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A Food-Grade System for Inducible Gene Expression in *Lactobacillus plantarum* Using an Alanine Racemase-Encoding Selection Marker

Tien-Thanh Nguyen,[†,‡] Geir Mathiesen,[§] Lasse Fredriksen,[§] Roman Kittl,[†] Thu-Ha Nguyen,[∥] Vincent G. H. Eijsink,[§] Dietmar Haltrich,[†] and Clemens K. Peterbauer*[,†]

[†]Food Biotechnology Laboratory, Department of Food Sciences and Technology, University of Natural Resources and Life Sciences, Vienna, Austria

[§]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway

[‡]Food Technology Laboratory, School of Biotechnology and Food Technology, Hanoi University of Science and Technology, Hanoi, Vietnam

[∥]Research Centre Applied Biocatalysis, Graz, Austria

ABSTRACT: Food-grade gene expression systems for lactic acid bacteria are useful for applications in the food industry. We describe a new food-grade host/vector system for *Lactobacillus plantarum* based on pSIP expression vectors and the use of the homologous alanine racemase gene (*alr*) as selection marker. A new series of expression vectors were constructed by exchanging the erythromycin resistance gene (*erm*) in pSIP vectors by the *L. plantarum* WCFS1 *alr* gene. The vectors were applied for the overexpression of β-galactosidase genes from *L. reuteri* L103 and *L. plantarum* WCFS1 in an *alr* deletion mutant of *L. plantarum* WCFS1. The expression levels obtained in this way, i.e. without the use of antibiotics, were comparable to the levels obtained with the conventional system based on selection for erythromycin resistance. The new system is suitable for the production of ingredients and additives for the food industry.

KEYWORDS: inducible expression system of protein, pSIP, β-galactosidase, food-grade, alanine racemase, *Lactobacillus*

INTRODUCTION

Bacteria from the genera *Lactococcus* and *Lactobacillus* have considerable potential as safe cell factories of ingredients for food applications.[1,2] Several gene expression systems have been developed based on genes and promoters involving sugar utilization, heat shock resistance and bacteriocin production.[3−6] Two well-known inducible expression systems developed for lactic acid bacteria (LAB) are based on quorum-sensing mechanisms involved in regulation of bacteriocin production. These systems are derived from the genes involved in the production of nisin in *Lactococcus lactis*[6] or sakacin A and P (pSIP vectors) in *Lactobacillus sakei*.[7,8] The pSIP vectors have been used to express high amounts of heterologous proteins such as β-glucuronidase, aminopeptidase, amylase and β-galactosidases in lactobacilli.[7−11] However, due to the use of an erythromycin antibiotic resistance gene (*erm*) as selection marker, the potential of the pSIP system for food applications has been limited.

Food-grade selection markers can be classified as dominant markers or complementation markers.[3] Dominant markers usually confer a new ability to the host strain such as bacteriocin immunity/resistance,[12−14] heat-shock resistance,[15] or sugar utilization abilities.[16,17] Dominant markers have the advantage that they potentially may be used in a wide variety of strains, but the number of suitable food-grade markers is limited.[3] Furthermore, these markers have certain potential disadvantages such as a narrow spectrum of application (e.g. bacteriocin immunity genes[12,13]), necessary manipulation of the growth medium (carbohydrate source, bacteriocins) or the necessity to use unfavorable cultivation conditions for the host strain (e.g., elevated temperature,[15]). Selection markers based on complementation do not require supplements in the cultivation medium; their disadvantage is that they require the use of specially adapted host strains.

In order to develop a food-grade complementation-based host/marker system, a gene on the host chromosome is mutated or deleted, and a wild type copy is inserted into the expression vector. A gene which is chosen as complementation marker usually has a decisive influence on the growth of host strain. Examples of such genes include the thymidylate synthase gene (*thyA*),[18] and genes involved in lactose conversion, such as lactose phosphotransferase (*lacF*)[19,20] or phospho-β-galactosidase (*lacG*).[21] The enzyme alanine racemase converts L-alanine to D-alanine, which is crucial for cell wall biosynthesis, and is thus an essential enzyme for growth of prokaryotic cells.[22] In lactococci and lactobacilli, alanine racemase activity is encoded by a single gene, *alr*.[22,23] D-Alanine is not a common ingredient of large-scale fermentation media, and previous studies have shown that the *alr* gene has considerable potential as a food-grade selection marker in lactic acid bacteria.[24]

The goal of the present study was to develop a food-grade expression system based on the pSIP expression vectors,[8] using the *alr* gene as selection marker in *L. plantarum* WCFS1. The new host/vector systems were applied to overproduce β-galactosidases from *L. plantarum* WCFS1 and *L. reuteri* L103, both dimeric proteins encoded by two overlapping genes called *lacLM*.[9,25] The expression levels obtained with the newly developed food-grade system were

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Table 1. Strains and Plasmids Used in This Work[a]

strains or plasmids	relevant characteristics	refs
Strains		
Lactobacllus plantarum		
WCFS1	wild type	26
TLG01	double cross over mutant Δalr, Cm^r, D-alanine auxotroph	this work
TLG02	Δ*alr*, D-alanine auxotroph	this work
E. coli		
MB2159	D-alanine auxotroph, cloning host	27
TOP10	cloning host	Invitrogen
Plasmids		
pNZ5319	Cm^r, Em^r, containing *lox66*-P_{32}-*cat*-*lox71* fragment	30
pNZ5348	Em^r, *Cre*-recombinase expression	30
pTLG01	Cm^r, Em^r, pNZ5319 derivative for knocking out *alr* from *L. plantarum* WCFS1 genome	this work
pSIP403	Em^r, pSIP401 derivative, *gusA* controlled by P_{sppA}	8
pSIP409	Em^r, pSIP401 derivative, *gusA* controlled by P_{sppQ}	8
pLp_0373sNuc	pSIP401 derivative, Em^r	10
pEH9R	Em^r, pSIP409 derivative, containing *lacLM* from *L. reuteri* L103	9
pEH9P	Em^r, pSIP409 derivative, containing *lacLM* from *L. plantarum* WCFS1	9
pEH3R	Em^r, pSIP403 derivative, containing *lacLM* from *L. reuteri* L103	9
pEH3P	Em^r, pSIP403 derivative, containing *lacLM* from *L. plantarum* WCFS1	9
pSIP609	pSIP409 derivative, *alr* replaced with *erm*	this work
pSIP609R	pSIP409 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. reuteri* L103	this work
pSIP609P	pSIP409 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. plantarum* WCFS1	this work
pSIP603R	pSIP403 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. reuteri* L103	this work
pSIP603P	pSIP403 derivative, *alr* replaced with *erm*, *gusA* replaced by *lacLM* from *L. plantarum* WCFS1	this work

[a] Cm^r, Erm^r, chloramphenicol and erythromycin resistance; *cre*, cre-recombinase encoding gene; *alr*, alanine racemase encoding gene; *erm*, erythromycin resistance gene.

compared to the levels obtained using the conventional pSIP system, which is based on the use of an erythromycin resistance gene for selection.[9] These new vectors open up the possibility to use the pSIP expression system for food-related applications.

MATERIALS AND METHODS

Bacterial Strains and Media. The bacterial strains, plasmids and primers used in this study are listed in Tables 1 and 2. *L. plantarum* WCFS1[26] was grown in MRS medium (Oxoid, Basingstoke, U.K.) at 37 °C without agitation. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA) and *E. coli* MB2159 (D-alanine auxotrophe)[27] were used as cloning hosts and cultivated in Luria−Bertani (LB) medium at 37 °C with shaking at 200 rpm. Solid media were prepared by adding 1.5% agar to the respective media. Unless otherwise stated, the antibiotic concentrations were 10 μg/mL of chloramphenicol (*Cm*) for *Lactobacillus* and *E. coli*, and 5 μg/mL or 200 μg/mL of erythromycin for *Lactobacillus* or *E. coli*, respectively. *E. coli* MB2159 and *L. plantarum* strains TLG01 and TLG02 (Table 1) were cultivated as the wild types except for addition of 200 μg/mL D-alanine (Sigma, St. Louis, MO) to the respective media.

DNA Manipulation. Genomic DNA of *L. plantarum* WCFS1 was isolated by using the E.Z.N.A. bacterial DNA kit (Omega Bio-Tek Inc., Doraville, GA) according to the instructions of the manufacturer. Plasmids were isolated using NucleoSpin Plasmid Kit (Mini-Prep) (Macherey-Nagel GmbH & Co., Düren, Germany) or Jetstar (Midi-Prep) plasmid purification system (Genomed GmbH, Löhne, Germany) as recommended by the supplier. PCR amplification of DNA was performed with the hot start KOD polymerase (Toyobo, Osaka, Japan) and primers purchased from Operon Biotechnologies GmbH (Cologne, Germany). All amplified sequences were verified by DNA sequencing. PCR products and restriction enzyme digested DNA were purified with Nucleo-Spin Extract II kit (Macherey-Nagel GmbH & Co.), and the DNA concentration was measured using the Quant-it assay (Invitrogen). Samples for plasmid copy number determination were taken after 6 h (OD_{600nm} ∼ 3.0) and 24 h (OD_{600nm} ∼ 9), DNA isolation was done by phenol−chloroform−extraction. qPCR amplification and detection was done using an iCycler thermal cycling system and myIQ single Color Real-Time PCR Detection system (BioRad, Hercules, CA). The qPCR reactions (25 μL total volume) were prepared in triplicate in 96 well plates (BioRad) that were sealed with optical adhesive covers (Microseal "B" film, BioRad). Each reaction included an optimized concentration for each of forward and reverse primers (see Table 2), 12.5 μL of IQ SYBR Green Super mix (BioRad), and 2.5 μL of DNA template. Negative controls, prepared by replacing the DNA template with water, were included to confirm the absence of DNA contaminations in the reagents. qPCR reactions were done as follows: initial denaturation at 95 °C for 3 min followed by 50 cycles of 20 s at 95 °C, 20 s at 60 °C, and 10 s at 72 °C. The fluorescence signal was collected at the end of each extension step at 72 °C. Afterward, the temperature was increased from 55 to 95 °C at a rate of 0.2 °C/s to establish the melting curve. Threshold cycle values (C_t) were automatically determined by the software BioRad MyIQ optical system Version 2.0.

L. plantarum competent cells were prepared and electroporated as described by Josson and co-workers.[28] Chemically competent cells of *E. coli* TOP10 were supplied by Invitrogen. Competent cells of *E. coli* MB1259 were prepared and transformed according to the method of Inoue et al.[29]

Construction of the *alr* Deletion Mutant. The integrative vector for deletion of the alanine racemase gene (*alr*) from *L. plantarum* WCFS1 genome was constructed as described by Lambert and co-workers.[30] The two flanking fragments upstream and downstream (∼1 kb)

Table 2. Primers Used in This Study[a]

primer	sequence (5′−3′)
T1	CAGCACTTGTGCGTCCTATGC
T2	CGAGCATTGCCTAGCCACC
T3	GCATACATTATACGAACGGTAGATTT AACCATCACAAATTGCCTCTTTC
T4	CGGTACAGCCCGGGCATGAG ATAGATTAAGTTTTATCAGACGATAGGTT
T5	TCAACTAGCTTACCTAATCGTCGTC
T6	AGCGATATTCACGATTTACAGCC
is128	AAATCTACCGTTCGTATAATGTATGC
is129	CTCATGCCCGGGCTGTACCG
T7	TATGCGTGCGGGATCCC (*Bam*HI)
T8	GCGGTGCTCCCCAATTACAACCATGTAATCTCTCCTGAAGTTAAACGATT
T9	ATGGTTGTAATTGGGGAGCAC
T10	TTAATCTATATAAACTCTCGGCACTCGT
T11	GAGTGCCGAGAGTTTATATAGATTAATTCTATGAGTCGCTTTTTTAAATT
T12	CACTTTTGATAATCGATATGGTAAACT (*Cla*I)
16S_f	TGATCCTGGCTCAGGACGAA
16S_r	TGCAAGCACCAATCAATACCA
LacReu_f	CCA GAT TCC GTG GTA TTA CCT TTG TG
LacReu_r	TAC TACT ACG TCA CGC CAT TGA GGA AC

[a] The restriction sites are underlined.

of the *alr* gene were PCR amplified using genomic DNA from *L. plantarum* as template and the two primer pairs, T2 and T3 and T4 and T5, respectively (Table 2). The *lox66*-P_{32}-*cat*-*lox71* cassette (∼1.2 kb) was amplified from the pNZ5319 template[30] using the primers is128 and is129 (Table 2). The flanking regions were fused with the *lox66*-P_{32}-*cat*-*lox71* fragment by overlap extension PCR (SOE-PCR) using primer pair T2 and T5. Subsequently, the fused fragment was ligated to a ∼2.7 kb fragment obtained from *Swa*I−*Ecl*136II digestion of pNZ5319, yielding pTLG01 (Table 1). Plasmid DNA was purified by phenol−chloroform extraction and sodium acetate/isopropanol precipitation from the cloning host (*E. coli* TOP 10) and resuspended in 50 μL of nuclease-free ddH_2O. Four micrograms (4 μg) of plasmid DNA were used for transformation into *L. plantarum*. Selection of the double crossover mutant (named *L. plantarum* TLG01), marker excision by Cre-dependent recombination of the *lox*-sites and selection of the *alr* deletion mutant (*L. plantarum* TLG02) were done using the previously described method.[30] Successful double crossover plus subsequent marker excision should result in a deletion of the entire *alr* coding sequence in the chromosome of *L. plantarum*. Selected progeny was checked by PCR amplification with the primers T1 and T6 primers (Table 2) and named *L. plantarum* TLG02.

Construction of β-Galactosidase Expression Vectors. The food-grade expression vector was constructed based on the pSIP vector series (Figure 1A)[9] by replacing the erythromycin resistance gene (*erm*) with the alanine racemase gene (*alr*) from *L. plantarum* WCFS1 genome. For that purpose, the pSIP derivative pLp_0373sNuc[10] was used as template to amplify upstream and downstream flanking regions of *erm* with primer pairs T7 and T8 and T11 and T12, respectively (Table 2). The *alr* gene was amplified using primers T9 and T10. In order to allow seamless replacement of the *erm* gene, the *alr* gene was fused by SOE-PCR to the two fragments located upstream and downstream of the *erm* gene. Using the In-Fusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA), this fused fragment was ligated to a ∼5.8 kb fragment obtained upon cleavage of pSIP409[8] (Table 1) with *Bam*HI−*Cla*I, resulting in plasmid pSIP609 (Table 1). The net effect of these manipulations is an exchange of the coding region of the *erm* gene in pSIP409 with the coding region of the *alr* gene, without any additional changes in upstream our downstream sequences.

Fragments containing the *lacLM* genes from *L. reuteri* L103 and *L. plantarum* WCFS1 fused to promoters P_{sppQ} (pSIP409) or P_{sppA} (pSIP403) were obtained by digesting plasmids pEH9R, pEH9P, pEH3R and pEH3P[9] with *Pst*I and *Xma*I (Figure 1A). These fragments were ligated into the ∼5.5 kb *Pst*I−*Xma*I digested fragment of pSIP609 yielding 4 plasmids named pSIP609R, pSIP609P, pSIP603R and pSIP603P (Figure 1B and Table 1). These expression vectors were constructed in *E. coli* MB2159 before electroporation into the D-alanine auxotroph expression host *L. plantarum* TLG02.

Segregational Stability of *alr*- and *erm*-Based Plasmids. The wild type *L. plantarum* WCFS1 harboring pEH9R and mutant *L. plantarum* TLG02 harboring pSIP609R were cultivated in nonselective and selective medium (MRS with and without 5 μg/mL erythromycin or 200 μg/mL D-alanine, respectively) at 37 °C without agitation. Under these conditions, about 7 generations of growth passed in 12 h. Bacterial cultures were diluted into fresh medium every 12 h for a total cultivation time of 144 h (12 transfers); samples were taken after six transfers (42 generations) and at the end of cultivation (84 generations). The number of cells that still contained the expression plasmids was estimated by plating dilution series of the culture on selective plates.[31] The effect of induction on the segregational stability of the plasmids was tested by supplying SppIP (25 ng/mL; CASLO Laboratory, Lyngby, Denmark) to the culture. The percentage of plasmid-harboring cells and the β-galactosidase activity from these samples were determined regularly.

Expression of β-Galactosidases with *alr*-Based Vectors. Overnight cultures of *L. plantarum* harboring the pSIP derived novel plasmids were diluted in fresh prewarmed MRS medium (for *erm*-based systems, 5 μg/mL of erythromycin was added) to an OD_{600nm} ∼ 0.1 and incubated at 30 °C. The cells were induced at an OD_{600nm} ∼ 0.3 by adding the peptide pheromone (SppIP) to a final concentration of 25 ng/mL. Cells were harvested at OD_{600nm} of 1.8 to 2.0. Bacterial cells were pelleted from 10 mL of culture by centrifugation at 3500*g* for 10 min at 4 °C, washed with buffer P (50 mM of sodium phosphate buffer, pH 6.5, 20% of glycerol and 1 mM DTT),[32] and resuspended in 500 μL of the same buffer. The cells were disrupted by ∼1 g of glass beads (0.5 mm) using a Precelly 24 glass bead mill (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Cell-free extracts obtained after 5 min of centrifugation at 9000*g* (4 °C) were used for activity assays and protein concentration determination.

Enzyme Assay and Protein Determination. β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG)

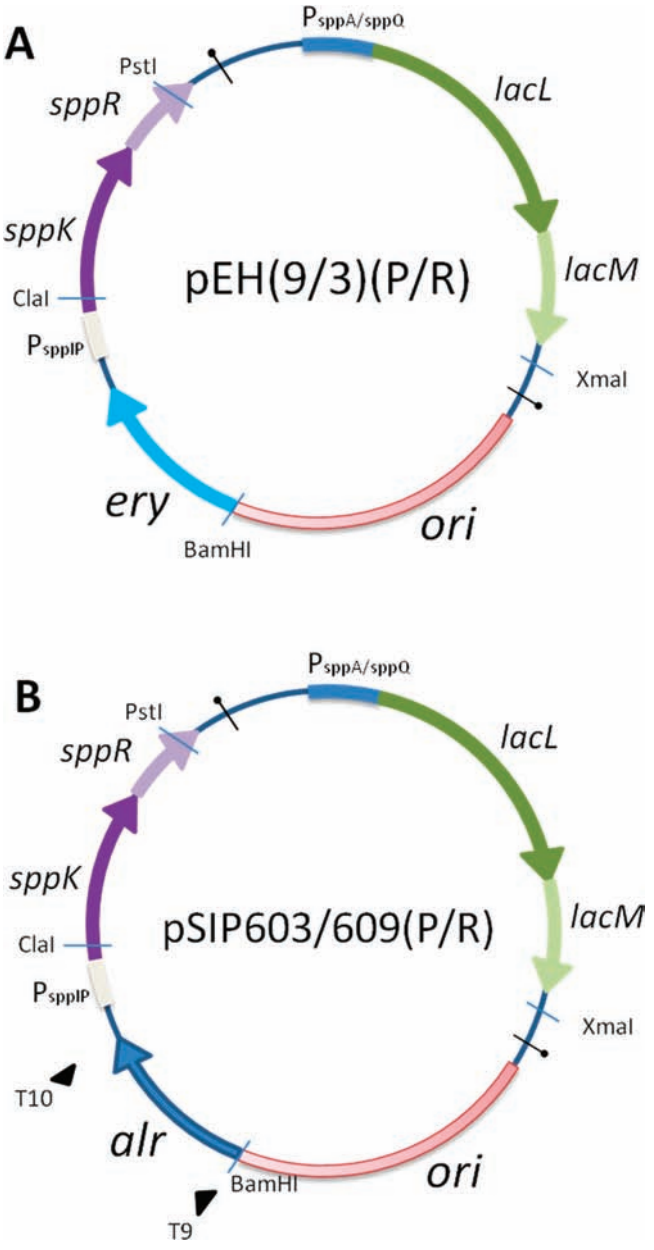

Figure 1. Expression vectors for *lacLM* based on the erythromycin resistance gene (*erm*)[9](A) and the alanine racemase (*alr*) gene (B) as selection markers. *SppK* and *sppR*, denoting a histidine kinase and a response regulator, respectively, are regulated by P_{sppIP}. The structural genes (overlapping genes *lacLM* from *L. plantarum*, indicated by "P", or *L. reuteri*, indicated by "R") are controlled by the inducible promoter P_{sppA} (pSIP403 derivatives) or P_{sppQ} (pSIP409 derivatives).

as described previously.[32] Protein concentration was determined by using the method of Bradford[33] with bovine serum albumin (BSA) as standard.

RESULTS

Construction *alr*⁻ Mutant Strain. After transforming *L. plantarum* WCSF1 with the (nonreplicating) vector pTLG01 chloramphenicol-resistant progeny (on MRS-agar + D-alanine) was replica-plated on MRS-agar containing chloramphenicol (without D-alanine) and MRS-agar with D-alanine and erythromycin to check for D-alanine-auxotrophy and erythromycin-sensitivity (indicating replacement of the genomic *alr* gene by the chloramphenicol-resistance gene as well as loss of the plasmid-backbone). Six out of ten tested transformants displayed the desired phenotype; two of these were checked by PCR and confirmed as gene replacements. Marker excision of the *lox*-sequence-flanked chloramphenicol-resistance gene was done by transformation with pNZ5348 containing the gene for the Cre-recombinase. About 20% of the transformants displayed the desired Erm^R, Cm^S phenotype and marker excision was confirmed by PCR. Loss of pNZ5348 was then induced by cultivation in the absence of erythromycin. The resulting strain, *L. plantarum* TLG02, was unable to grow within 24 h at 37 °C in both liquid and solid MRS medium. In contrast, growth of the mutant strain in the presence of D-alanine was identical to the wild type *L. plantarum* WCFS1 (data not shown).

pSIP-Derived Expression Vectors for the New Host Strain. New expression vectors were constructed based on the pSIP-vector series by exchanging the *erm* gene with the genomic *alr* gene (Figure 1A, 1B) as described in Materials and Methods. The expression of *alr* is controlled by the native erythromycin promoter which is derived from a *Lactobacillus reuteri* strain.[34]

The *lacLM* genes from *L. plantarum* and *L. reuteri*[9] were cloned into these *alr*-based plasmids resulting in 4 plasmids in which the transcription of *lacLM* genes is controlled by the promoters P_{sppA} (pSIP603P and pSIP603R, respectively) or P_{sppQ} (pSIP609P and pSIP609R, respectively) (Figure 1B).

Plasmid Segregational Stability and Plasmid Copy Numbers. The segregational stability of the *alr*- and *erm*-based vectors was tested using pSIP609R(*alr*) in *L. plantarum* TLG02 and pEH9R-(*erm*) in *L. plantarum* WCFS1 as representatives. The strains were cultivated for an estimated 84 generations (144 h) at 37 °C in nonselective and selective media, followed by replica plating of diluted cultures in order to determine the fractions of cells retaining the plasmid. The fraction of cells retaining pEH9R after 84 generations in nonselective medium (MRS without erythromycin) was 3%, whereas in selective medium 82% of the colonies still contained the plasmid. Interestingly the pSIP609R plasmid showed better segregational stability: after 84 generations it was retained in approximately 76% and 100% of cells of *L. plantarum* TLG02 under nonselective and selective conditions, respectively (Table 3). Induction of expression of the *lacLM* genes generally led to reduced plasmid stability, a phenomenon that is most clearly visible under nonselective conditions (Table 3). Table 3 also shows that plasmid loss is reflected in gradual loss of the β-galactosidase activity in cell extracts. For example, for *L. plantarum* TLG02/pSIP609R grown in nonselective medium and in the presence of SppIP, only 17% of the cells still harbored the plasmid after 84 generations, resulting in a β-galactosidase activity amounting to production of 5.0 U/mg, as compared to 26.0 U/mg after 7 generations.

Plasmid copy numbers were determined in the same strains both in the exponential growth phase after six hours (OD_{600nm} of 3.0) and in the stationary phase after about 24 h (OD_{600nm} of 9) by comparing the genomic 16S rDNA-encoding gene with the plasmid-borne *L. reuteri LacLM* gene using quantitative Real-Time PCR. The average copy number for pEH9R was 5.4 for the first sample (exponential phase) and 1.3 for the second sample during the stationary phase. pSIP609R had average copy numbers of 2.9 and 1.4 for the two samples, respectively, with ratios of pSIP609R/pEH9R of 0.54 and 1.07, respectively.

Expression of *Lactobacillus* β-Galactosidases in *alr*-Based Vectors and Comparison with *erm*-Based Vectors. On the SDS−PAGE of cell-free extracts (Figure 2), the two extensive bands (LacL and LacM) at approximately 75 kDa and 37 kDa,

Table 3. Segregational Stability of *alr*-Based and *erm*-Based Plasmids in Different Media[a,b]

	7 generations		42 generations		84 generations	
plasmid/medium[a]	remaining plasmid (%)	sp act. (U/mg)	remaining plasmid (%)	sp act. (U/mg)	remaining plasmid (%)	sp act. (U/mg)
pEH9R/MRS	–	–	37	–	3	–
pEH9R/MRS+SppIP	–	26.5	14	0.6	0	0.1
pEH9R/MRS+ery	–	–	89	–	82	–
pEH9R/MRS+ery+SppIP	–	32.0	88	27.7	79	16.1
pSIP609R/MRS	–	–	100	–	100	–
pSIP609R/MRS+SppIP	–	27.9	100	23.9	100	20.3
pSIP609R/MRS+D-alanine	–	–	90	–	76	–
pSIP609R/MRS+D-alanine+ SppIP	–	26.0	56	17.5	17	5.0

[a] *L. plantarum* WCFS1 harboring pEH9R and *L. plantarum* TLG02 harboring pSIP609R were cultivated in selective and nonselective medium with and without SppIP (the final concentration of SppIP was 25 ng/mL). The D-alanine concentration was 200 μg/mL, the erythromycin concentration was 5 μg/mL. [b] The strains were cultivated at 37 °C without agitation. Every 12 h, 7 generations passed. The fraction of plasmid containing cells was calculated by dividing the number of CFU on selective medium with the number of CFU on nonselective medium. A symbol "–" stands for nondetermined.

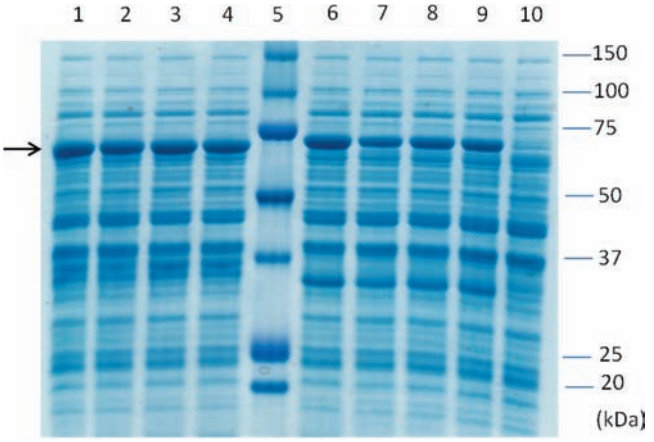

Figure 2. SDS–PAGE of cell-free extracts of strain *L. plantarum* TLG02 and the wild type strain harboring different expression vectors harvested at OD_{600} ~ 1.8–2. Lane 1, WCFS1/pEH3P; lane 2, TLG02/pSIP603P; lane 3, WCFS1/pEH9P; lane 4, TLG02/pSIP609P (cultures in lanes 1–4 were induced); lane 5, Precision Protein standard (Biorad); lane 6, WCFS1/pEH3R; lane 7, TLG02/pSIP603R; lane 8, WCFS1/pEH9R; lane 9, TLG02/pSIP609R (lanes 5–9 induced); lane 10, WCFS1/pEH9P (noninduced). The arrow indicates the band representing the large subunit LacL of the heterologously expressed β-galactosidase.

respectively, indicate the high production levels of two beta-galactosidases in new *alr*-based vectors which are relatively comparable to the levels obtained with *erm*-based expression vectors expressing the same genes. These high expression levels were confirmed by measured enzyme activities (Table 4). In general, the obtained specific enzyme activities (U/mg protein) were slightly higher with the conventional system (*erm* based vectors in *L. plantarum* WCFS1) and the magnitude of this difference varied between the pairs of constructs. The largest difference was observed for pSIP403 derivatives used to express *L. reuteri lacLM*, where the conventional system (WCFS1/pEH3R) yielded a specific activity that was almost twice as high as the activity obtained with the TLG02/pSIP603R system (Table 4). Non-induced cells harboring the *alr*-based vectors generally yielded lower background activities than noninduced cells harboring the *erm*-based vectors in three out of four cases (Table 4), and this may be beneficial in cases in which strict control is desirable.

Table 4. β-Galactosidase Activity in Cell-Free Lysates of Induced and Noninduced *L. plantarum* WCFS1 and *L. plantarum* TLG02 Carrying Various Plasmids

	sp act. (U/mg protein)[a]		
strain/plasmid	induced	noninduced	induction factor[b]
TLG02/pSIP603P	87.50 ± 7.38	1.15 ± 0.09	76
WCFS1/pEH3P	99.35 ± 3.04	1.67 ± 0.03	59
TLG02/pSIP603R	61.25 ± 3.17	0.51 ± 0.10	119
WCFS1/pEH3R	109.29 ± 2.09	2.51 ± 0.11	44
TLG02/pSIP609P	79.84 ± 2.86	0.51 ± 0.16	158
WCFS1/pEH9P	90.57 ± 3.86	0.45 ± 0.02	200
TLG02/pSIP609R	64.74 ± 3.57	0.50 ± 0.10	129
WCFS1/pEH9R	71.68 ± 1.10	0.72 ± 0.01	99

[a] Data are expressed as means ± standard deviation from three independent cultivations. [b] The induction factors are calculated by dividing the mean specific activities (U/mg) of induced and noninduced cultures.

To get better insight into the fermentation characteristics of the two systems, two of the strains, *L. plantarum* WCFS1 carrying pEH9 and *L. plantarum* TLG02 carrying pSIP609R, respectively, were cultivated in 200 mL of MRS broth at 30 °C without agitation. The medium for the former strain contained 5 mg/mL erythromycin; both cultures were induced at an OD_{600} of 0.3 with 25 ng/mL SppIP. After induction, cells were sampled every two hours for activity analysis and biomass determination. Figure 3 gives an overview of the wet biomass (g/L), β-galactosidase activity (U/mL fermentation and U/mg protein), and specific biomass activity (activity in 1 g of wet biomass) (kU/g) during the fermentation period for both systems. The growth rate of bacteria harboring the *erm*-vector (pEH9R) was slightly lower than of those containing an *alr*-vector (pSIP609R). For example, at 12 h after induction WCFS1/pEH9R yielded 7.8 g/L wet biomass, while TLG02/pSIP609R yielded 8.6 g/L at the same sampling time. The amount of enzyme increased from the induction time and reached maximum values at 8–10 h after induction. Cells carrying the *alr*-based vector gave slightly higher yields compared to cells carrying the *erm*-based vector (approximately 33 and 29 U/mL fermentation, respectively). The specific activity of the enzyme was similar in both systems

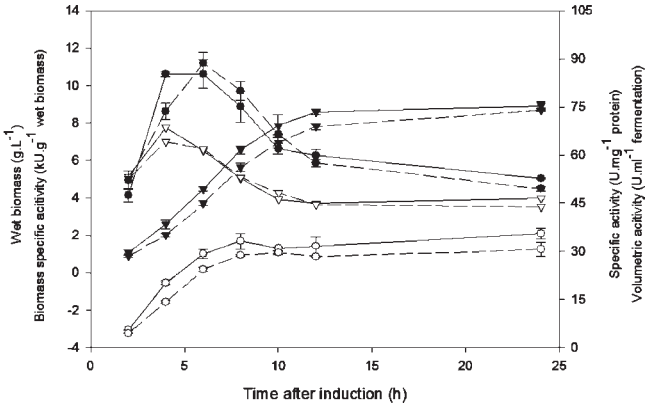

Figure 3. Fermentations of *L. plantarum* TLG02/pSIP609R (solid lines) and *L. plantarum* WCFS1/pEH9R (dashed lines) *L. plantarum* in MRS at 30 °C. For *L. plantarum* WCFS1/pEH9R the medium contained erythromycin (5 μg/mL). The cells were induced at $OD_{600} \sim 0.3$. Wet biomass (g/L fermentation broth) (▼), volumetric activity (U/mL fermentation broth) (○), specific activity (U/mg protein) (●), and specific biomass activity (kU/g wet biomass) (▽) were determined for the interval after induction. The data are mean values of 3 cultivations, and error bars indicate the standard deviations.

and reached the highest value approximately 6 h after induction (~85 U/mg). The relationship between volumetric activity (U/mL) and biomass (g/L) is expressed as specific biomass activity. Specific biomass activities were almost identical for both systems and reached maxima of 7.8 kU/g and 7 kU/g for for *alr*-based and *erm*-based systems, respectively, 4 h after induction (Figure 3).

■ DISCUSSION

We have constructed a new host/vector system for inducible gene expression in *Lactobacillus* using pSIP-type vectors and the *alr* gene as a selection marker. The Δ*alr* mutant *L. plantarum* TLG02 was developed using the Cre-*lox* system for complete *alr* gene replacement in wild type *L. plantarum* WCFS1. This approach prevents the reintegration of the *alr* marker back into the host chromosome as has been observed during early work on *alr*-based selection in lactic acid bacteria where the gene was only partially deleted from the host chromosome.[24] The segregational stability of one of the *alr*-based vectors was evaluated for 84 generations and compared to that of the corresponding *erm*-based vector. The results (Table 3) showed that the *alr*-based vector was more stable than the *erm*-based vector, under both selective and nonselective conditions. Interestingly, when the expression system was activated by SppIP pheromone, the *erm*-based plasmid was lost from the bacterial cells more quickly (Table 3), indicating that expression of the target gene exerts a considerable metabolic load on the cells. Most interestingly, this effect was not observed for the *alr*-based plasmid in the mutant strain under selective conditions, meaning that, in this case, all cells maintained the plasmid. Since selective conditions are easy to maintain for a complementation marker, the *alr*-based plasmids offer clear advantages for heterologous expression under industrial conditions.

We have previously overexpressed *lacLM* genes from four *Lactobacillus* species in the closely related hosts *L. plantarum* WCFS1 and *L. sakei* Lb790 using *erm*-based pSIP vectors.[9] The highest expression levels were obtained for the *lacLM* genes from *L. reuteri* L103 and *L. plantarum* WCFS1, hence these genes were used to test β-galactosidase overproduction in the new *alr*-based expression systems. The specific activity data (Table 4) show that the expression levels of *lacLM* genes with *alr*-based vectors were comparable to the levels obtained with the *erm*-based vectors, although they were generally slightly lower. The β-galactosidase activities in noninduced cultures were also higher using the conventional system, especially with the pSIP603 series, where *lacLM* expression is controlled by the leakier of the two promoters, P_{sppA} (Table 4). We found that *L. plantarum* WCFS1 carries an average number of 5.4 copies of pEH9R during the exponential growth phase, compared to 2.9 copies of pSIP609R in *L. plantarum* TLG02. This difference essentially disappears as the cultures reach the stationary phase, where both plasmids are present in their respective host strains at average copy numbers of about 1.3. The higher copy number of pEH9R during the first hours of cultivation may account for the observed higher enzyme activities. In the wild type strain, a single copy of the *alr* gene is required for growth, and additional copies may not offer any advantage to the cells. In contrast, in an antibiotic-containing environment a higher copy number of the resistance gene may benefit the cells, which could exert selection pressure toward keeping multiple copies of the *erm*-based vectors. The replacement of an antibiotic selection marker by a non-antibiotic one has been reported as a possible reason for a reduction in plasmid copy number.[35]

In the fermentation experiments, *L. plantarum* TLG02 harboring pSIP609R (*alr*) grew slightly faster than *L. plantarum* WCFS1 harboring pEH9R (*erm*), and the former strain showed a higher wet biomass yield (Figure 3) and higher volumetric activity (U/mL) (Figure 3). It is possible that the synthesis of the *erm*-gene product and/or the resulting methylation of 23S rRNA puts a higher physiological stress on the bacteria than synthesis of alanine racemase and racemization of L-alanine (i.e., normal, wild type metabolism).

Recent studies on the use of β-galactosidases from probiotic lactobacilli, in particular *L. reuteri*, for application in the production of prebiotic galacto-oligosaccharides (GOS) have shown promising results.[32,36] We show here that high amounts of *L. reuteri* β-galactosidases may be produced using the new *alr*-based system (~33 U/mL fermentation). While this production level is slightly higher than the level obtained with a corresponding *erm*-based pSIP vector (Figure 3), it is still considerably lower than the yields obtained with *E. coli*/pET21 systems (which may amount to as much as 110 U/mL[25]). However, as the *erm*-based pSIP systems, this latter system depends on the use of antibiotics in the fermentation medium.[25] Recently, Maischberger and coworkers showed that expression of the same *L. reuteri lacLM* gene with the food grade *lacF*-based NICE system yielded production levels of approximately 8 U/mL of fermentation,[37] which is much lower than the yields obtained with the *alr*-based system in this study. The *lacF*-based expression systems require that lactose must be used as carbon source, whereas *alr*-based systems are carbon source independent, providing more options for fermentation in industrial scale. A potential additional disadvantage of the *erm*-based systems is that the selective agent, erythromycin, can be degraded at acidic pH,[38,39] which is unfavorable in fermentations of lactic acid bacteria without pH control.

Summarizing, we present in this study a new version of the pSIP expression vector series for lactic acid bacteria based on using alanine racemase as a food grade complementation marker. When tested for overexpression of lactobacillal β-galactosidases, these systems showed high production levels. Overcoming

certain drawbacks of the original *erm*-based systems, these new *alr*-based host/vector systems have a potential to be used on an industrial scale for the production of recombinant proteins with food applications. Currently, we are working on development of an economical and effective medium for industrial fermentation using these new systems.

AUTHOR INFORMATION

Corresponding Author

*Department für Lebensmittelwissenschaften und -technologie, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Wien, Austria. E-mail: clemens.peterbauer@boku.ac.at. Tel: +43-1-47654-6144. Fax: +43-1-47654-6199.

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REFERENCES

(1) Giraffa, G.; Chanishvili, N.; Widyastuti, Y. Importance of lactobacilli in food and feed biotechnology. *Res. Microbiol.* **2010**, *161* (6), 480–7.

(2) Kleerebezem, M.; Hols, P.; Hugenholtz, J. Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. *Enzyme Microb. Technol.* **2000**, *26* (9−10), 840–8.

(3) de Vos, W. M. Safe and sustainable systems for food-grade fermentations by genetically modified lactic acid bacteria. *Int. Dairy J.* **1999**, *9*, 3–10.

(4) de Vos, W. M. Gene expression systems for lactic acid bacteria. *Curr. Opin. Microbiol.* **1999**, *2*, 289–95.

(5) Diep, D. B.; Mathiesen, G.; Eijsink, V. G.; Nes, I. F. Use of lactobacilli and their pheromone-based regulatory mechanism in gene expression and drug delivery. *Curr. Pharm. Biotechnol.* **2009**, *10* (1), 62–73.

(6) Kuipers, O. P.; de Ruyter, P. G.; Kleerebezem, M.; de Vos, W. M. Controlled overproduction of proteins by lactic acid bacteria. *Trends Biotechnol.* **1997**, *15* (4), 135–40.

(7) Sorvig, E.; Gronqvist, S.; Naterstad, K.; Mathiesen, G.; Eijsink, V. G.; Axelsson, L. Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiol. Lett.* **2003**, *229* (1), 119–26.

(8) Sorvig, E.; Mathiesen, G.; Naterstad, K.; Eijsink, V. G.; Axelsson, L. High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. *Microbiology* **2005**, *151* (Part 7), 2439–49.

(9) Halbmayr, E.; Mathiesen, G.; Nguyen, T. H.; Maischberger, T.; Peterbauer, C. K.; Eijsink, V. G.; Haltrich, D. High-level expression of recombinant β-galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* using a Sakacin P-based expression system. *J. Agric. Food Chem.* **2008**, *56* (12), 4710–9.

(10) Mathiesen, G.; Sveen, A.; Piard, J. C.; Axelsson, L.; Eijsink, V. G. Heterologous protein secretion by *Lactobacillus plantarum* using homologous signal peptides. *J. Appl. Microbiol.* **2008**, *105* (1), 215–26.

(11) Straume, D.; Axelsson, L.; Nes, I. F.; Diep, D. B. Improved expression and purification of the correctly folded response regulator PlnC from lactobacilli. *J. Microbiol. Methods* **2006**, *67* (2), 193–201.

(12) Allison, G. E.; Klaenhammer, T. R. Functional analysis of the gene encoding immunity to lactacin F, *lafI*, and its use as a *Lactobacillus*-specific, food-grade genetic marker. *Appl. Environ. Microbiol.* **1996**, *62* (12), 4450–60.

(13) Takala, T. M.; Saris, P. E. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene *nisI*. *Appl. Microbiol. Biotechnol.* **2002**, *59* (4−5), 467–71.

(14) von Wright, A.; Wessels, S.; Tynkkynen, S.; Saarela, M. Isolation of a replication region of a large lactococcal plasmid and use in cloning of a nisin resistance determinant. *Appl. Environ. Microbiol.* **1990**, *56* (7), 2029–35.

(15) El Demerdash, H. A.; Heller, K. J.; Geis, A. Application of the *shsp* gene, encoding a small heat shock protein, as a food-grade selection marker for lactic acid bacteria. *Appl. Environ. Microbiol.* **2003**, *69* (8), 4408–12.

(16) Boucher, I.; Parrot, M.; Gaudreau, H.; Champagne, C. P.; Vadeboncoeur, C.; Moineau, S. Novel food-grade plasmid vector based on melibiose fermentation for the genetic engineering of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **2002**, *68* (12), 6152–61.

(17) Leenhouts, K.; Bolhuis, A.; Venema, G.; Kok, J. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* **1998**, *49* (4), 417–23.

(18) Sasaki, Y.; Ito, Y.; Sasaki, T. *ThyA* as a selection marker in construction of food-grade host-vector and integration systems for *Streptococcus thermophilus*. *Appl. Environ. Microbiol.* **2004**, *70* (3), 1858–64.

(19) MacCormick, C. A.; Griffin, H. G.; Gasson, M. J. Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. *FEMS Microbiol. Lett.* **1995**, *127* (1−2), 105–9.

(20) Platteeuw, C.; van Alen-Boerrigter, I.; van Schalkwijk, S.; de Vos, W. M. Food-grade cloning and expression system for *Lactococcus lactis*. *Appl. Environ. Microbiol.* **1996**, *62* (3), 1008–13.

(21) Gosalbes, M. J.; Esteban, C. D.; Galan, J. L.; Perez-Martinez, G. Integrative food-grade expression system based on the lactose regulon of *Lactobacillus casei*. *Appl. Environ. Microbiol.* **2000**, *66* (11), 4822–8.

(22) Hols, P.; Defrenne, C.; Ferain, T.; Derzelle, S.; Delplace, B.; Delcour, J. The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. *J. Bacteriol.* **1997**, *179* (11), 3804–7.

(23) Palumbo, E.; Favier, C. F.; Deghorain, M.; Cocconcelli, P. S.; Grangette, C.; Mercenier, A.; Vaughan, E. E.; Hols, P. Knockout of the alanine racemase gene in *Lactobacillus plantarum* results in septation defects and cell wall perforation. *FEMS Microbiol. Lett.* **2004**, *233* (1), 131–8.

(24) Bron, P. A.; Benchimol, M. G.; Lambert, J.; Palumbo, E.; Deghorain, M.; Delcour, J.; De Vos, W. M.; Kleerebezem, M.; Hols, P. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. *Appl. Environ. Microbiol.* **2002**, *68* (11), 5663–70.

(25) Nguyen, T. H.; Splechtna, B.; Yamabhai, M.; Haltrich, D.; Peterbauer, C. Cloning and expression of the β-galactosidase genes from *Lactobacillus reuteri* in *Escherichia coli*. *J. Biotechnol.* **2007**, *129* (4), 581–91.

(26) Kleerebezem, M.; Boekhorst, J.; van Kranenburg, R.; Molenaar, D.; Kuipers, O. P.; Leer, R.; Tarchini, R.; Peters, S. A.; Sandbrink, H. M.; Fiers, M. W.; Stiekema, W.; Lankhorst, R. M.; Bron, P. A.; Hoffer, S. M.; Groot, M. N.; Kerkhoven, R.; de Vries, M.; Ursing, B.; de Vos, W. M.; Siezen, R. J. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (4), 1990–5.

(27) Strych, U.; Penland, R. L.; Jimenez, M.; Krause, K. L.; Benedik, M. J. Characterization of the alanine racemases from two mycobacteria. *FEMS Microbiol. Lett.* **2001**, *196* (2), 93–8.

(28) Josson, K.; Scheirlinck, T.; Michiels, F.; Platteeuw, C.; Stanssens, P.; Joos, H.; Dhaese, P.; Zabeau, M.; Mahillon, J. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. *Plasmid* **1989**, *21* (1), 9–20.

(29) Inoue, H.; Nojima, H.; Okayama, H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **1990**, *96* (1), 23–8.

(30) Lambert, J. M.; Bongers, R. S.; Kleerebezem, M. Cre-*lox*-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **2007**, *73* (4), 1126–35.

(31) Lederberg, J.; Lederberg, E. M. Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.* **1952**, *63* (3), 399–406.

(32) Nguyen, T. H.; Splechtna, B.; Steinbock, M.; Kneifel, W.; Lettner, H. P.; Kulbe, K. D.; Haltrich, D. Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54* (14), 4989–98.

(33) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–54.

(34) Axelsson, L. T.; Ahrne, S. E.; Andersson, M. C.; Stahl, S. R. Identification and cloning of a plasmid-encoded erythromycin resistance determinant from *Lactobacillus reuteri*. *Plasmid* **1988**, *20* (2), 171–4.

(35) Dong, W. R.; Xiang, L. X.; Shao, J. Z. Novel antibiotic-free plasmid selection system based on complementation of host auxotrophy in the NAD de novo synthesis pathway. *Appl. Environ. Microbiol.* **2010**, *76* (7), 2295–303.

(36) Splechtna, B.; Nguyen, T. H.; Steinbock, M.; Kulbe, K. D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54* (14), 4999–5006.

(37) Maischberger, T.; Mierau, I.; Peterbauer, C. K.; Hugenholtz, J.; Haltrich, D. High-level expression of *Lactobacillus* β-galactosidases in *Lactococcus lactis* using the food-grade, nisin-controlled expression system NICE. *J. Agric. Food Chem.* **2010**, *58* (4), 2279–87.

(38) Lorian, V.; Sabath, L. D. Effect of pH on the activity of erythromycin against 500 isolates of gram-negative bacilli. *Appl. Microbiol.* **1970**, *20* (5), 754–6.

(39) Volmer, D. A.; Hui, J. P. Study of erythromycin A decomposition products in aqueous solution by solid-phase microextraction/liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1998**, *12* (3), 123–9.