS AND METHODS

Bacterial strain and growth conditions

Two strains of *Lactobacillus casei* were used in this study: S1, *L. casei* (ATCC 393); S2, *L. casei*, a potential probiotic, obtained from the laboratory collection; this strain was identified by sequencing the 16S rRNA gene (BankIt1773923 BL2 KP123430). All strains were stored on De Man, Rogosa, Sharpe (MRS; LAB M, Bury, UK) broth added with 33% of sterile glycerol at -20°C. Working cultures were grown at 37°C in modified MRS broth at pH of 6.4 for 24 h, which contained 0.5% maltose, 1% peptone, 0.5% yeast extract, 1 ml Tween 80, 0.2% K_2HPO_4, 0.2% ammonium citrate, 0.02% $MgSO_4$ and 0.005% $MnSO_4$. The pH of the modified MRS was 6.4 and, unless otherwise stated, it was maintained constant by the on-line addition of 1 M NaOH. In this study, the cells were grown at 37°C on MRS for 24 h.

Stress conditions

Cells were recovered by centrifugation and re-suspended to an optical density at 600 nm of 0.5 immediately before heat shock treatment at 45, 55, and 65°C. One culture (1 ml) was maintained at 37°C as a control, and three cultures were shifted to 45, 55 and 65°C. After 10 min, heat shock was stopped by placing samples on ice for 5 min.

Determination of the lethal temperature of *Lactobacillus*

100 µl of a bacterial suspension was inoculated with an optical density at 595 nm of about 0.6 prepared from a bacterial culture in MRS incubated overnight. These bacterial suspensions were treated at temperatures ranging from 45 to 95°C with an interval of 10°C for 10 min. All suspensions were prepared at the same time; one sample of each is removed after every increase of 10°C. All experiments were performed in triplicate.

Enumeration of cells

To determine the number of cultivable cells, decimal dilutions were performed in series in sterile saline solution. A volume of 0.1 ml of each dilution was then spread on the surface of three plates of MRS at 37°C. After 24-48 h of incubation, the enumeration of CFU/g of the appropriate dilution were made. All experiments were performed in triplicate.

After incubation and enumeration, we observed temperatures for which a bacterial growth is possible. Thus, we performed the heat shock at three temperatures: 45, 55 and 65°C.

Total protein extraction

Total proteins of two strains of *Lactobacilli* were prepared according to the method described previously (Sabri et al., 2000). Cultures of 24 h at 37°C were centrifuged (7000 ×g for 10 min at 4°C) and the cells were then washed three times in 40 ml of sterile saline solution

(0.9% NaCl) and resuspended in 5 ml of sterile saline solution. Cells were disrupted by lysosyme solution (10 mg/ml) and incubated at 37°C for 30 min. 30 µl of loading buffer was added to the solution and the pellet was incubated in a water bath at 100°C for 5 min. Supernatant was collected and further centrifuged at 100,000 ×*g* for 40 min at 4°C. The concentration of the total proteins in the final preparation was determined using the Bradford assay (Bradford, 1976).

Secreted protein extraction

Cultures of 24 h at 37°C were centrifuged (7000 ×*g* for 20 min) and subsequently filtered through a 0.45-µm pore-size filter. Proteins from the cell-free culture supernatants were then precipitated by addition of 10% (vol/vol) trichloroacetic acid and recovered by centrifugation at 7000 ×g for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and protein were precipitated by addition of 20 ml of cold acetone. After centrifugation at 7000 ×g for 20 min, the pellets were washed once with cold acetone, air-dried and re-suspended in 25 µl of PBS. The concentration of the secreted proteins in the final preparation was determined using the Bradford assay (Bradford, 1976).

Sample preparation and SDS-PAGE

Protein pellet were denatured with sample containing β-mercaptoethanol (Sigma, Chemical Co., St Louis, MO, USA). These protein samples were subjected to SDS-PAGE analysis using a Mini Protean 3 gel Unit (BIORAD; Richmond, CA) on 15% (w/v) polyacrylamide gels (Laemmli, 1970). Wide range protein markers (from 212 to 6.5 kDa) were used as molecular weight standards (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK). Proteins were visualized by Biosafe colloidal Coomassie blue (Sigma, Chemical Co., St Louis, MO, USA) according to the manufacturer's instructions.

Western blotting

20 µg of total proteins were analyzed by SDS PAGE as reported above and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham), using the Mini Trans-Blot equipment (BioRad Inc.), at 90 mA, 4°C, for 16 h in transfert Buffer. The membrane was incubated in TBST buffer (20 mM Tris base, 137 mM NaCl, 0.15% Tween 20) + nonfat dry milk 4% for 1 h at room temperature, with orbital shaking, then incubated in rabbit polyclonal anti-GroEL antibodies (Abcam) diluted 1:10000 in blocking buffer, at room temperature for 1 h. After three washes with TBST, the membrane was incubated with a 1:10000 dilution of the secondary antibody conjugated with horseradish peroxidase (GE Healthcare), in blocking buffer at room temperature for 1 h. After three washes with TBST, the bound antibodies were revealed with revelation solution (tris HCl pH 9, 0.1 M $MgCl_2$, 1 M Nacl, NBT, BCIP).

Statistical analysis

The statistical analysis was performed on SPSS v.17.0 statistics software. The statistical differences and significance were assessed by ANOVA test. $P < 0.05$ was considered significant.

RESULTS

Effect of temperature on survival of *Lactobacillus*

The survival of *L. casei* at different temperatures was investigated. Our results show that the two strains used remained cultivable up to 65°C. The viability was studied for an initial population of about 10^8 CFU/g. It was shown that both strains can survive at 75°C for 10 min (Figure 1). Remarkabely, at 45°C, we observed an increase of the optical density of the culture (Figure 1).

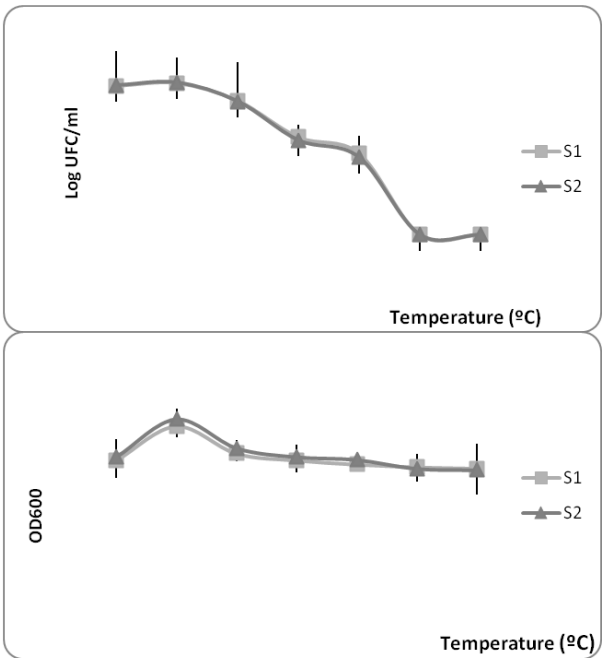

Figure 1. Effect of heat stress on the survival of *L. casei ATCC 393* (S1) and *L. casei* BL2 KP123430(S2) after 48 h of incubation at pH 7.

Analysis of total proteins

Total protein of *Lactobacilli* strains before and after heat shock were analyzed by SDS-PAGE. They displayed different profiles before and after exposure of heat shock. Before heat stress, the strains S1 and S2 of *L. casei* had the same total protein profile. Five clear bands were detected in each profile at 27, 35, 38 43, 90 kDa (Figure 2). After heat stress, some modifications were observed in total protein profile in both strains. However, at 45 and 55ºC, the expression of band corresponding to protein of 35 kDa was significantly decreased. Interestingly, bands corresponding to molecular weights of 38 and 43 kDa were significantly slightly increased at 55°C (Figure 2). At 65°C, the protein synthesis was completely stopped; all bands were not visible after heat shock to this temperature for 10 min.

Analysis of secreted proteins profiles

Secreted protein of Lactobacilli strains displayed different

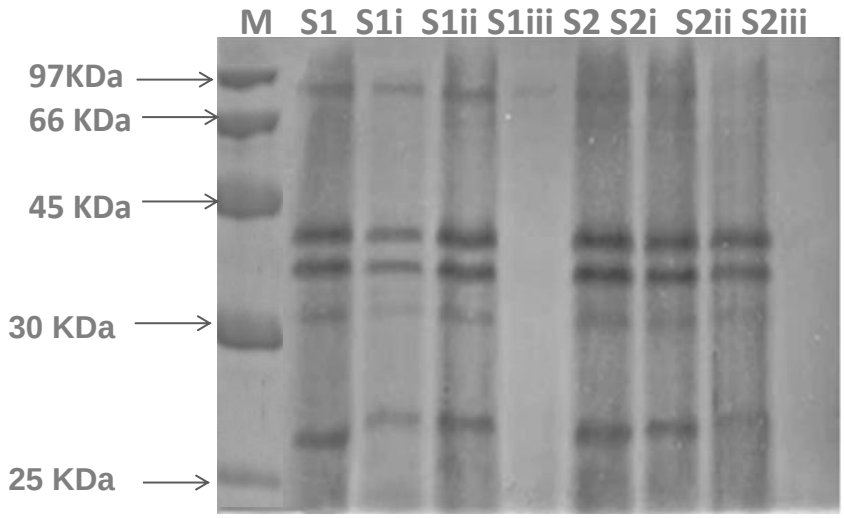

Figure 2. Total protein profile of two strains of *L. casei* subjected to heat stress: M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK), S1: *L. casei ATCC339*; S2: *L. casei* BL2. Si, ii and iii: strains subject respectively, at temperatures of 45, 55 and 65°C.

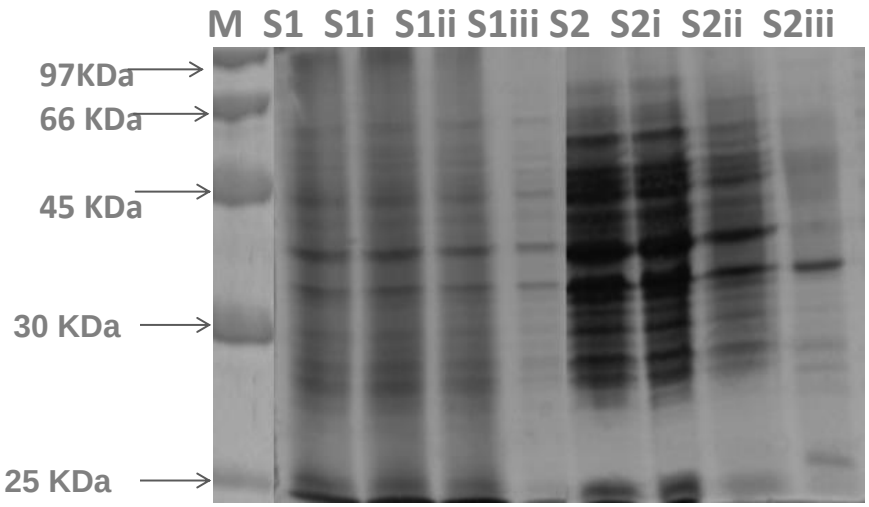

Figure 3. Secreted protein profile of two strains of *L. casei* subjected to heat stress: M: High-Range Rainbow (Amersham, Little Chalfont, Buckinghamshire, UK), S1: *L. casei ATCC339*; S2: *L. casei* BL2. Si, ii and iii: strains subject respectively, at temperatures of 45, 55 and 65°C.

profiles before and after exposure to heat shock (Figure 3). *Lactobacillus* presents five or six major proteins in their profiles. After heat stress, both strains have a different extracellular protein profile, whereas, bands corresponding to protein of molecular weight of 65 kDa were not visible at 55 and 65°C for S2. In comparison, the intensity of this band for S1 was increased in intensity after heat stress. In addition, we have observed the level intense of one band corresponding to molecular weight of 35 kDa for S2. Interestingly, bands corresponding to molecular weights of 25 and 27 kDa approximately were slightly increased at 55 and 65°C.

GroEL expression

As shown in the Figure 4, the expression of protein was greater at 45°C (column S1i and S2i) and the higher levels were found in both strains at 55°C. However, the proteins were not visible at 65°C (Figure 4).

Figure 4. Western blot analysis of GroEL present in total protein extracts *L. casei* detected with antibodies raised against GroEL., M: high-range rainbow (Amersham, Little Chalfont, Buckinghamshire, UK), S1: *L. casei ATCC339*; S2: *L. casei* BL2. Si, ii and iii: strains subject, respectively, at temperatures of 45, 55 and 65°C.

DISCUSSION

The results show that Gram positive bacteria, such as *Lactobacilli*, are able to survive heat shock up to 75°C. In addition, we found that *L. casei* cells remained cultivable at temperature of 65°C, which demonstrates that bacteria are able to withstand such adverse environments (Van de Guchte et al., 2002). It has been shown that organisms respond to environmental stress by modifying the rate of synthesis of certain proteins (Hennequin et al., 2001). In our study, we have shown that the heat shock induced alterations of secreted and total proteins and induced elevated GroEL expression. The marked increase on both GroEL expression and protein synthesis may suggest that *Lactobacillus* species are able to grow and survive under suboptimal conditions during food and beverage fermentations. Desmond et al. (2004) revealed by 2D-PAGE that the chaperone protein GroEL was among the most strongly expressed proteins in the cell under heat adaptation conditions; indeed, densitometry analyses indicated an approximately 50-fold increase in cells that were pre-adapted to heat shock. Kim et al. (2001) demonstrated that the transcriptional activity of both chaperones is increased dramatically in response to heat shock and was increased to a lesser extent by ethanol stress. Other studies have shown that culture of log-phase *Lactobacillus* cultures were subjected to heat stress, a 15-fold increase in GroEL synthesis was observed when compared with only 1.5-fold increase in protein synthesis in stationary phase cultures (Prasad et al., 2003).

These chaperones play a key role in the maturation of synthesized proteins and are pivotal in the degradation or refolding of denatured proteins (Rechinger et al., 2000). Thus, these heat-shock proteins can also be induced by multiple stresses, such as acid, heat, bile salts, high pressure stress and so on (De Angelis et al., 2004). Several studies have been done in the elucidation of the major chaperones belonging to the Hsp60 (GroEL) and Hsp70 (DnaK) families because protein folding has been recognized as one of the central problems in biology (Narberhaus, 2002). The study of GroESL-overproducing *L. lactis* and *L. paracasei NFBC 338* demonstrated that technologically sensitive cultures can be potentially manipulated to become more robust for survival under harsh conditions, such as food product development and gastrointestinal transit (Desmond et al., 2004). The main function of these HSPs appeared to be the prevention of the accumulation of unfolded protein intermediates during periods of stress (Veinger et al., 1998). Although, the HSPs are always constitutively expressed, the rate of synthesis is significantly enhanced under stress (Ang et al., 1991).

The production of the HSPs is the origin of the beneficial effect of heat stress in the conservation of *Lactococcus*. Indeed, the physiological and biochemical riposte of the unicellular organisms to all occasional thermal elevation is universal. Some works have been done on *L. lactis* (Boutibonnes et al., 1995) which shows that the temporary exhibition of the cells to a temperature of 40-45°C provoked a disturbance followed by an adjustment of the metabolism. Thus, production of the majority of the usual proteins in stalled synthesis of HSP is unregulated. It has been demonstrated for *E. coli*, that a thermal treatment of 8 h has a beneficial effect on the cryodessiccation and the lyophilization (Joe et al., 2000).

In conclusion, our data suggest that overexpression of stress-induced proteins has the potential to improve the performance of lactobacilli strains. In particular, the GroES/EL chaperone complex can be exploited to prepare *Lactobacillus* for industrial processes. Indeed, the innate probiotic characteristics of the strain, such as adherence to the host cell wall and acid tolerance during gastric transit, may also be improved by the overexpression of GroESL.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Pr. Gaspar Perez Martinez [Laboratory of Lactic Acid Bacteria and Probiotics of the Institute of Agro-chemistry and Food Technology (Spanish National Research Council] for sequencing the 16S rRNA gene of *L. casei*.

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