Journal of Food Protection, Vol. 74, No. 4, 2011, Pages 631–635
doi:10.4315/0362-028X.JFP-10-276

Research Note

Autoinducer-2–like Activity in Lactic Acid Bacteria Isolated from Minced Beef Packaged under Modified Atmospheres

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MS 10-276: Received 2 July 2010/Accepted 2 January 2011

ABSTRACT

Fifteen fingerprints (assigned to *Leuconostoc* spp., *Leuconostoc mesenteroides, Weissella viridescens, Leuconostoc citreum,* and *Lactobacillus sakei*) of 89 lactic acid bacteria (LAB) isolated from minced beef stored under modified atmospheres at various temperatures were screened for their ability to exhibit autoinducer-2 (AI-2)–like activity under certain growth conditions. Cell-free meat extracts (CFME) were collected at the same time as the LAB isolates and tested for the presence of AI-2–like molecules. All bioassays were conducted using the *Vibrio harveyi* BAA-1117 (sensor 1^-, sensor 2^+) biosensor strain. The possible inhibitory effect of meat extracts on the activity of the biosensor strain was also evaluated. AI-2–like activity was observed for *Leuconostoc* spp. isolates, but none of the *L. sakei* strains produced detectable AI-2–like activity. The AI-2–like activity was evident mainly associated with the *Leuconostoc* sp. B 233 strain, which was the dominant isolate recovered from storage at 10 and 15°C and at the initial and middle stages of storage at chill temperatures (0 and 5°C). The tested CFME samples displayed low AI-2–like activity and inhibited AI-2 activity regardless of the indigenous bacterial populations. The LAB isolated during meat spoilage exhibited AI-2–like activity, whereas the LAB strains retrieved depended on storage time and temperature. The production of AI-2–like molecules may affect the dominance of different bacterial strains during storage. The results provide a basis for further research concerning the effect of storage temperature on the expression of genes encoding AI-2 activity and on the diversity of the ephemeral bacterial population.

Quorum sensing is a cell-to-cell signaling mechanism that allows bacterial populations to sense their environment and coordinate gene expression *(33)*. Various bacterial behaviors are regulated by quorum sensing, including symbiosis, virulence, antibiotic biosynthesis, bioluminescence, sporulation, motility, plasmid transfer, and biofilm formation *(1, 6, 11)*. Among the several signaling molecules that have been identified, autoinducer (AI)-1 quorum sensing signaling molecules (*N*-acyl homoserine lactones) are produced and used by gram-negative bacteria primarily for intraspecies communication. AI-2 signaling molecules (furanosyl borate diesters) are produced by both gram-positive and gram-negative bacteria and are thought to serve as a universal signal for both intra- and interspecies communication *(1)*. Gram-positive bacteria produce and use autoinducing peptides *(18)*. Other molecules chemically similar to *N*-acyl homoserine lactones have been described, e.g., 2(5H)-furanones, which were released by *Lactobacillus helveticus* that was exposed to oxidative and heat stresses *(21)*. The 2(5H)-furanones were released during different growth phases by gram-positive bacteria such as *Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus sanfranciscensis,* and *Enterococcus faecalis (30)*.

AI-1 and AI-2 signaling compounds are present and/or increase their concentrations in various food ecosystems such as meat, milk, and vegetables as the number of spoilage bacteria increases *(4, 16, 17, 22, 24)*. These compounds may be produced by the specific spoilage organisms or a smaller fraction of them, called ephemeral spoilage organisms *(1)*. However, no direct correlations have been found between the presence of signaling compounds and the presence of specific or ephemeral spoilage organisms (mainly gram-negative bacteria), which represent most of the microbial community generally associated with these food products when stored under aerobic conditions *(23)*. The bacterial strains isolated from these products have been tested for the production of these signaling compounds *(8, 12, 14, 16)*. Similar studies have not been conducted with lactic acid bacteria (LAB), which are the specific spoilage organisms on meat stored under modified atmospheres *(23)*.

The objective of the present study was to determine whether the ephemeral LAB isolated throughout spoilage of minced beef stored under modified atmospheres at various temperatures exhibit AI-2–like activity. Cell-free meat extracts (CFMEs) were collected at the same time as were samples for microbiological analysis and isolate recovery. These CFMEs were evaluated for the presence of AI-2–like

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activity that could be correlated with the indigenous microbial population.

MATERIALS AND METHODS

Bacterial strains and culture conditions. From the 89 strains of LAB used in this study, 15 fingerprints were obtained. These strains were isolated from minced beef stored under modified atmospheres (40% CO_2, 30% O_2, 30% N_2) at 0, 5, 10, and 15°C *(2)*. The strains were identified using pulsed-field gel electrophoresis (PFGE) and 16S rRNA gene sequence analysis according to the methods of Doulgeraki et al. *(9)*. Throughout the storage period, relevant petri dish cultures from the highest dilution of the minced beef samples were kept. At the end of the storage period, LAB strains were isolated from three time points (initial, middle, and final stages of storage) considering the growth kinetic parameters related to LAB populations, i.e., LAB were recovered from the lag phase (initial stage), the middle of the exponential growth phase (middle stage), and the early stationary phase (final stage of storage). Isolated LAB were purified by successive subculture in de Man Rogosa Sharpe (MRS) agar (Biolife, Milano, Italy) and stored at −80°C in MRS broth (Biolife) supplemented with 20% (vol/vol) glycerol (Merck, Darmstadt, Germany). Before experimental use, each strain was grown twice in quarter-strength brain heart infusion (BHI) broth (Lab M, Bury, UK) at 30°C with agitation (160 rpm).

The *Vibrio harveyi* BAA-1117 (*luxN*::Tn*5* sensor 1^- sensor 2^+) biosensor strain, which only senses the AI-2 molecule, and the AI-2–producing *V. harveyi* BAA-1119 (*luxL*::Tn*5* AI-1^- AI-2^+) strain were used for the AI-2 activity bioassay; both strains were purchased from LGC Promochem (Teddington, Middlesex, UK) *(3)*. The *V. harveyi* strains were stored at −80°C in cryovials (Lab M). The working stock cultures were streaked onto autoinducer bioassay (AB) plates, and cells from a single colony were grown for 16 h at 30°C with agitation (160 rpm) in AB medium. The AB medium was prepared as described by Lu et al. *(17)*.

An exogenous source of AI-2–like molecules in the inhibition assays was used. The cell-free culture supernatant (CFCS) from *Salmonella enterica* serovar Typhimurium strain 4/74 ($CFCS_{ST}$) had previously produced AI-2 in our laboratory.

Preparation of CFCSs. LAB isolates were grown in quarter-strength BHI broth to avoid the effects of glucose repression on the luminosity of the *V. harveyi* BAA-1117 biosensor strain *(8)*. The isolates were incubated at 30°C with agitation (160 rpm) until early stationary phase (about 20 h). $CFCS_{LAB}$ was prepared by removing the cells from the growth medium by centrifugation at 5,000 × *g* for 15 min at 4°C in a Heraeus Fresco 21 microcentrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cleared culture supernatants were filter sterilized with 0.2-μm-pore-size filters (Whatman, Clifton, NJ) and stored at −20°C until the AI-2 activity bioassays were performed.

Preparation of CFMEs. CFMEs were collected throughout minced beef storage at the same time as the LAB isolates were recovered (i.e., initial, middle, and final stages of storage). Five-gram portions of minced beef samples were homogenized with 10 ml of Ringer solution (Lab M). The CFMEs were obtained by centrifugation at 5,000 × *g* for 15 min at 4°C in a Heraeus Multifuge 1S-R centrifuge (Thermo Electron) and filtered through 0.2-μm-pore-size filters (Whatman) as described by Nychas et al. *(22)*. The supernatants were stored at −20°C until the assays were performed.

Bacterial enumeration. A detailed description of the methodology used for the enumeration of the total viable bacteria and LAB in this work was presented elsewhere *(2)*. LAB counts were determined on MRS agar (Biolife) overlaid with the same medium and incubated at 30°C for 72 h.

AI-2 activity bioassay. The AI-2 activity bioassay was performed as described by Surette and Bassler *(28)*. An overnight culture of *V. harveyi* BAA-1117 was diluted 1:5,000 with fresh AB medium. Ninety microliters of this cell suspension was mixed with 10 μl of the tested sample (i.e., $CFCS_{LAB}$ or CFME) in a 96-well polystyrene microplate (μ-Clear, Greiner Bio-One, Munich, Germany). Ten microliters of sterile growth medium (quarter-strength BHI) was used as the negative control *(15)* when screening CFCS and 10 μl of CFME of the 0-h minced beef sample was used as the negative control when screening CFME. The CFCS (10 μl) of *V. harveyi* BAA-1119 was used as the positive control to verify the bioassays.

To identify inhibition of luminescence caused by the CFME in the biosensor strain *V. harveyi* BAA-1117, an equal volume (50 μl) of meat extract and CFCS of an AI-2 producer (*Salmonella* Typhimurium) were mixed, and the AI-2 activity bioassay was performed *(17)*. The $CFCS_{ST}$ was used as a positive control (50 μl of $CFCS_{ST}$ and 50 μl of AB medium).

The microplates were incubated at 30°C, and luminescence was measured every 30 min with a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT) until the negative control exhibited an increase in luminescence *(8)*. AI-2–like activity is expressed as relative AI-2–like activity, which was calculated as the ratio of luminescence of the test sample ($CFCS_{LAB}$ or CFME) to that of the control (negative) sample. The inhibition of the AI-2–like activity was expressed as a percentage of luminescence relative to the corresponding positive control: 100 − [(relative light unit of sample/relative light unit of positive control) × 100] *(17)*. All bioassays were conducted in triplicate.

Statistical analysis. Statistical analysis was performed with a nonparametric one-way analysis of variance. Differences among replicates were considered nonsignificant ($P > 0.05$).

RESULTS AND DISCUSSION

To our knowledge, no researchers have documented AI-2 production in LAB isolated from meat and/or meat products. In a few studies, the production of AI-2 signaling molecules was found in LAB isolated from milk, dairy products, and human or animal gastrointestinal tract. These LAB were probiotic strains of *Lactobacillus* (*L. rhamnosus* GG, *L. salivarious* UCC118, *L. acidophilus* NCFM, and *L. johnsonii* NCC533) isolated from human intestine or human feces *(20)*. Several strains of *L. rhamnosus* and *Lactobacillus casei* and strains *L. plantarum* NCIMB 8826 Int-1, *L. johnsonii* VPI 11088, and *Lactococcus lactis* MG1363 originally isolated from human gastrointestinal tract and/or dairy products also produce AI-2 molecules *(8)*. AI-2 signals also were produced by the pathogen *Streptococcus suis* serotype 2, which is commonly associated with disease in pigs and humans *(15)*.

Recent reports have associated meat spoilage with quorum sensing compounds *(1)*. Because LAB are considered the ephemeral and specific spoilage organisms that contribute to spoilage of modified-atmosphere-packaged meat products, the AI-2 signals have been proposed as potential compounds that may be involved directly or

indirectly with spoilage. In this study, 89 $CFCS_{LAB}$ and 13 CFME samples were tested for the production of AI-2–like activity and the presence of the AI-2–like signaling molecules, respectively. The AI-2 activity bioassay used relies on the ability of the *V. harveyi* BAA-1117 biosensor strain to produce light in response to AI-2. The tested $CFCS_{LAB}$ were collected from equal numbers of isolates (*Leuconostoc* spp., *Leuconostoc mesenteroides, Weissella viridescens, Leuconostoc citreum,* and *Lactobacillus sakei*) recovered from initial, middle, and final stage of minced beef storage. From those isolates, 15 fingerprints were obtained. Identical isolates were tested and verified for presence or absence of relative AI-2–like activity. The isolates exhibiting AI-2–like activity are shown in Table 1. The $CFCS_{LAB}$ extracted from the *Leuconostoc* sp. type B 233 isolate expressed AI-2–like activity ranging from 12.41- to 26.84-fold compared with the negative control. No significant differences ($P > 0.05$) in AI-2–like activity were found among these identical strains regardless of the stage of storage (initial, middle, and final) and the storage temperature of the minced meat. This AI-2–like activity may explain why these bacteria can survive at the last stages of storage. The *Leuconostoc* spp. (B 232 and B 240) and *L. mesenteroides* (B 243) strains also expressed AI-2–like activity (Table 1). Quantification of AI-2 signaling molecules was not possible because there is no linear relationship between luminescence values and AI-2 signaling molecule concentrations *(31)*. Eleven fingerprints assigned to *L. sakei* (B 222, B 227, B 236, B 237, B 238, B 239), *W. viridescens* (B 234 and B 235), *Leuconostoc* sp. (B 241), *L. citreum* (B 258), and *L. mesenteroides* (B 242) did not express detectable AI-2–like activity under standard growth conditions. The isolates were propagated under certain growth conditions to promote growth and the ability of the biosensor strain to detect AI-2. AI-2 production is affected by the growth medium and external environmental factors such as temperature *(7, 29),* and components of the culture medium may promote false-negative or false-positive results *(8)*. The *luxS* genes are subject to catabolic repression by glucose; consequently, AI-2 activity cannot be detected when cells with these genes are grown in the presence of glucose *(1)*. The *luxS* gene is responsible for the production of AI-2 signaling molecules and is present in the genomes of a wide variety of gram-negative and gram-positive bacteria *(13, 33)*. Various LAB, such as *L. mesenteroides, Lactobacillus gasseri*, *L. plantarum*, *Lactococcus lactis,* and *Leuconostoc oenos,* possess a *luxS* gene *(11)*. However, many gram-positive bacteria communicate via quorum sensing autoinducing peptides, which are not detected by the AI-2 biosensor strain *(27)*. Among LAB, some strains of *L. sakei* produce this category of signaling molecules, which induce bacteriocin (sakacin P) production *(5, 10, 19)*. The absence of an AI-2 production mechanism and/or the presence of autoinducing peptides in the tested isolates would explain the results reported in this study.

All the tested CFME samples had low AI-2–like activity ranging between 0.47 and 2.24 compared with the control (negative) sample (Table 2). The control sample was CFME from the 0-h minced beef sample, which had AI-2–like activity similar to that of CFME from a ''clean'' meat sample (obtained as previously described by Nychas et al. *(22)*) and sterile growth medium (data not shown). Similar results, i.e., very low levels of AI-2 activity (less than onefold induction of luminescence compared with the negative control), have been reported in a recent study with beefsteak, beef patties, chicken breast, and turkey patties, although the indigenous population loads in that study were high (6.4 to 8.0 log CFU/ml) *(17)*. The low AI-2 activity found in CFME in comparison with those from the LAB raises questions concerning the contribution of these compounds to growth of the specific LAB during meat storage and to the spoilage process. No evidence indicates that the LAB populations were related to AI-2 activity, a possible inhibitory effect of CFME should be considered. The CFME could have inhibited the ability of the biosensor strain to react to AI-2 activity, which was determined by mixing equal volumes of the CFCS of the AI-2-producing *Salmonella* Typhimurium strain with the CFME and performing the AI-2 activity bioassay. In this study, the inhibitory effect ranged from approximately 51.11 to 91.09% (Table 2). Comparable results also were reported previously, when meat matrices were tested for inhibition of AI-2–like activity. Beefsteak and beef patties produced high levels of inhibition, 90.6 and 84.4%, when indigenous bacterial populations were 7.4 and 6.4 log CFU/ml, respectively *(17)*. Various compounds from food matrices may lead to incorrect results and false conclusions *(17, 25)*. Previous findings suggest that the presence of fatty acids (linoleic acid, oleic acid, palmitic acid, and stearic acid) isolated from ground beef and poultry meat can inhibit AI-2 activity *(25, 32)*. Food additives such as sodium propionate, sodium benzoate, sodium acetate, and sodium nitrate also may influence AI-2 production *(17)*.

In this study, the majority of the LAB produced AI-2 activity. Among the 89 isolated LAB with 15 different fingerprints, e.g., B 232, B 233, B 240, and B 243, obtained by PFGE analysis *(9),* 76.4% (68) of the isolates produced AI-2–like activity. Although the LAB isolated at the same storage times and temperatures were identical and displayed similar activity patterns, the hypothesis that these signal compounds affect the dominance of these particular strains cannot be supported with confidence, and further data are needed. At chill temperatures (0 and 5°C), isolates with 11 different fingerprints were recovered *(9),* whereas at relative high temperatures (10 and 15°C) the strain diversity was reduced to 5 different fingerprints *(9)*. Two fingerprints, B 233 assigned to *Leuconostoc* sp. and B 237 assigned to *L. sakei,* were common among those isolates obtained at both chill and relative high temperatures. At the initial stage of storage (day 0), two *Leuconostoc* spp. strains (B 232 and B 233) were recovered, and both exhibited AI-2–like activity (Table 1). At 10 and 15°C, *Leuconostoc* sp. B 233 was the dominant strain, whereas at 0 and 5°C the same strain was prevalent in the initial and middle stages of storage. Forty-four (95.7%) of the tested LAB isolated at 10 and 15°C exhibited AI-2–like activity, whereas only 18 (48.6%) of the LAB isolated at 0 and 5°C displayed AI-2–like activity. Twenty-three (95.8%) and 21 (95.5%) isolates recovered

TABLE 1. *Representative lactic acid bacteria exhibiting AI-2–like activity at each storage period*

Temp (°C)	Storage period	No. of isolates	Strains exhibiting AI-2	No. of identical isolates exhibiting AI-2	AI-2–like activity of strains[a]
	Day 0, initial flora	6	*Leuconostoc* spp. (B 233)	5	25.90 ± 11.60 A
			Leuconostoc spp. (B 232)	1	2.23 ± 0.32 B
0	Initial	5	*Leuconostoc* spp. (B 233)	2	13.28 ± 1.79 A
	Middle	6	*Leuconostoc* spp. (B 233)	5	14.81 ± 1.32 A
	Final	5		0	
5	Initial	6	*Leuconostoc* spp. (B 233)	4	22.11 ± 2.13 A
	Middle	6	*Leuconostoc* spp. (B 233)	5	18.03 ± 0.85 A
	Final	9	*Leuconostoc* spp. (B 233)	2	13.86 ± 1.89 A
10	Initial	6	*Leuconostoc* spp. (B 233)	6	13.97 ± 4.73 A
	Middle	8	*Leuconostoc* spp. (B 233)	8	13.41 ± 1.58 A
	Final	10	*Leuconostoc* spp. (B 233)	8	12.41 ± 0.53 A
			L. mesenteroides (B 243)	1	3.24 ± 0.74 B
15	Initial	6	*Leuconostoc* spp. (B 233)	6	25.73 ± 10.73 A
	Middle	8	*Leuconostoc* spp. (B 233)	8	24.71 ± 9.41 A
	Final	8	*Leuconostoc* spp. (B 233)	6	26.84 ± 13.12 A
			Leuconostoc spp. (B 240)	1	3.01 ± 1.14 B
Total		89		68	

[a] AI-2–like activity was calculated as the ratio of the luminescence of the test sample ($CFCS_{LAB}$) to that of the control (negative) sample and is presented as the mean ± standard deviation ($n = 3$). Values with the same letter are not significantly different ($P > 0.05$).

from 10 and 15°C, respectively, were positive for AI-2–like activity. The isolates that exhibited positive response in the AI-2 activity bioassay were characterized as *Leuconostoc* spp. (B 233 and B 240) and *L. mesenteroides* (B 243), and those that did not exhibit AI-2–like activity were characterized as *W. viridescens* (B 234) and *L. sakei* (B 237) *(9)*. Seven (43.8%) and 11 (52.4%) of the LAB isolates recovered at 0 and 5°C, respectively, exhibited AI-2–like activity; those isolates were all identified as *Leuconostoc* sp. (B 233). The isolates that did not exhibit any light induction at chill temperatures belonged to 10 different fingerprints: *L. sakei* (B 226, B 227, B 236, B 237, B 238, B 239, and B 241), *L. mesenteroides* (B 242), *L. citreum* (B 258), and *W. viridescens* (B 235) *(9)*. These isolates were recovered mainly from the final stages of meat storage (Table 1), where only a small fraction of isolates recovered at 5°C produced luminescence.

Nychas et al. *(22)* reported the effect of CFME containing quorum sensing molecules on the kinetic parameters of gram-negative bacteria isolated from meat, suggesting that these signals may contribute at least to the physiological behavior of bacteria during the spoilage process. Considering the potential role of these molecules for modulating microbial persistence and growth, Soni et al. *(26)* reported that the presence of AI-2 molecules promoted the survival of *Escherichia coli* O157:H7 cells, whereas the protective effect of AI-2 molecules was negated in the presence of ground beef extracts that produced significant inhibitory activity. Nevertheless, data concerning the effect of AI-2 molecules on bacterial growth and their role in food

TABLE 2. *Relative CFME AI-2–like activity, bacterial counts, and inhibition of AI-2–like activity at each storage period*

Temp (°C)	Storage period	Relative AI-2–like activity of CFME[a]	Bacterial counts (log CFU/g)	% inhibition of AI-2–like activity[b]
	Day 0, initial flora		5.26 ± 0.13	89.50 ± 0.37
0	Initial	1.07 ± 0.43	5.10 ± 0.11	84.70 ± 0.04
	Middle	1.21 ± 0.30	6.31 ± 0.24	82.92 ± 4.47
	Final	1.24 ± 0.13	7.54 ± 0.11	85.35 ± 3.30
5	Initial	1.78 ± 1.23	5.60 ± 0.39	75.76 ± 2.03
	Middle	1.49 ± 0.12	6.74 ± 0.37	81.30 ± 2.88
	Final	1.00 ± 0.53	7.24 ± 0.08	91.09 ± 0.49
10	Initial	0.59 ± 0.12	5.97 ± 0.42	83.87 ± 4.31
	Middle	0.56 ± 0.18	7.02 ± 0.17	81.62 ± 4.89
	Final	0.47 ± 0.17	8.56 ± 0.15	51.11 ± 4.89
15	Initial	2.24 ± 1.22	6.86 ± 0.08	83.55 ± 1.48
	Middle	1.01 ± 0.54	7.17 ± 0.04	85.61 ± 2.98
	Final	1.69 ± 0.91	8.44 ± 0.01	78.45 ± 1.07

[a] Relative AI-2–like activity was calculated as the ratio of the luminescence of the test sample (CFME) to the control (negative) sample and is presented as mean ± standard deviation ($n = 3$).

[b] Inhibition of AI-2–like activity was expressed as a percentage relative to the activity of the corresponding positive control.

spoilage are scarce *(1)*. Further studies are needed to explore the possible effect of these molecules produced by the ephemeral spoilage organisms on the dominance of different bacterial strains during food storage and the probability that temperature strongly affects the expression of genes encoding molecules that produce AI-2 activity and thus affects the diversity of the LAB population.

ACKNOWLEDGMENT

This work was supported by the European Union project ProSafeBeef (Food-CT-2006-36241) within the 6th Framework Programme.

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