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N-Acyl-homoserine Lactones from *Enterobacter sakazakii* (*Cronobacter* spp.) and Their Degradation by *Bacillus cereus* Enzymes

Francisca Diana da Silva Araújo,[†] Luciana Maria Ramires Esper,[‡,§] Arnaldo Yoshiteru Kuaye,[‡] Marcelo Palma Sircili,[∥] and Anita Jocelyne Marsaioli*[,†]

[†]Chemistry Institute, University of Campinas, POB 6154, 13084-971, Campinas, SP, Brazil

[‡]Department of Food Technology, Faculty of Food Engineering, University of Campinas, POB 6121, Campinas, SP, Brazil

[∥]Laboratory of Bacteriology, Butantan Institute, 05503-900, São Paulo, SP, Brazil

Supporting Information

ABSTRACT: A chemical study of acyl-homoserine lactones (acyl-HSLs) produced by *Enterobacter sakazakii* resulted in the identification of three molecules: (*S*)-*N*-heptanoyl-HSL, (*S*)-*N*-dodecanoyl-HSL and (*S*)-*N*-tetradecanoyl-HSL. Mixed cultures of *E. sakazakii* and *Bacillus cereus* depleted *E. sakazakii* acyl-HSLs, suggesting acyl-HSL degradation by *B. cereus* hydrolases (hydrolysis of the lactone or amide moiety). The expression of *B. cereus* acyl-HSL lactonase and acyl-homoserine acylase was confirmed by monitoring the biotransformation of (*S*)-*N*-dodecanoyl-HSL into (*S*)-*N*-dodecanoyl-homoserine, dodecanoic acid and homoserine in the presence of *B. cereus* whole cells, using electrospray-mass spectrometry (ESI-MS).

KEYWORDS: *Enterobacter sakazakii (Cronobacter spp.), Bacillus cereus, N-acyl-homoserine lactones, foodborne diseases, enzymes*

INTRODUCTION

Enterobacter sakazakii (*Cronobacter* spp.), a Gram-negative bacteria of the Enterobacteriaceae family,[1,2] is an opportunistic pathogen associated with meningitis, necrotizing enterocolitis and sepsis in neonates and immunocompromised patients, consequently gaining the attention of public health authorities and researchers in different countries.[2−5]

Research concerning outbreaks of *E. sakazakii* infections in hospitals indicated powdered infant formula as a common contamination source, both in closed bottles or as reconstituted milk.[2−5] The presence of *E. sakazakii* in milk processing equipment and utensils may be due to its ability to adhere and form biofilms on many surfaces, including silicone, latex, polycarbonate (used in the bottle manufacture) and stainless steel.[2,4]

Bacterial processes involved in biofilm formation, virulence and bioluminescence, among other phenotypic expressions, are mediated by quorum sensing, a mechanism based on the production, release and detection of signaling molecules of low molar mass, the autoinducers (AI). Extracellular concentrations of AI are related to the microorganism population density, and, upon reaching a concentration threshold, these signaling molecules are detected by the cells initiating a coordinated action.[6−8]

Quorum sensing mechanisms are at the basis of bacterial contamination in the food industry and in hospitals. Among others, these include acyl-homoserine lactones (acyl-HSL) (AI-1) in Gram-negative bacteria, oligopeptides in Gram-positive bacteria, autoinductor-2 (AI-2) in Gram-positive and Gram-negative interspecies communication, butyrolactones in *Streptomyces*, fatty acids, usually classified as diffusible signal factors, in *Xanthomonas fastidiosa*, diketopiperazines in *Proteus mirabilis*, and AI-3/epinefrin/norepinefrin, which is present in several bacterial species (e.g., *Erwinia carotovora*, *Chromobacterium violaceum* and *Ralstonia solacearum*).[7−9]

Lehner et al.[4] investigated the ability of *E. sakazakii* to produce acyl-HSL using thin layer chromatography and detected two molecules: 3-oxo-hexanoyl-HSL and 3-oxo-octanoyl-HSL. Pinto et al.[10] also detected short chain acyl-HSL in *E. sakazakii*, through bioassays with *C. violaceum* CV026. However, isolation and full chemical characterization of these molecules are missing.

Interference in quorum sensing mechanisms, known as quorum quenching, involves, among other strategies, degradation of acyl-HSL molecules, decreasing the concentration of these molecules in the immediate vicinity of the bacterial cells, and has been proposed as a promising alternative to control bacterial virulence.[11−13]

Quorum quenching by acyl-HSL enzymatic degradation can be promoted by acyl-HSL acylases that cleave the acyl chain to produce homoserine lactone and fatty acids and by acyl-HSL lactonases that cleave the lactone ring.[11−13] Acyl-HSL degradation activity was initially discovered in species of *Bacillus* sp.[14] and *Variovorax paradoxus*[15] but is relatively widespread among diverse bacteria.

Bacillus cereus, a Gram-positive bacterium and member of the Bacillaceae family, is a microorganism that produces acyl-HSL lactonase and peptidases.[16−19] This bacterium is responsible for two types of food poisoning, the emetic and the diarrheal syndromes, and a variety of local and systemic infections, such as meningitis.[20,21] Similar to *E. sakazakii*, *B. cereus* also affects milk quality and its derivatives,[22] mainly due to its ability to

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form biofilms and spores that are heat-resistant, surviving food processing and remaining for long periods in dried foods.[23] Both the genus *Bacillus* and the genus *Enterobacter* occur in dairy environments and in pasteurized liquid milk products and are responsible for food spoilage together with other microorganisms coexisting in biofilms.[24,25] The existence of multiple species biofilms has motivated the present investigation on cell-to-cell communication via the acyl-HSL of a Gram-negative pathogenic bacterium (*E. sakazakii*) in the presence of a cooccurring Gram-positive bacterium (*B. cereus*).

MATERIALS AND METHODS

General Experimental Procedures. All solvents were treated with anhydrous Na_2SO_4 and distilled. The reagents were from Aldrich, Sigma and Merck. Column chromatography purifications were carried out with silica gel (Acros, 0.035−0.070 mm) and thin layer chromatographic analyses with silica gel 60 F254 plates (Merck), visualized with UV light (254 nm) or sprayed with a solution of $KMnO_4$ and *p*-anisaldehyde (5%), acetic acid (50 mL), and concentrated sulfuric acid (1 mL) and heated to ∼100 °C with a heat gun.

GC−MS analyses. GC−MS analyses (70 eV) were carried out with an Agilent 6890 chromatograph coupled to a Hewlett-Packard 5973 mass detector, equipped with a 30 m × 0.25 mm × 0.25 μm i.d. DB5 fused silica capillary column. Mass spectra were recorded over the 40−600 amu range at 3.54 scans/s. Helium was the carrier gas at a flow rate of 1 mL/min. The injector temperature was kept at 250 °C. The initial oven temperature was 100 °C, increasing at 10 °C/min to 290 °C, then held for 10 min. Samples (1 mg/mL) of 1 μL were injected using a 10:1 split ratio.

Chiral GC-FID Analyses. Chiral GC-FID analyses were carried out with an Agilent 6890 chromatograph with a 25.0 m × 0.25 mm × 0.25 μm chiral capillary column (Chrompack CP Chirasil cyclodextrin CB coating 7502). High purity hydrogen was the carrier gas at a flow rate of 1 mL/min. Samples of 1 μL were injected, with a 1:100 split ratio. For *N*-heptanoyl-HSL the temperature program was 50−180 °C (2 °C/min), held for 5 min at 180 °C, with the detector at 250 °C and the injector at 220 °C. For *N*-dodecanoyl-HSL the isothermal temperature was 180 °C for 150 min, the detector was at 240 °C and the injector was at 280 °C. For *N*-tetradecanoyl-HSL the isothermal temperature was 180 °C for 200 min, the detector was at 220 °C and the injector was at 240 °C.

ESI-MS Analyses. All experiments were performed using a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany). Nitrogen gas was used for nebulization, desolvation and collision-induced dissociation (CID). The samples were diluted in methanol, adding 0.1% of ammonium hydroxide for the negative mode, and submitted to direct injection at atmospheric pressure and at a flow rate of 10 μL/min. The positive ionization mode was best to detect the (*S*)-*N*-dodecanoyl-HSL, sheat gas = 8, spray voltage = 3.5 kV, capillary voltage = 43 V, capillary temperature = 275 °C. The negative ionization mode was more appropriate to monitor the degradation of acyl-HSL, with sheat gas = 10, spray voltage = 3.8 kV, capillary voltage at −38 V and capillary temperature 275 °C. Full scan experiments (range m/z 50−800) were performed in both the linear trap as well as the Orbitrap. Masses were acquired as profile data at a resolution of 30 000 at m/z 400. The automatic gain control (AGC) ion population target in full scan MS was 30 000 for LTQ-MS and 300 000 for Orbitrap-MS, and the ion population target for MS*n* was set to 10 000 for LTQ-MS. Mass data were analyzed by Xcalibur 2.0.5 software (Thermo Electron Corporation).

Bacteria Strains and Cultivation Media. *E. sakazakii* DUP 18623 was isolated from infant feeding bottles from a hospital nursery. *B. cereus* ATCC 14579 was obtained from the Laboratory of Hygiene and Legislation of the State University of Campinas (UNICAMP). Both strains were kept in Eppendorf tubes containing BHI (brain heart infusion) and glycerol (80:20) at −80 °C. *B. cereus* CCT 4060 was purchased from the André Tosello Culture Collection, Campinas, Brazil, and was kept in Luria−Bertani medium (LB) adding 1% peptone (Oxoid), 0.5% NaCl (Sigma) and 0.5% yeast extract (Oxoid) to 100 mL of sterile water.

Agrobacterium tumefaciens NTL4(pZLR4) was kept in LB medium plus gentamicine (50 μg/mL). *C. violaceum* CV026 strain also was grown in LB medium supplemented with 20 μL/mL kanamycin at 25 °C for 24 h. Solid media were prepared with 2% bacteriological agar (Sigma).

Bioassays with *Agrobacterium tumefaciens* NTL4(pZLR4). *E. sakazakii*, *B. cereus* ATCC 14579 and mixed cultures of *E. sakazakii* and *B. cereus* ATCC 14579 were grown in slants and inoculated in 2 mL of LB liquid medium in a BOD (biochemical oxygen demand) incubator at 28 °C for 24 h. Cultivation media with the pure strains of microorganisms and in mixed cultures were maintained at pH 7.0 in all experiments. Assays were performed in test tubes. Test tube 1 (negative control): 20 μL of *A. tumefaciens* NTL4(pZLR4), 20 μL of X-Gal (50 mg/mL in DMF), 2 mL of LB. Test tube 2: 20 μL of *A. tumefaciens* NTL4(pZLR4), 20 μL of *E. sakazakii*, 20 μL of X-Gal, 2 mL of LB medium. Test tube 3: 20 μL of *A. tumefaciens* NTL4(pZLR4), 20 μL of *B. cereus* ATCC 14597, 20 μL of X-Gal, 2 mL of LB medium. Test tube 4: 20 μL of *A. tumefaciens* NTL4(pZLR4), 20 μL of *E. sakazakii* + *B. cereus* ATCC 14597, 20 μL of X-Gal, 2 mL of LB medium. All experiments were performed in duplicate (two different batches). The test tubes were incubated in BOD at 30 °C, and were visually evaluated after 24 h.[26,27]

General Procedures for Extract Preparations. *E. sakazakii*, *B. cereus* ATCC 14579 and a mixed culture (*E. sakazakii* and *B. cereus* ATCC 14579) in 10 mL of LB were kept in a BOD at 30 °C for 24 h. These were then transferred to separate Erlenmeyers (2 L) containing liquid LB (1 L) and incubated at 30 °C for 24 h under stirring. After this time the mixtures were centrifuged at 5000 rpm for 20 min and NaCl was added to the aqueous solution that was then extracted with ethyl acetate (3 × 300 mL). The combined organic phases were dried over anhydrous magnesium sulfate and filtered, and the solvent was evaporated under reduced pressure, producing crude extracts of *E. sakazakii* (87.52 mg), *B. cereus* ATCC 14579 and mixed cultures of *E. sakazakii* and *B. cereus* ATCC 14579 (89.15 mg). A control experiment was set with sterile LB culture medium (1 L) which was extracted with ethyl acetate as described above, producing 80.50 mg. The microorganism culture extracts were dissolved in ethanol (2 mg/mL), and 20 μL of each solution was used in the bioassays with *A. tumefaciens* NTL4(pZLR4) to detect the expression of β-galactosidase.

Purification of the Acyl-HSLs of *E. sakazakii* and Mixed Culture Extracts. Cultivation and extraction with ethyl acetate was repeated eight times yielding 0.70 g (*E. sakazakii*) and 0.71 g (*E. sakazakii* + *B. cereus* ATCC 14579) of crude extracts. These extracts were fractionated by silica column chromatography (15 g of silica, 2 cm column diameter, particle size 0.035 to 0.070 mm), eluting with hexane, ethyl acetate, methanol and mixtures of these solvents of gradually increasing polarity. All fractions (10 mL) were monitored by TLC. Additionally the fractions were dissolved in ethanol (2 mg/mL) and monitored with *A. tumefaciens* NTL4(pZLR4) and *C. violaceum* CV026 biosensors. These bioassays were performed by adding 20 μL of a cell suspension of the biosensor to 20 μL of solution in test tubes containing 2 mL of LB medium, which were incubated at 25 °C for 24 h. A control experiment for *C. violaceum* CV026 biosensor was performed by adding 20 μL of cell suspension, grown for 24 h, to 20 μL of ethanolic solution of the synthetic (*S*)-*N*-heptanoyl-HSL solution (2 mg/mL). The development of a violet color indicated the presence of short chain acyl-HSL. The control experiment for *A. tumefaciens* NTL4(pZLR4) biosensor was performed by adding 20 μL of cell suspension grown for 24 h to 20 μL of synthetic (*S*)-*N*-dodecanoyl-HSL solution (2 mg/mL). The development of a blue-green color indicated the presence of acyl-HSL.

Coinjection of Synthetic Standards with Natural Products. Structures of acyl-HSLs detected in the fractions of the *E. sakazakii* crude extract by GC−MS were confirmed by coinjections with synthetic standards previously synthesized and fully characterized in our laboratory.[28,29]

Determination of the Absolute Configuration of Acyl-HSLs. The absolute configuration of the natural acyl-HSLs was determined

by GC-FID equipped with a Chrompack Chirasil cyclodextrin column, applying the chromatographical conditions previously described, to discriminate both enantiomers of the racemic synthetic and (*S*) enantiomer standards: 66.54 min = (*R*)-*N*-heptanoyl-HSL, 66.75 min = (*S*)-*N*-heptanoyl-HSL;[28] 77.49 min = (*R*)-*N*-dodecanoyl-HSL, 78.75 min = (*S*)-*N*-dodecanoyl-HSL;[29] and 169.81 min = (*R*)-*N*-tetradecanoyl-HSL, 172.60 min = (*S*)-*N*-tetradecanoyl-HSL.[29] The natural product was determined by injecting the fractions and standards using the same conditions and by coinjecting with synthetic racemic product.

(*S*)-*N*-Dodecanoyl-HSL Biotransformation by *B. cereus* ATCC 14579 and CCT 4060. *B. cereus* ATCC 14579 was inoculated into test tubes containing 10 mL of LB liquid medium and kept in a BOD at 30 °C for 24 h. After the incubation period, the test tubes were centrifuged at 5000 rpm for 20 min to separate the cells. To 50 mL Falcon tubes were added sterile phosphate buffer (5 mL, pH 7.0), wet cells (100 mg) and (*S*)-*N*-dodecanoyl-HSL (1 mg dissolved in ethanol). The bioreaction cell-free solution (filtrated) was monitored for 8 h by ESI-MS−MS in the negative mode, but there was signal suppression (sample D). Therefore, bioreactions using two different strains of *B. cereus* (ATCC 14579 and CCT 4060) were monitored for three hours and the resulting suspensions were stirred at 220 rpm and 29 °C for 1 h and then extracted with ethyl acetate (3 × 5 mL). The combined organic phases were dried over anhydrous magnesium sulfate, filtered and the solvent was evaporated under reduced pressure (sample E). Control experiments were done with sterile phosphate buffer (5 mL) and wet cells (100 mg) (control 1) and with sterile phosphate buffer (5 mL) and substrate (1 mg dissolved in ethanol) (control 2), and were extracted with ethyl acetate as described above. The sample E and control experiments were analyzed by electrospray ionization mass spectrometry (ESI-MS) and also monitored by TLC. The same procedure was repeated with *B. cereus* CCT 4060.

Samples for ESI-MS Analyses. Metanolic solutions A, B and C containing homoserine lactone, (*S*)-*N*-dodecanoyl-homoserine lactone and dodecanoic acid were prepared (Table 1) to optimize the ESI-MS analyses and MS^2 (fragmentation pattern, daughter ions). Sample D was prepared by adding 500 μL of methanol to 500 μL of the biotransformation reaction liquid medium (filtered, details in the biotransformation reaction section). The resulting solution was split into two samples of 500 μL for the negative and positive detection modes. Sample E and the controls were prepared by adding 10 μL of a stock solution (1 mg of ethyl acetate extract of the biotransformation reaction, in 1 mL of methanol) to 100 μL of methanol (details in the biotransformation reaction section).

Table 1. Samples Used in ESI-MS Analyses and MS^2 Optimization[a]

sample	HSL[b]	2[c]	5[d]
A	422.48	271.33	383.88
B	211.30	678.56	
C	1026.65		192.00

[a]Values in μmol/L. [b]Homoserine lactone. [c](*S*)-*N*-Dodecanoyl-homoserine lactone. [d]Dodecanoic acid.

RESULTS AND DISCUSSION

Detection of Acyl-HSL in *E. sakazakii*. The *E. sakazakii* cell suspension furnished a blue-green solution in the presence biosensor *A. tumefaciens* NTL4(pZLR4) and X-gal (Figure 1a). This color suggested that *A. tumefaciens* NTL4(pZLR4) detected *E. sakazakii* acyl-HSL by expressing a phenotypic galactosidase which hydrolyzed the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) added to the reaction, yielding the indigo blue derivative.[27,30] Since naturally occurring *E. sakazakii* β-galactosidase could hydrolyze X-gal and produce a false positive result, *A. tumefaciens* NTL4(pZLR4) biosensor was employed with ethyl acetate extracts of *E. sakazakii*, carrying

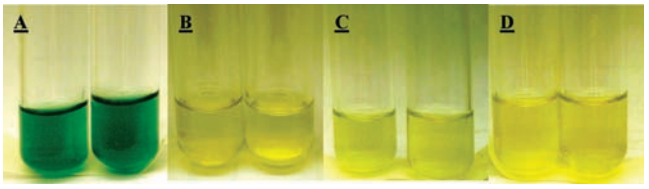

Figure 1. Bioassays with *A. tumefaciens* NTL4(pZLR4). (A) *E. sakazakii* cell suspension. (B) *B. cereus* ATCC 14579 cell suspension. (C) *E. sakazakii* and *B. cereus* ATCC 14579 mixed cell suspension. (D) Negative control. Assays in duplicate (two different batches). Blue-green color reveals the expression of galactosidase due to the presence of acyl-HSL. This enzyme hydrolyzes X-Gal added to the reaction medium producing a colored compound.[30] Yellow is the color of the natural medium.

no enzymes, yielding a blue-green color in the culture medium after 24 h, confirming the presence of acyl-HSL, which is consistent with the results employing cell suspensions (Figure 2a). Negative control experiments with *A. tumefaciens* NTL4(pZLR4)

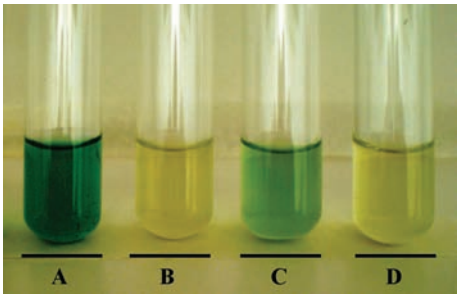

Figure 2. Bioassays with *A. tumefaciens* NTL4(pZLR4). (A) (*S*)-dodecanoyl-HSL (positive control). (B) Negative control. (C) *E. sakazakii* ethyl acetate extract. (D) *E. sakazakii* and *B. cereus* ATCC 14579 ethyl acetate extract. Assays in duplicate (two different batches). Blue-green color reveals the expression of galactosidase due to the presence of acyl-HSL. This enzyme hydrolyzes X-Gal added to the reaction medium producing a colored compound.[30] Yellow is the color of the natural medium.

were performed with cell suspensions in LB medium containing X-gal, and the blue-green color was not observed.

Notwithstanding this evidence there are limitations, and one must be cautious in the interpretation of data based on acyl-HSL biosensors. Therefore the isolation and identification of the acyl-HSLs produced by *E. sakazakii* was undertaken.

Identification of *E. sakazakii* Acyl-HSLs. Fractions F50−57, eluted with hexane/ethyl acetate (50%), and F58−64, eluted with pure ethyl acetate, produced positive response with *A. tumefaciens* NTL4(pZLR4). Additionally fractions F50 and F56−62 responded to *C. violaceum* CV026, indicating the presence of short chain acyl-HSL.

The bioactive fractions were analyzed by GC−MS, monitoring acyl-HSL characteristic fragments at *m/z* 185, 143, 128, 102, 101, 100 and 85. *N*-Heptanoyl-HSL (**1**) (Figure 3) ($M^{+\bullet}$ *m/z* 213) was detected in fractions F56−F62 (Figure 1S of the Supporting Information). An unsubstituted lateral chain was suggested by the decreasing relative abundances of fragments $-CH_2CH_2CH_3$, at *m/z* 156 (13%), and $-CH_2CH_3$, at *m/z* 170 (4%). The base peak at *m/z* 143 was characteristic of the acyl-HSL odd electron cation fragment formed during the McLafferty rearrangement. *N*-dodecanoyl-HSL (**2**) (Figure 3) ($M^{+\bullet}$ *m/z* 283) was detected in fractions F56−F62 (Figure 2S

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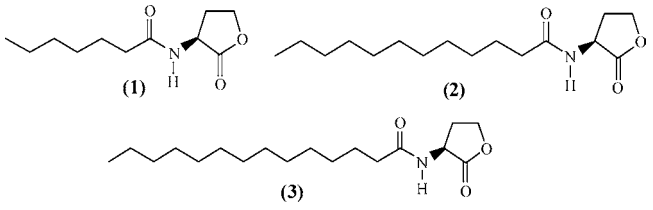

Figure 3. (*S*)-*N*-Acyl-homoserine lactones identified in *E. sakazakii* (*Cronobacter* spp.) extracts: (*S*)-*N*-heptanoyl-HSL (**1**), (*S*)-*N*-dodecanoyl-HSL (**2**) and (*S*)-*N*-tetradecanoyl-HSL (**3**).

of the Supporting Information), and *N*-tetradecanoyl-HSL (**3**) (Figure 3) ($M^{+\bullet}$ m/z 311) was present in F62−F63 (Figure 3S of the Supporting Information), both displaying the acyl-HSL characteristic fragmentation pattern at m/z 143.[27] The detected compounds were coinjected with synthetic standards, revealing perfect chromatographic and mass fragmentation pattern matchings (Figures 4S−6S of the Supporting Information).

Acyl-HSLs Absolute Configuration. The absolute configuration of the acyl-HSL produced by *E. sakazakii (Cronobacter* spp.*)* was determined using a gas chromatography equipped with a Chrompack chiral column (Chirasil cyclodextrin CB) and a flame ionization detector. Racemic (±)-*N*-heptanoyl-HSL standard was used to optimize the analytical conditions to have a good enantiomeric resolution where (*R*) eluted in 66.54 min and (*S*) eluted in 66.74 min. Synthetic (*S*)-*N*-heptanoyl-HSL eluted in 66.78 min, and this was close to the elution time of the *N*-heptanoyl-HSL in fraction F59 (66.73 min). A coinjection of the natural product with the racemic synthetic standard produced an enhancement of the relative abundance of the (*S*) (66.73 min) peak in relation to the (*R*) (66.53 min) enantiomer, confirming the presence of (*S*)-*N*-heptanoyl-HSL. The (*S*) configuration of the *N*-dodecanoyl-HSL in F63 and *N*-tetradecanoyl-HSL in F55 was also confirmed (Figure 7S of the Supporting Information).

Detection of Acyl-HSL in Mixed Cultivation of *E. sakazakii* and *B. cereus* ATCC 14579. A negative response from *A. tumefaciens* NTL4(pZLR4) biosensor was obtained with the *B. cereus* ATCC 14579 cell suspension (Figure 1b), a Gram-positive bacterium employing cell-to-cell chemical communication different from acyl-HSL. This biosensor had an analogous negative response with the mixed cell suspension and ethyl acetate extracts of both *B. cereus* and *E. sakazakii* (Figures 1c and 2d). These results were interpreted as depletion of the *E. sakazaki* acyl-HSLs by *B. cereus* enzymes. To confirm this hypothesis we have searched for acyl-HSL in the ethyl acetate extract of the *E. sakazakii* and *B. cereus* ATCC 14579 mixed culture and investigated hydrolase activity in different strains of *B. cereus*.

A dereplication strategy was applied to the chromatographic fractions with *A. tumefaciens* NTL4(pZLR4) and *C. violaceum* CV026 revealing that fraction M42 had a positive response with *A. tumefaciens* NTL4(pZLR4) (Figure 8S of the Supporting Information). The GC−MS analysis in the SIM mode (single ion monitoring, m/z 143) revealed the presence of trace amounts of *N*-dodecanoyl-HSL (**2**) (Figure 9S of the Supporting Information), confirming that most acyl-HSL were depleted by *B. cereus* ATCC 14579. This degradation phenomenon of Gram-negative bacterium quorum signal molecules, acyl-HSLs, by *B. cereus* strains of (Gram-positive) producing acyl-HSL lactonases was already reported in the literature for *Erwinia carotovora* and *Yersinia enterocolitica*.[19] However acyl-HSL degradation could follow an *N*-acyl bond hydrolysis, thus demanding a better investigation of the acyl-HSL depletion mechanism.

Acyl-HSL Depletion Mechanism by *B. cereus* Enzymes. To detect such enzymatic reactions we first selected and optimized the ESI-MS−MS spectrometry methodology by using a mixture of acyl-HSL, a fatty acid and homoserine in different concentrations (Table 1). The homoserine and acyl-HSL were best detected in the positive mode at 422.48 and 271.33 μmol/L levels. However, the fatty acid and, consequently, the opened acyl-HSL (this standard was not available) were best detected in the negative mode at 383.88 μmol/L. The bioreaction to detect acyl-HSL lactonase and/or acyl-HSL acylase activities in *B. cereus* to explain their degradation in mixed cultivation of *E. sakasaki* and *B. cereus* was set with (*S*)-*N*-dodecanoyl-HSL and *B. cereus* cells in buffer solution. The bioreaction cell-free solution was monitored by ESI-MS−MS in the negative mode for 8 h but (*S*)-*N*-dodecanoyl-HSL (**2**), *N*-dodecanoyl homoserine (**4**), dodecanoic acid (**5**) and homoserine (**6**) (Figure 4b) were not detected due to signal suppression. Consequently bioreactions using two different strains of *B. cereus* (ATCC 14579 and CCT 4060) were monitored for three hours, being extracted with ethyl acetate prior to negative mode ESI-MS−MS analyses.

The bioreaction with *B. cereus* CCT 4060 and (*S*)-*N*-dodecanoyl-HSL (**2**) produced *N*-dodecanoyl homoserine (**4**), suggested by the presence of the quasi-molecular ion (M − H) at m/z 300.2183 (Figure 4b), indicating that 18 amu had been added to the (*S*)-*N*-dodecanoyl-HSL by enzymatic action. This is in agreement with *N*-dodecanoyl-homoserine (**4**), confirming the presence of acyl-HSL lactonase (Figure 4c).

The MS^2 fragmentation of the quasi-molecular ion m/z 300.2183 produced ions at m/z 282.2068, characteristic of loss of a water molecule, at m/z 256.2275, assigned to the loss of CO_2, at m/z 198.1857, assigned to N−C bond cleavage, and at m/z 118.0504, corresponding to the loss of the homoserine moiety (Figure 5a).

Additionally the quasi-molecular ions at m/z 199.1702 and 118.0507 revealed the formation of dodecanoic acid (**5**) and homoserine (**6**), respectively, which are the expected products of an acyl-homoserine acylase on *N*-dodecanoyl-homoserine (**4**) (Figure 4c). These compounds were not detected in the ethyl acetate extracts of the control reactions with and without *B. cereus* CCT 4060 cells in phosphate buffer (Figure 4a and Figure 10S of the Supporting Information) The MS^2 fragmentation of the ion at m/z 118.0507 produced the ion at m/z 100.0401 by loss of a water molecule (Figure 5c). These results are also in agreement with the presence of an acyl-homoserine acylase (Figure 4c).

Furthermore the genes coding for acyl-HSL inactivating enzymes were detected and studied,[18] showing that they are present in various microorganisms; moreover, mining sequenced genome databases revealed organisms possessing conserved domains for acyl-HSL lactonases and acylases (*Deinococcus, Streptomyces, Hyphomonas, Photorhabdus* and *Ralstonia*), however, *Bacillus* showed domain only for acyl-HSL lactonase.[31] The acylase activity has never been reported in *Bacillus* before, but, based on enzymatic promiscuity,[32] that refers to one enzyme accepting more than one substrate, and on the fact that peptidases were detected in *B. cereus* genome,[16] our results are novel but not unexpected.

Finally, in *B. cereus* ATCC 14579 acyl-HSL lactonase was detected, however, no acyl-homoserine acylase activity was observed.

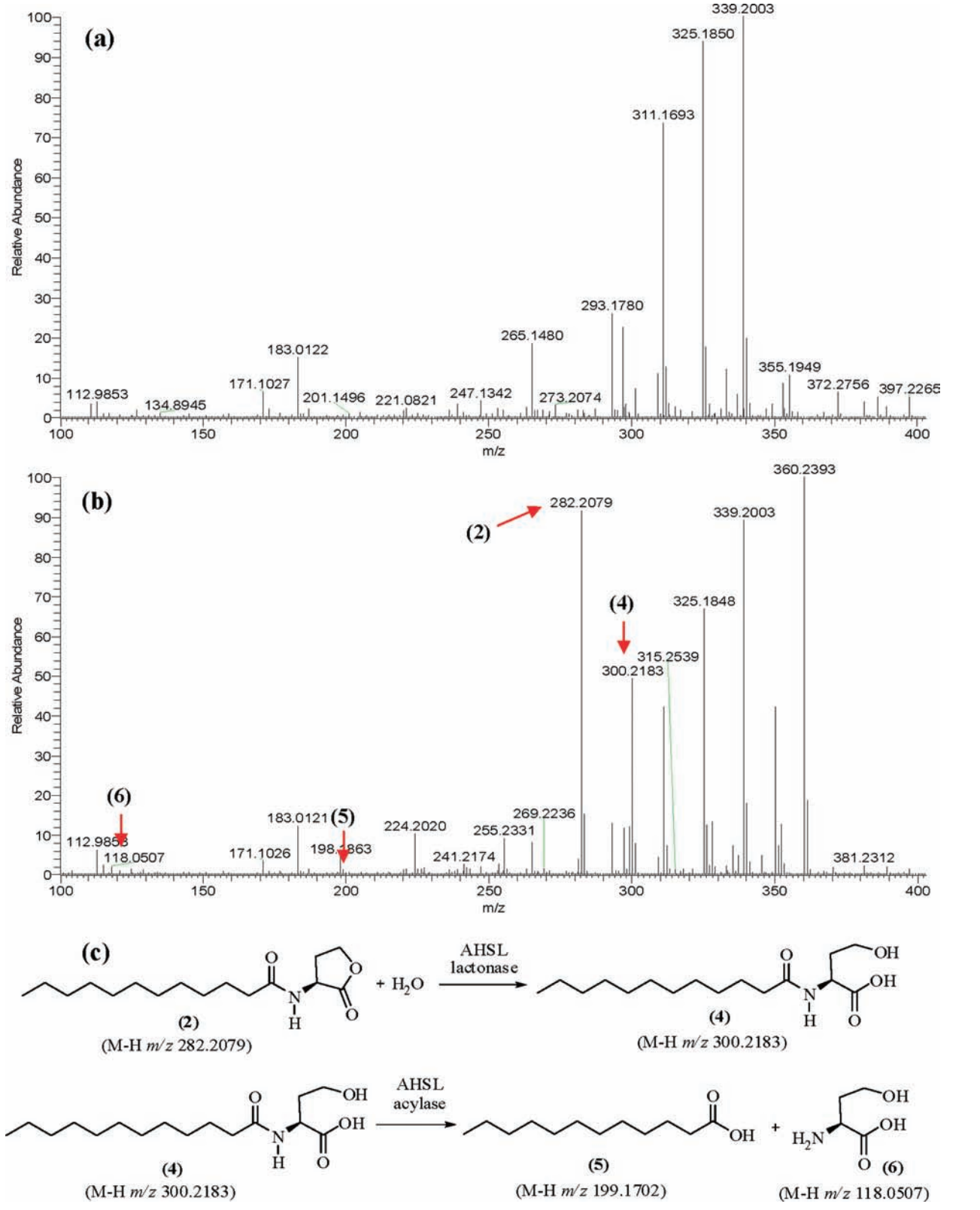

Figure 4. Bioreaction monitoring with ESI-MS. (a) Spectrum of control experiment (ethyl acetate extract of sterile phosphate buffer and wet cells). (b) Spectrum and compounds **2**, **4**, **5** and **6** indicated with arrows. (c) Schematic origin of **4**, **5** and **6**.

In conclusion, this work revealed that the *E. sakazakii* strain produces homoserine lactones (*S*)-*N*-heptanoyl-HSL, (*S*)-*N*-dodecanoyl-HSL and (*S*)-*N*-tetradecanoyl-HSL, results not before reported in the literature. The identification and characterization of these structures was the first important step, however, further research will be necessary to identify the functions regulated by these molecules in *E. sakazakii*.

The present work also provides evidence of acyl-HSL-degrading capacities during the cocultivation of a Gram-negative bacterium (*E. sakazakii*) with a Gram-positive bacterium

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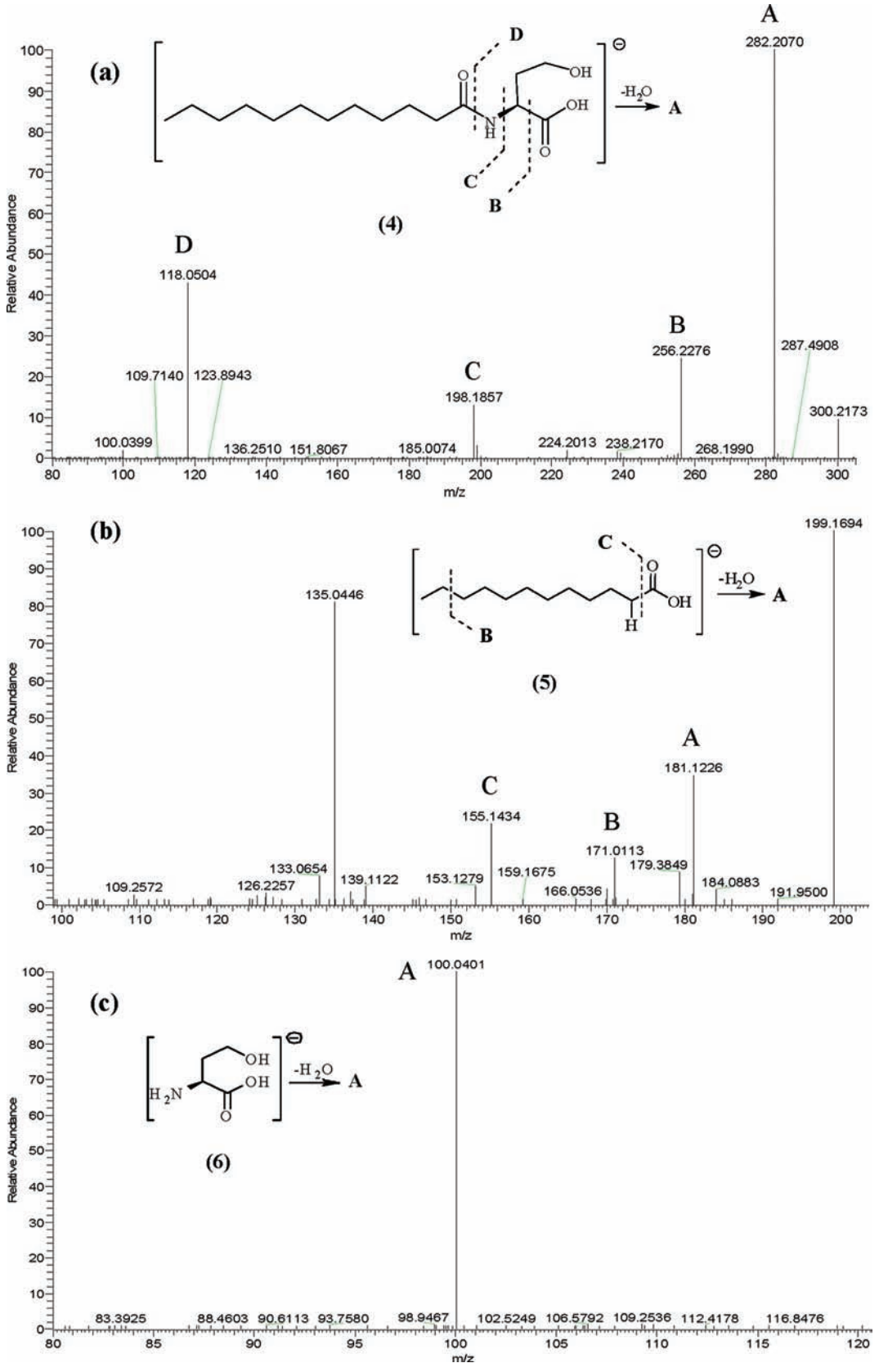

Figure 5. (a) MS^2 of fragment at m/z 300.2183; (b) MS^2 of fragment at m/z 199.1702 and (c) MS^2 of fragment at m/z 118.0507.

(*B. cereus*). This phenomenon was further investigated revealing that a cascade of enzymatic reactions with an acyl-HSL lactonase and acyl-homoserine acylase (or peptidase) in synchronized operation are responsible for degradation by *B. cereus*, leading to a disruption of the acyl-HSL communication system.

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■ ASSOCIATED CONTENT

Supporting Information

Additional chromatograms and mass spectra, and bioassay with *A. tumefaciens* NTL4(pZLR4) for *E. sakazakii* and *B. cereus* ATCC 14579 mixed culture extract fractions (Figures 1S–9S). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +55 19 35213067. Fax: +55 19 35213023. E-mail: anita@iqm.unicamp.br.

Present Address

[§]Department of Bromatology, Faculty of Pharmacy, Fluminense Federal University, Niteroi, RJ, Brazil.

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■ ABBREVIATIONS USED

HSL, homoserine lactone; X-gal, 5-bromine-4-chloro-3-indolyl-β-D-galactopyranoside; BOD, biochemical oxygen demand

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