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Screening and Identification of Effective Thermotolerant Lactic Acid Bacteria Producing Antimicrobial Activity Against *Escherichia coli* and *Salmonella* sp. Resistant to Antibiotics

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ABSTRACT

Two hundred fifty six isolates of lactic acid bacteria (LAB) displaying antimicrobial activity were isolated from chicken intestine. Twenty effective isolates of LAB show bacteriocin – like activity (BLA) against the target strains resistant to antibiotics (*Escherichia coli* and *Salmonella sp.*) to varying degrees. Broad spectrum inhibition specific (SIS) were found from 6 h cultivation while the longer cultivation time of 12 and 24 h appeared either the loss of BLA or the inhibition activity against the different target strain. Only 3 effective thermotolerant isolates, i.e., KUB-AC5, KUB-AC16 and KUB-AC20, exerted broad SIS against all target strains except *Salmonella sp.* S001. However, BLA belonging to KUB-AC5 could not inhibit growth of *E. coli* E006 and E010 whereas KUB-AC20 did not inhibit only *E. coli* E006. Based on biochemical, physiological and molecular systematic method, these isolates were classified as *Lactobacillus reuteri*.

Key words : lactic acid bacteria, bacteriocin–like activity, antimicrobial activity, *Lactobacillus reuteri*

INTRODUCTION

Lactic acid bacteria (LAB) has been used as a probiotic product in animal such as chicken, pig (Salminen and Marteau, 1997). It produces variety of antimicrobial substances which have beneficial health effects in the host by improving the integrity of the intestine microflora. LAB produces a variety of low molecular mass compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide and other metabolites. Many of these metabolites are bacteriocin, siderophore, reuterin, and benzoic acid (Helander *et al.*, 1997). These compounds effect to various microorganisms including those pathogens . So far a number of antibiotics have been applied to chicken in order to to prevent the infection and the increasing dosage of the antibiotics used caused the development of resistant bacterial strains in domestic animals. Therefore, the objective of this paper was (i) to investigate the antimicrobial activity producing LAB from chicken intestine against the antibiotics

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resistant pathogenic strains of *Escherichia coli* and *Salmonella sp.* and (ii) to identify the effective isolates selected.

MATERIALS AND METHODS

Sample sources

Lactic acid bacteria were isolated by direct plating method from 10 fresh chicken intestines samples collecting from the area around Bangkok.

Bacterial strains and media

Twelve strains of *Escherichia coli* and 3 strains of *Salmonella* resistant to some antibiotics (Table 2) from infectious chicken used as the target strains were obtained from Betagro Agro-Group Public Company Limited, Samutprakarn, Thailand. These strains were maintained in the NB medium (Difco) containing 20% glycerol at –80°C. For routine work, they were kept as nutrient agar (Difco) slant at +4°C.

Table 1 Primers used for PCR reaction corresponding to 3' and 5' end of 16S RNA and sequencing experiments.

Primer	Purpose	Type	Hits	Sequence (5' –3')	Reference
1101A	PCR product	Forward	At 3' end of 16 sRNA	AACGAGCGCAAC CC	Oyaizu, 1992
1407B	PCR product	Reverse	At 3' end of 16 sRNA	GACGGGCGGTGT GTAC	Oyaizu, 1992
8UA	PCR product	Forward	At 5' end of 16 sRNA	AGAGTTTGATCCT GGCTCAG	Kawamura et al. 1995
519C	PCR product	Reverse	At 5' end of 16 SRNA	ATTACCGCCGCTG CTG	Kawamura et al. 1995
M13 universal primer	Sequencing	Forward	PUC 18	CGACGTTGTAAA ACGACGGCCAGT	Supplied by Hokkaido system science Co., Ltd., Hokkaido, Japan.
M13 reversal primer	Sequencing	Reverse	PUC18	TTTCACACAGGA AACA	Supplied by Hokkaido system science Co., Ltd., Hokkaido, Japan

MRS agar with 1.6% bromcresol purple in 95% ethanol was utilized for isolation of lactic acid bacteria (LAB) from chicken intestine samples (de Man *et al.*, 1960). Soft nutrient agar were prepared by adding 0.7% (w/v) agar to liquid medium used for target strains preparation.

E. coli JM109 used as a host cell for PCR cloning and sequence analysis was cultivated in L medium containing 50 μg/ml of ampicillin at 37°C (Sambrook *et al.*, 1989).

Detection and isolation of bacteria producing bacteriocin - like activity (BLA)

A 10% (w/v) sample in diluent (0.8% w/v NaCl) was homogenized (Seward Stomacher 400 Lab System) and 10-fold serially diluted. Detection of antimicrobial activity was carried out by the spot on lawn method. Multiple plates of serial dilution were overlaid with target strains and incubated under anaerobic condition for 24 h at 37° and 45° C. The colonies producing zones of growth inhibitor in the indicator lawn were selected and isolated from within the agar using a sterilized Pasture pipette to remove a small plug containing the colony of interest. The agar plug was grown in MRS broth medium over night and streaked out on MRS agar medium to get the pure culture. Cell free culture fluid (CF) of each colony (in MRS medium at 37°C for 24 h) was prepared as a sample. A paper disk (6 mm diameter) with an adsorbed aliquot of 25 μl of sample was put on NA medium containing a target strain at the concentration of about 10^7 cfu/ml. After incubation for 24 h at 37°C, the antimicrobial activity of each isolate was evaluated based on the formation of a clear zone around the paper disk.

The subsequent screening for the positive isolates displaying bacteriocin - like activity (BLA) was carried out by using the paper disk method (Hoover and Harlander, 1993). CF in MRS broth at 37°C for 6, 12, 24 h was prepared as samples. A paper disk (6 mm diameter) with an adsorbed aliquot of 25 μl of sample adjusted to pH 6.0 was put on an MRS agar and overlaid with NA medium containing a target strain at the concentration of about 10^7 cfu/ml. After incubation for 24 h at 37° C, the BLA of each isolate was evaluated based on the formation of a clear zone around the paper disk.

Morphology and motility

Cell shape and gram stain of 24 h cultures grown on MRS medium were examined by electron microscope (JSM 5410 LV, Joel). The motility was investigated by the method of Atlast (1995).

Determination of growth on various temperature, pH and NaCl concentration

One colony from a 1 day culture was inoculated into MRS medium and grown for 24 and 48 h at 15°, 37°, 45° and 50°C; pH 4.5, 9.6 and %NaCl of 6.5 and 18. The growth was evaluated by measuring its turbidity at 660 nm (Koch, 1981).

Biological tests

API 20E and API 50CH (API Laboratory Products Ltd. Biomerieus Saa, France) were used to determine the biochemical and carbohydrate fermentation patterns, respectively. The experiment was performed at 37°C. The results of biochemical test and carbohydrate fermentation were determined after 24 and 48 hrs.

Analytical methods

Lactic acid was determined using HPLC method. A 24 h culture grown on MRS medium with 2% glucose was filtered through 0.25 μm membrane and analyzed using HPLC method. Sulfonate divinyl benzene-styrene HPX-87H column was used. The running condition was performed as instructed by BIO-RAD. Tartaric acid was used as an internal standard. Glucose content was determined according to the method of

Miller (1959).

Plasmid DNA and oligonucleotide primers

pUC18 was used as a plasmid DNA vector for PCR cloning and sequencing work. Plasmid DNA was constructed to be a T-vector by firstly making blunt end restriction reaction with SmaI and then a single T was added at the 3' end of DNA with Taq DNA polymerase as described by Smith (1997). All oligonucleotide primer sequences used were listed in Table 1.

Extraction of DNA

Chromosomal and plasmid DNA were isolated by magnetic extraction. The genomic DNA extraction mag extractor kit and plasmid purification extraction kit were used according to the manufacturer (Toyobo Co. Ltd.).

PCR reaction

For PCR products at the 3' and 5' end of 16S RNA, genomic DNA amplification was performed on a programable thermocycle (Astec program temp control system PC-800). PCR reaction was performed by the modified method of Newton and Graham (1994). Each reaction tube contained 50 µl of a solution of 1x amplification buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.1% Triton X-100, Toyobo, Co. Ltd.), 200 µmol of each deoxynucleotide triphosphate (Toyobo Co. Ltd.), 20 pmol of primer and 1 U of Taq polymerase (Toyobo Co. Ltd.). About 200 ng of target DNA were added to the mixture. All amplfication were performed using 1 cycle of 5 min denaturation at 94°C and further 35 cycles as follows: 1 min denaturation at 94°C, 2 min primer annealing at 55 C and 2 min primer extension at 72°C. After the 35th cycle, the extension reaction was continued for another 15 min at 72°C to ensure that the final extension step was completed.

For PCR cloning, whole cell PCR reaction was used for screening of clones containing positive fragments. Each clone was picked up and suspended in PCR solution. M13 universal and reversal primer were used. The reaction condition was performed as the following: 1 cycle of 3 min cell breaking at 94°C; 30 cycles of 30 sec denaturation at 94°C, 30 sec primer annealing at 55°C, and 30 sec primer extension at 72°C.

Cloning and sequencing of the 16S rRNA gene

The amplified products at both 3' and 5' end were cloned into pUC 18 T vector by standard method (Sambrook *et al.*, 1989) and screened as the method mentioned above. The sequence was determined by using thermo sequenase fluorescent labelled primer cycle sequencing kit (Pharmacia Biotech) with an automatic sequencer (Model ALF express, Phamacia Biotech). The comparisons of the relative nucleotide sequence of unknown strain were determined by performing sequence database searches and the sequences of closely related strains were retrieved from GenBank. Sequences were aligned and calculated as % identity with the Blast search program.

DNA base composition

The DNA G+C content (mol%) was determined by the modified method of Hou *et al.*(1998). 50 µl of sample were analyzed using HPLC (Shimadzu, SPD-M10A). The column TSK gel ODS – 120A (Tosoh) was used. The analysis was performed at room temperature and 10mM phosphate buffer pH 3.5 as mobile phase with the flow rate of 1 ml/min.

RESULTS

Screening for lactic acid bacteria producing antimicrobial substances

Two hundred fifty six LAB strains isolated from chicken intestines in the market around

Bangkok, Thailand were primary examined for the antimicrobial activity by the spot on lawn method. The *E. coli* strain E012 was used as a target strain since it was resistant to broad spectrum of antibitic substances used as shown in Table 2. 24 isolates were selected according to high antimicrobial activity of more than 80% as shown on Figure 1. They were designed as AC1-24. However, the isolate AC7, AC12, AC15 and AC23 died out during storage. Therefore only 20 isolates were further studied for BLA production against all target strains.

The BLA of LAB was extracellularly produced either in the logarithmic growth phase as a typical growth associated pattern (Ohmomo *et al*., 2000) or in the stationary phase (Schved *et al*., 1993). The maximum production mostly displayed at 6 and 12 h cultivation. Therefore, the CF of 20 isolates were decided to prepare at 6, 12 and 24 h. The BLA from 6 h cultivation of each isolates showed more broad spectrum inhibition specificity (SIS) against the targets strains than the ones from 12 and 24 h. The result showed that none of them inhibited growth of *Salmonella* S001. According to inhibition activity, they were devided into 3 groups. The group I, II and III were designated high , medium and low SIS respectively.

The group I, high SIS, showed inhibition activity to 9–11 target strains. They were AC5, AC8, AC16 and AC20 as shown in Table 3. At 6 h cultivation , they all inhibited growth of the target strain; E001, E002, E003, E005, E007, E011, S002

Table 2 Drug resistance of 15 target strains . AM, Amoxycillin; CL, Colistin; ER, Erythromycin; GM, Gentamicin; KA, Kanamycin; NE, Neomysin; FD, Nitrofurantoin; PN, Pennicillin; SxT, Sulfamethoxazole + Trimetroprim; ST, Streptomycin; TR, Oxytetracycline (Terramycin); EN, Enrofloxacin; NF, Norfloxacin; CP, Cephalothin; AMC, Amoxycillin and C, Chloramphenicol. X, resistance.

Code	Bacterial strain	Drug sensitivity															
		AM	CL	ER	GM	KA	NE	FD	PN	SxT	ST	TR	EN	NF	CP	Amc	C
E001	*E. coli*			X		X	X	X	X	X	X	X	X	X	X	X	X
E002	*E. coli*			X		X	X	X	X	X	X	X	X	X		X	X
E003	*E. coli*			X		X	X	X	X	X	X	X	X	X	X	X	X
E004	*E. coli*			X		X									X	X	
E005	*E. coli*			X	X	X	X	X	X	X	X	X					X
E006	*E. coli*			X	X	X	X	X	X	X	X	X				X	
E007	*E. coli*			X	X	X	X	X	X	X	X	X			X		
E008	*E. coli*						X		X	X	X	X			X		X
E009	*E. coli*			X	X				X	X	X						
E010	*E. coli*	X		X				X	X	X	X	X					X
E011	*E. coli*			X					X		X		X	X	X		X
E012	*E. coli*	X	X	X	X	X	X	X	X	X	X	X	X	X		X	
S001	*Salmonella*			X				X	X								
S002	*Salmonella*			X					X								
S003	*Salmonella*	X		X				X	X								

Table 4 Inhibition activity of cell-free culture fluid at 37°C from the isolates belonging to group II against 15 target strains. Inhibition activity expressed as diameter of clear zone in mm unit. -, no activity.

Cultivation time (h)	LAB	The target strain														
		E001	E002	E003	E004	E005	E006	E007	E008	E009	E010	E011	E012	S001	S002	S003
6 h	AC1	-	8	-	-	-	-	9	-	-	9	8	-	-	8	8
	AC2	-	11	-	-	10	-	-	-	-	11	-	-	-	9	8
	AC3	-	-	-	-	9	9	-	-	-	9	8	-	-	8	8
	AC4	-	11	-	-	9	-	-	-	-	11	-	-	-	9	8
	AC6	-	11	-	-	9	-	-	-	-	12	-	-	-	9	8
	AC9	-	-	-	-	8	8	-	-	-	-	9	-	-	8	7.5
	AC10	-	12	-	-	9	-	-	-	-	12	-	-	-	8	8
	AC11	-	11	-	-	9	-	-	-	-	9	-	-	-	10	8
	AC14	-	9	-	-	8	-	-	-	-	8	-	-	-	9	8
12 h	AC1	-	8	8	-	-	-	-	-	-	-	-	8	-	-	-
	AC2	-	11	-	-	-	-	-	-	-	-	-	-	-	8	-
	AC3	-	8	-	-	-	-	-	-	-	8	-	8	-	-	-
	AC4	-	12	-	-	-	-	-	-	-	10	-	-	-	10	-
	AC6	-	12	-	-	-	-	-	-	-	11	-	-	-	10	-
	AC9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AC10	-	9	-	-	-	-	-	-	-	-	-	-	-	9	-
	AC11	-	9	-	-	-	-	-	-	-	-	-	-	-	11	-
	AC14	-	-	-	-	-	-	-	-	-	-	-	-	-	12	-
24 h	AC1	8	9	-	-	-	-	-	-	-	-	-	-	-	-	-
	AC2	-	9	-	-	-	-	-	-	-	10	-	-	-	10	-
	AC3	8	8	-	-	-	-	-	-	-	8	-	-	-	-	-
	AC4	-	9	-	-	-	-	-	-	-	9	-	-	-	10	-
	AC6	-	10	-	-	-	-	-	-	-	10	-	-	-	8	-
	AC9	8	9	9	-	-	-	-	-	-	8	-	-	-	-	-
	AC10	-	-	-	-	-	-	-	-	-	9	-	-	-	9	-
	AC11	-	-	-	-	-	-	-	-	-	9	-	-	-	9	-
	AC14	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-

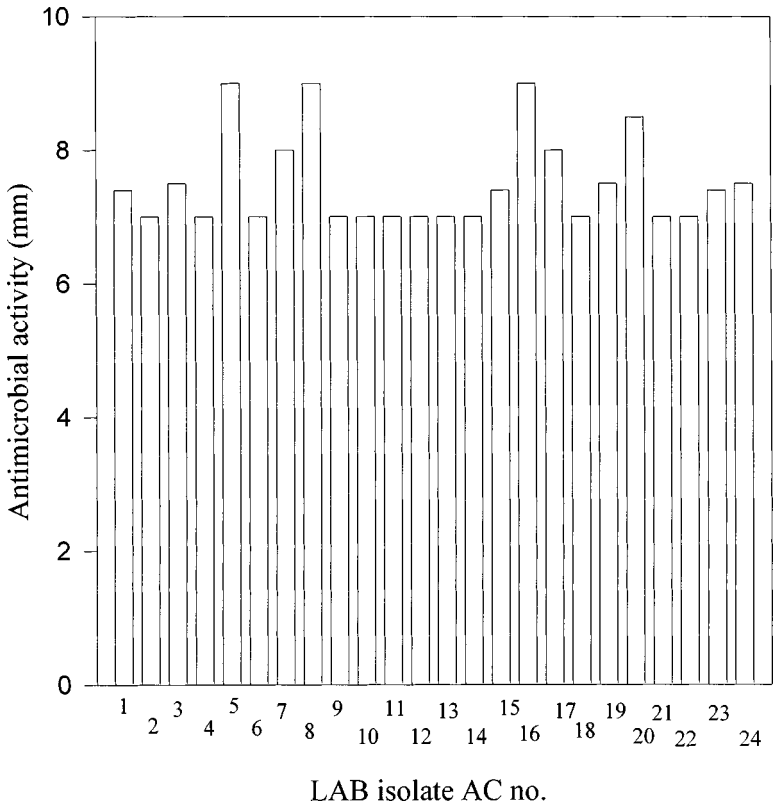

Figure 1 The antimicrobial activity of the selective LAB isolates against the target strain Escherichia coli E012. Antimicrobial activity was defined as diameter (mm) of clear zone.

and S003. Neither of them did to E010 and S001. However, when the cultivation time was prolonged to 24 h, the inhibition activity of isolate AC8, AC16 and AC20 did appear against E010. This kind of result also happened to the isolate AC5 against E004, E008 and E012, the isolate AC8 against E008 and the isolate AC16 against E006 and E008. In the other hand, the activity of the same isolates disappeared when the cultivation time was longer as shown on the activity against E001, E003, E004, E005, E007, E008, E009, E011 and S002.

The isolates belonging to group II played medium SIS against 5–8 target strains as shown on Table 4. They were AC1–4, AC6, AC9–11 and AC14. A 6 h CF from every isolate inhibited only the growth of *Salmonella* S002 and S003. However when the cultivation time was prolonged to 12 and 24 h, the activity against the target strain S003 disappeared. Considering the activity against the target strain *E. coli*, the CF from every isolate could not inhibit the target strain E004, E008 and E009 whereas those from some LAB isolates did inhibit to E002, E003, E005, E006, E007, E010 E011and E012. The inhibition activity of some isolates i.e. AC1, AC3 and AC9 showed up after 12 or 24 h against the target strain E001 E002 and E012. Similar to the isolates belonging to group I, the activity may disappear after prolonging incubation time to 12 and 24 h as shown in Table 4.

The isolates belonging to group III showed

Table 3 Inhibition activity of cell-free culture fluid at 37°C from the isolates belonging to group I against 15 target strains. Inhibition activity expressed as diameter of clear zone in mm unit. -, no activity.

Cultivation time (h)	LAB	The target strain														
		E001	E002	E003	E004	E005	E006	E007	E008	E009	E010	E011	E012	S001	S002	S003
6 h	AC5	9	9	9	-	11	-	9	-	8	-	11	0	-	8	8
	AC8	8	9	9	9	9	-	9	-	8	-	11	9	-	8	8
	AC16	8	9	9	8	8	-	9	-	-	-	10	9	-	9	8
	AC20	8	9	9	8	9	-	8	8	-	-	10	0	-	8	8
12 h	AC5	8	9	8	8	-	-	8	8	8	-	-	9	-	8	9
	AC8	9	8	8	8	-	-	-	8	-	-	-	9	-	8	9
	AC16	8	8	8	8	-	8	-	8	-	-	8	9	-	8	9
	AC20	8	8	8	8	-	-	-	8	-	-	-	9	-	8	9
24 h	AC5	8	9	8	-	-	-	8	-	-	-	8	8	-	-	8
	AC8	8	9	9	-	-	-	9	-	-	8	8	9	-	-	8
	AC16	-	8	8	-	-	-	8	-	-	10	8	9	-	-	8
	AC20	-	8	-	-	-	-	-	8	-	9	-	8	-	-	8

lowest SIS against only 1–4 target strains (Table 5). The 6 h CF of the isolates AC13, AC17–19, AC22 and AC24 inhibited the growth of target strain E002, E010 and S002 except the CF of AC13 which appeared later after 24 h for strain E002 but not E010 whereas the one of AC21 did to E006, E012, S002 and S003. At 12 and 24 h cultivations, all of them except AC13 still exhibited the inhibition activity against S002 but not to the target strain E002 and E010. Considering AC21, the inhibition activity against E012, S002 and S003 disappeared after 12 h cultivation. However the activity from some isolates did appear after 12 and 24 h cultivation for example the activity of AC17, AC18, AC19, AC22 and AC24 against E001, E006, E007 or E008. All isolates showed no inhibition activity against the target strain E004, E009, E011 and S001.

Identification of selected strains

Four effective isolates from group I were selected for further study. As shown in Table 6, all isolates were gram positive, nonmotile, non-sporeforming and not catalase producing. The isolate AC5, AC16 and AC20 were short rod, pair-forming whereas the isolate AC8 was long rod and chain-forming. The isolate AC5, AC16 and AC20 produced 64.6, 63.5 and 46.4% of lactic acid from glucose with gas production indicating that they were heterofermentative lactic acid bacteria. However % yield of lactic acid obtained from the isolate AC8 was higher to 99.8% without gas formation. Thus, it was classified as a homofermentative lactic acid bacteria. They all would be classified as genus *Lactobacillus* (Wood and Holzapfel, 1995).

Growth of all strains were inhibited at 15°C, in MRS broth with NaCl concentration up to 18% and the pH adjusted to 9.6. The isolate AC5, 16 and 20 were able to grow at high temperature up to 45–50°C and low pH of 4.5 whereas the isolate AC8 could not. Therefore, the isolates AC5, AC16 and AC20 were catagorized as thermotolerant strains. Since we are more interested in the thermotolerant strains, therefore only the isolates AC5, AC16 and AC20 were further studied for biochemical test and phylogenetic relation based on sequence analysis of 16 S rRNA and %Mol G+C contents.

The ability to utilize various carbon sources was determined using API 50 CHL test strip (Atrih *et al*., 1993; Vescovo *et al*., 1996). They all could produce acid from ribose, galactose, glucose, maltose, lactose, melibiose, sucrose, raffinose and gluconate (Table 7). Initial identifications made by API database correlation indicated 99.8% similarity to *L. fermentum.*

Partial sequencing on signature regions at 3' and 5' end of the 16S rDNA of these strains was carried out as an alternative approach to strain identification. The PCR products obtained by amplification were about 306–308 and 561–565 bp at 3' and 5' end of 16 rrs genes respectively. They were sequenced and compared directly with the GenBank databases. The closet matches for these three strains were different from those determined by the API method. 99% and 95–96% identity to *L. reuteri* DSM 20016 T was obtained at 3' and 5' end respectively.

Three isolates were also investigated for G+C contents (Table 6). The mol% G+C content was 41–42 which was closed to *L. reuteri* Type strain DSM 20016 (40–42%) (Wood and Holzapfel, 1995)

DISCUSSION

The selected 20 LAB isolates were able to inhibit the growth of 12 *E. coli* strains and 3 *Salmonella* sp. strains to varying degrees. These results were interesting because the strain inhibition against gram-negative bacteria (eg. *E. coli* and

Table 5 Inhibition activity of cell-free culture fluid at 37 C from the isolates belonging to group III against 15 target strains. Inhibition activity expressed as diameter of clear zone in mm unit. -, no activity.

Cultivation time (h)	LAB	The target strain														
		E001	E002	E003	E004	E005	E006	E007	E008	E009	E010	E011	E012	S001	S002	S003
6 h	AC13	-	-	-	-	-	-	8	-	-	-	-	-	-	8	8
	AC17	-	9	-	-	-	-	-	6.5	-	11	-	-	-	8	-
	AC18	-	9	-	-	-	-	-	-	-	11	-	-	-	10	-
	AC19	-	12	-	-	-	-	-	6.5	-	9	-	-	-	10	-
	AC21	-	-	-	-	-	8	-	-	-	-	-	8	-	7	7
	AC22	-	10	-	-	-	-	-	6.5	-	10	-	-	-	10	-
	AC24	-	8	-	-	-	-	-	-	-	11	-	-	-	10	-
12 h	AC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AC17	7	-	-	-	-	-	-	8	-	-	-	-	-	9	-
	AC18	7	-	-	-	-	-	-	-	-	-	-	-	-	9	-
	AC19	6.5	-	-	-	-	-	-	8	-	-	-	-	-	9	-
	AC21	-	-	-	-	-	7	-	-	-	-	-	-	-	-	-
	AC22	7	8	-	-	8	-	-	-	-	-	-	-	-	9	-
	AC24	7.5	-	-	-	8	-	-	-	-	-	-	-	-	9	-
24 h	AC13	8	8	8	-	-	-	-	-	-	-	-	-	-	-	-
	AC17	-	-	-	-	-	-	-	6.5	-	-	-	-	-	9	-
	AC18	7	-	-	-	-	6.5	-	-	-	-	-	-	-	9	-
	AC19	7	-	-	-	-	-	-	-	-	-	-	-	-	9	-
	AC21	-	-	-	-	-	7	-	-	-	-	-	-	-	-	-
	AC22	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-
	AC24	-	-	-	-	-	-	6.5	-	-	-	-	-	-	10	-

Salmonella sp.) were not from acidity effect. This did not agree with the result of Jin *et al.* (1996) which reported that all effective *Lactobacillus* isolated from chicken intestine showed the inhibition of the pathogenic bacteria as *E. coli* and *Salmonella sp.* due to the production of organic acid but not from hydrogen peroxide and bacteriocin. Our results did agree with the result of McGroarty and Reid (1988) that *L. rhamnosus* GR-1 were found to exert an inhibitory effect on *E. coli* Hu 734 and ATCC 25922. Their data also indicated that the inhibitor was not due to lactic acid or hydrogen peroxide but substance having molecular weight greater than 12,000–14,000 dalton.

Among the target strains used in this study, it was found that *E. coli* E006 and *Salmonella sp.* S001 were the most resistant strains whereas *E. coli* E002 and *Salmonella sp.* S002 were most sensitive. The result above also showed that the BLA of some LAB strains after 12 and 24 h cultivation disappeared. This maybe due to the activity condition. The pH obtained from CF at 6 h was

Table 6 Phenotypic characteristics of the isolate AC5, AC8, AC16 and AC20.

Test	Characteristics and reaction			
	AC5	AC8	AC16	AC20
1. Morphology	Short rod, pair	Long rod, pair	Short rod, pair	Short rod, pair
2. Gram stain	+	+	+	+
3. Motility	-	-	-	-
4. Spore	-	-	-	-
5. Catalase	-	-	-	-
6. Growth at 15°C	-	-	-	-
37°C	+	+	+	+
45°C	+	-	+	+
50°C	+	-	+	+
pH 4.5	+	-	+	+
pH 9.6	-	-	-	-
%NaCl 6.5	-	+	+	-
%NaCl 18	-	-	-	-
7. % lactic acid from glucose	64.6	99.8	63.5	46.4
8. Gas from glucose	+	-	+	+
9. Acetoin production	+	+	+	+
10. β-glucosidase	-	-	-	-
11. β-glucuronidase	-	-	-	-
12. α-galactosidase	+	-	+	+
13. β-galactosidase	+	-	+	+
14. Alkaline phosphatase	-	+	-	-
15. Arginine dihydrolase	+	-	+	+
16. Leucine arylamidase	+	+	+	+
17. % Mol G+C content	41	ND	42	42

about 5 whereas those at 12 and 24 h were down to about 4 (data not shown). Giraffa *et al*. (1995) reported that the bacteriocin amounts from culture solution at pH 4–4.1 was two times lower than the one at pH 5.3–5.45. Therefore, the result of inhibition loss at 12–24 h maybe due to low BLA produced.

In the other hand, in some case the inhibition activity did appear after 12 or 24 h cultivation to the different target strains. It was possible that high BLA or more than one bacteriocin type was produced. Revol-Junelles *et al*. (1996) reported that *Leuconostoc mesenteroides* subsp. produced 2 kinds of bacteriocin. The first one, *Mesenteroides* FR52, produced a broad inhibitory spectrum including anti-listeria activity, while the other, Mesenteroides 52B was only active against *Leuconostoc* sp. In addition, the bacteriocin

Table 7 Carbohydrate fermentation of the thermotolerant LAB selected.

Test	The isolate			Test	The isolate		
	AC5	AC16	AC20		AC5	AC16	AC18
1. Glycerol	-	-	-	25. Esculin	-	-	-
2. Erythritol	-	-	-	26. Salicin	-	-	-
3. D-arabinose	-	-	-	27. Cellobiose	-	-	-
4. L-Arabinose	-	-	-	28. Maltose	+	+	+
5. Ribose	+	+	+	29. Lactose	+	+	+
6. D-xylose	-	-	-	30. Melibiose	+	+	+
7. L-xylose	-	-	-	31. Sucrose	+	+	+
8. Adonitol	-	-	-	32. Trehalose	-	-	-
9. β-methyl-D-xyloside	-	-	-	33. Inulin	-	-	-
10. Galactose	+	+	+	34. Melezitose	-	-	-
11. Glucose	+	+	+	35. Raffinose	+	+	+
12. Fructose	-	-	-	36. Starch	-	-	-
13. Mannose	-	-	-	37. Glycogene	-	-	-
14. Sorbose	-	-	-	38. Xylitol	-	-	-
15. Rhamnose	-	-	-	39. Gentiobiose	-	-	-
16. Dulcitol	-	-	-	40. D-turanose	-	-	-
17. Inositol	-	-	-	41. D-lyxose	-	-	-
18. Mannitol	-	-	-	42. D-tagatose	-	-	-
19. Sorbitol	-	-	-	43. D-fucose	-	-	-
20. Methyl-D-mannoside	-	-	-	44. L-fucose	-	-	-
21. Methyl-D-glucoside	-	-	-	45. D-arabitol	-	-	-
22. N-acethyl-glucosamine	-	-	-	46. L-arabitol	-	-	-
23. Amygdalin	-	-	-	47. Gluconate	+	+	+
24. Arbutin	-	-	-	48. 2-keto-gluconate	-	-	-
				49. 5-keto-gluconate	-	-	-

+, acid production; -, no acid produced

produced may exhibit synergism reaction. Hanlin *et al.* (1993) proposed that the combination of pediocin AcH and nisin inhibited more effectively to the growth of *Lactobacillus, Leuconostoc mesenteroides, listeria monocytogenes, Clostridium botulinum, C. perfringens* and *Listeria laramiei.* Therefore, with the support by above experiment, the BLA appearing at 12 or 24 h may be more than one type or synergism action enhancing the activity. Further work on purification and characterization should be addressed.

The isolates AC5, AC16 and AC20 were determined to be belonged to *L. fermentum* type strain ATCC 11742 by biochemical analysis. However, the results of mol% G+C content of 41–42 obtained were lower than the one of *L. fermentum* (52–54%) but it was closed to *L. reuteri* type strain DSM 20016 (40–42 %). In addition, % identities of 16S rRNA sequence from these three strains were also closed to *L. reuteri*. *L. reuteri* was earlier classified as *L. fermentum* type II and then it was changed to be the new specy of *L. reuteri* by Lerche and Reuter (Wood and Holzapfel, 1995). Therefore, these three strains were clearly classified as *L. reuteri.*

In conclusion, the results of the present study showed that (i) Three effective thermotolerant LAB strains AC5, AC16 and AC20 displayed broad inhibition spectrum against *E. coli* and *Salmonella* sp. resistant to various antibiotics by exerting BLA but not due to acidity. (ii) The most resistant target strains were *E. coli* E006 and *Salmonella sp*. S001. (iii) All effective thermotolerant were classified as *L. reuteri.* (iv) The proposed hypothesis on "the loss of BLA after prolonging cultivation time of 12 and 24 h may due to the effect of low pH causing low BLA and the inhibition activity against the different target strains after 12 and 24 h cultivation maybe due to more than one type produced with synnergisn action" should be addressed for further investigation. All three effective LAB strains designated KUB-AC5, KUB-AC16 and KUB-AC20 were kept at Department of Biotechnolgy, Faculty of Agro-Industry, Kasetsart University, Thailand.

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