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Repression of the Locus of the Enterocyte Effacement-Encoded Regulator of Gene Transcription of *Escherichia coli* O157:H7 by *Lactobacillus reuteri* Culture Supernatants Is LuxS and Strain Dependent[▽]

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Culture supernatants of *Lactobacillus reuteri* ATCC 55730 repressed *ler* expression in *Escherichia coli* O157:H7 cells, but neither the strain's isogenic *luxS* mutant nor the *L. reuteri* 100-23C wild-type strain and its *luxS* mutant elicited a comparable effect. Furthermore, the epinephrine-mediated induction of *ler* expression was repressed by secreted substance(s) of *L. reuteri* ATCC 55730.

Bacterial communication by secreted signaling molecules, known as quorum sensing (QS), impacts diverse cellular processes such as virulence gene expression (reviewed in references 12 and 33). Enterohemorrhagic *Escherichia coli* (EHEC) strains of serotype O157:H7, the causative agents of bloody diarrhea and hemolytic uremic syndrome in humans (11, 15), harbor QS-regulated virulence genes on a pathogenicity island termed the locus of enterocyte effacement (LEE) (23, 27) that are organized mainly into the five polycistronic operons *LEE1* to *LEE5* (6, 11). The first gene in *LEE1*, LEE-encoded regulator (*ler*), encodes the principal transcriptional activator of the LEE genes (5). The expression of *ler* was shown to be controlled by the bacterial QS molecule autoinducer 3 (AI-3) and the eukaryotic catecholamine hormones epinephrine and norepinephrine; thus, virulence properties such as the attachment to host cells are influenced by the cross talk between the bacteria and the host organism (24, 25). EHEC senses both AI-3 and catecholamines through the adrenergic two-component systems QseBC and QseEF, which in turn regulate LEE and flagellum gene expression via signal transduction cascades that are complex and not fully understood (3, 20, 26). An active LuxS synthase is the precondition for the formation of AI-3, as well as yet another QS signal, AI-2 (25). LuxS is a metabolic enzyme involved in the regeneration of the methyl donor *S*-adenosylmethionine in the activated methyl cycle of methionine metabolism (21). While AI-2, a furanosyl borate diester, is the actual product of LuxS activity, the molecular structure and synthesis of AI-3 are unknown (25). Although LuxS does not produce AI-3 directly, marked reduction of AI-3 activity of an EHEC *luxS* mutant has been observed, which could be restored by the expression of amino acid transporters and the addition of aspartate (31). LuxS homologues are widespread among both gram-negative and gram-positive bacteria, and AI-2 and AI-3 are, therefore, viewed as key mediators of intra- and interkingdom QS (12, 29). Recently, the LuxS-dependent induction of EHEC O157:H7 LEE genes by *Lactobacillus reuteri* was demonstrated (28). The stationary-phase supernatant of *L. reuteri* 100-23C, a rodent isolate (13), induced *ler* expression, which was abolished in an isogenic *luxS* mutant. Thus, it is very likely that the induction was mediated by AI-3-like molecules. In contrast to these findings, a recent publication showed that molecules secreted by probiotic *Lactobacillus acidophilus* La-5 led to reduced virulence gene expression of EHEC O157:H7, but whether this repression was LuxS dependent remained undetermined (14).

The aim of the present study was to investigate the LuxS/AI-3-dependent regulation of EHEC O157:H7 virulence genes by substances secreted by *L. reuteri* strains. We constructed an isogenic *luxS* mutant from the probiotic strain *L. reuteri* ATCC 55730 (BioGaia AB, Stockholm, Sweden) and compared the effects of supernatants of the wild type and the *luxS* mutant on EHEC *ler* transcription with the effects of supernatants of the corresponding strains of *L. reuteri* 100-23C. For this purpose, a novel fluorescence bioassay was developed by transcriptionally fusing the *ler* promoter with a green fluorescent protein (*gfp*) gene. The assay was validated by using supernatants of EHEC O157:H7 (the AI-3 producer) and *E. coli* DH5α (a natural *luxS* mutant), as well as growth medium with and without epinephrine.

Development and validation of a fluorescence bioassay that detected *ler* promoter activity. To measure the influence of external signals on *ler* transcription, we fused the *ler* promoter of EHEC O157:H7 EDL933 with the promoterless *gfp*(*ASV*) gene located on pJBA89 (1), a plasmid originally constructed to measure acyl homoserine lactone QS signals. To do this, we amplified the promoter sequence by using the primers PlerF (CTGAGAATTCTTAGAGATACTGGCTTTCAGGAAAC [the EcoRI recognition site is underlined]) and PlerR (CTGACGCATGCTTTAATATTTTAAGCTATTAGC [the SphI recognition site is underlined]). A fragment of pJBA89 containing *luxR* and the promoters P*luxR* and P*luxI* was removed and replaced with a 0.9-kb PCR product of the *ler* promoter by

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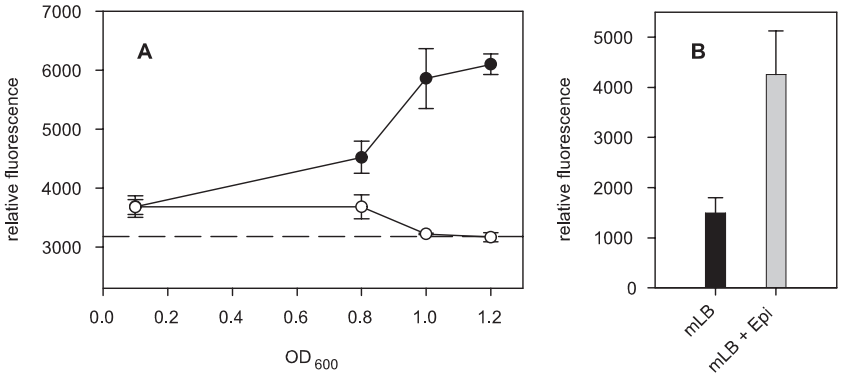

FIG. 1. Validation of the *E. coli* IJ01 fluorescence bioassay measuring *ler* promoter regulation. (A) Relative fluorescence induction obtained with culture supernatants of EHEC O157:H7 EDL933 (filled circles) and *E. coli* DH5α (open circles) of different growth phases and by mLB medium (negative control, dashed line). (B) Relative fluorescence values obtained with mLB medium (black bar) and mLB supplemented with 50 μM L-epinephrine (Epi, gray bar). Relative fluorescence values were calculated by dividing absolute fluorescence by the corresponding cell density (OD_{600}) values. Error bars denote standard deviations of three independent experiments performed in duplicate.

digestion with EcoRI and SphI and ligation. The correct insertion was verified by sequencing the resulting plasmid, pIJ001 (5.3 kb) (data not shown). *E. coli* MG1655 (8) was transformed with pIJ01 by electroporation (4), yielding *E. coli* IJ01. The *E. coli* K-12 derivative MG1655 was chosen as the host on the basis of the successful utilization of K-12 strains in gene expression studies using reporter fusions of EHEC genes (10, 18, 22, 23, 25), although the EHEC-specific transcription regulators GrlA/GrlR, EtrA/EivF, and Pch, which affect LEE gene expression, are not present in the K-12 background (32).

The applicability of *E. coli* IJ01 as the AI-3/epinephrine-responsive reporter organism in a fluorescence bioassay was established by using culture supernatants of EHEC O157:H7 strain EDL933 (with LuxS) (16) and *E. coli* strain DH5α (lacking LuxS; Promega), as well as growth medium with epinephrine (positive control) and without epinephrine (negative control). Supernatants were prepared by taking samples at several time points from the *E. coli* strains growing in modified LB (mLB) medium (with 4 g liter^{-1} NaCl) at 37°C, then removing cells by centrifugation and sterile filtering, and by adjusting the pH to 7.0 with 1 M NaOH. Prior to the start of fluorescence assays, *E. coli* IJ01 was inoculated in mLB medium containing 100 μg ml^{-1} ampicillin and grown aerobically at 30°C to an optical density at 600 nm (OD_{600}) of ≤0.2. After two further subculturing steps at 30°C, the culture was diluted 1:20 in fresh mLB medium, in mLB containing 50 μM L-epinephrine, or in *E. coli* supernatants, all preheated to 30°C. Aliquots (200 μl) were transferred to a microtiter plate, which was then incubated with shaking at 30°C. Fluorescence (515 nm) and OD_{600} values were measured for up to 5 h by using a Cary-Eclipse fluorescence reader (Varian) and a model 450 microplate reader (Bio-Rad). The regulation of *ler* was expressed as the relative fluorescence obtained by dividing the absolute fluorescence signals by the OD_{600} value of the *E. coli* reporter strain IJ01. For this and for all following experiments, comparable amounts of growth of *E. coli* strain IJ01 in the different supernatants were checked (OD_{600}) to eliminate cell density-dependent effects. As shown in Fig. 1A, the EDL933 supernatants of early to late stationary phase (OD_{600}, 0.8 to 1.2) significantly induced the relative fluorescence ($P < 0.05$) of the reporter strain IJ01 compared to those of strains grown in the mLB medium (negative control) and the DH5α supernatants. Similar results have already been demonstrated for EHEC, but maximum AI-3-mediated induction of the LEE genes was shown for mid-exponential culture supernatants (32). In addition, mLB with epinephrine significantly induced relative fluorescence compared to mLB without epinephrine ($P < 0.024$) (Fig. 1B). These results confirmed the applicability of the fluorescence assay for the detection of *ler* expression.

Construction of the *L. reuteri* ATCC 55730 *luxS* mutant strain *L. reuteri* LTH6560. An isogenic *luxS* mutant of *L. reuteri* strain ATCC 55730 was constructed by insertional inactivation, using the suicide vector pORI28 as described previously (30). An internal sequence of the *luxS* gene (bp 46 to 260 of lr0628; GenBank accession no. DQ233673) was amplified by using the primers luxSFor (TGACGAATTCTAAGCACCTTACGTTC GTTTAATTACC [the EcoRI recognition site is underlined]) and luxSRev (TGACGGATCCGTAATTAAGTGGAAACC AGTCGG [the BamHI recognition site is underlined]), cloned into pORI28 by using EcoRI and BamHI, and inserted in the chromosomal *luxS* open reading frame by homologous recombination. The correct localization of pORI28 in the *luxS* open reading frame and the singular insertion event in the chromosome of the obtained mutant strain *L. reuteri* LTH6560 were verified by PCR using the primers LuxCoF (GCACCTTACG TTCGTTTAATTACC) and LuxCoR (TCCCTTCATCAAGA ATCTTC), flanking the insertion site, and by Southern blot hybridization, respectively (data not shown).

Influence of *L. reuteri* supernatants on *ler* expression. Culture supernatants of the *L. reuteri* strains ATCC 55730, LTH6560, and 100-23C (13) and the *luxS* mutant of strain 100-23C (28) were tested for the ability to influence *ler* expression in the *E. coli* IJ01 bioassay. *L. reuteri* strains were grown anaerobically in modified MRS (mMRS) medium (9) at 37°C. Supernatants were prepared from cultures of different growth phases (OD_{600} of 0.1 to 2.5) by centrifugation, with pH adjustment to 7.0 and sterile filtration. mMRS medium (pH 7.0) was used as an AI-3-negative control. Prior to the start of the bioassay, *E. coli* IJ01 was cultured as described above and

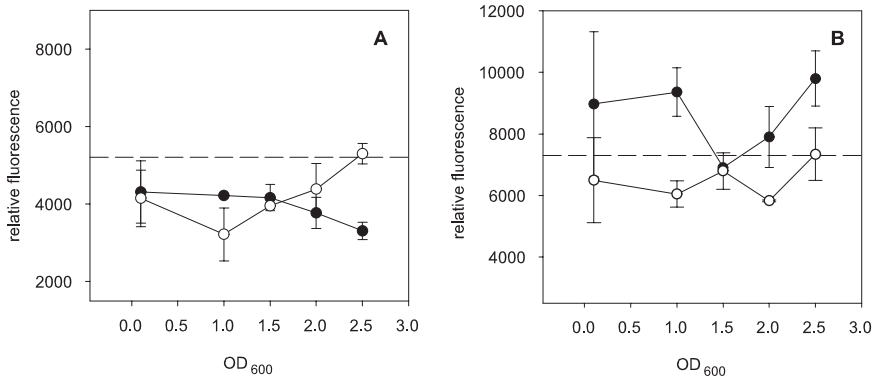

FIG. 2. Influence of culture supernatants of *L. reuteri* ATCC 55730 strains (A) and *L. reuteri* 100-23C strains (B) on *ler* expression, detected by applying the *E. coli* IJ01 fluorescence bioassay. Relative fluorescence inductions obtained with supernatants of the wild-type strains (filled circles) and of the corresponding isogenic *luxS* mutants (open circles). Dashed lines indicate the mean of the relative fluorescence induction of the negative control mMRS medium. Error bars denote standard deviations of three independent experiments performed in duplicate.

inoculated at a 1:20 dilution into *L. reuteri* supernatants, and the mMRS medium was adjusted to pH 7.0. Aliquots (200 μl) were transferred to a microtiter plate, and incubation and measurements were conducted as described above for the testing of *E. coli* supernatants.

The supernatants of the *L. reuteri* strains ATCC 55730 and 100-23C and the *luxS* mutants exhibited different effects on *ler* expression (Fig. 2). The *L. reuteri* ATCC 55730 (wild type) exponential-phase supernatants (OD_{600}, 0.1) caused lower relative fluorescence than the negative control mMRS medium and led to a constant decrease in fluorescence with increasing OD_{600} (Fig. 2A). At the stationary phase (OD_{600}, 2.5), *ler* expression was significantly repressed compared to that of the *luxS* mutant LTH6560 ($P < 0.005$), as well as that of the mMRS medium ($P < 0.0001$). Supernatants of strain LTH6560 caused levels of *ler* expression similar to that of the wild type at an OD_{600} of 0.1 and lower expression at an OD_{600} of 1.0; but in the stationary phase (OD_{600} of 2.5), the fluorescence exceeded the corresponding wild-type values, equalling the mMRS medium values. In contrast, the *L. reuteri* 100-23C (wild type) supernatants of exponential and early stationary phases (OD_{600} 0.1 and 1.0, respectively) induced levels of *ler* expression comparable to that of the *luxS* mutant and the mMRS medium, caused a minimum expression at an OD_{600} of 1.5, and induced *ler* expression at an OD_{600} of 2.5 compared to that of the *luxS* mutant and the mMRS medium (Fig. 2B). The 100-23C *luxS* mutant supernatants did not induce fluorescence above the negative control with mMRS medium.

Surprisingly, the supernatants of *L. reuteri* ATCC 55730 exhibited a negative regulatory effect on *ler* expression compared to those of the *luxS* mutant and growth medium alone, whereas *L. reuteri* 100-23C supernatants induced *ler* transcription as described previously (28). These results indicated a LuxS-dependent interference with AI-3-mediated QS. To further investigate the nature of repression, we added epinephrine to supernatants of *L. reuteri* grown to OD_{600} values of 2.0 and 2.5 and investigated *ler* regulation. mMRS medium (pH 7.0) containing 50 μM L-epinephrine and without epinephrine served as positive and negative control, respectively. The results depicted in Fig. 3 indicated a similar fluorescence induction for

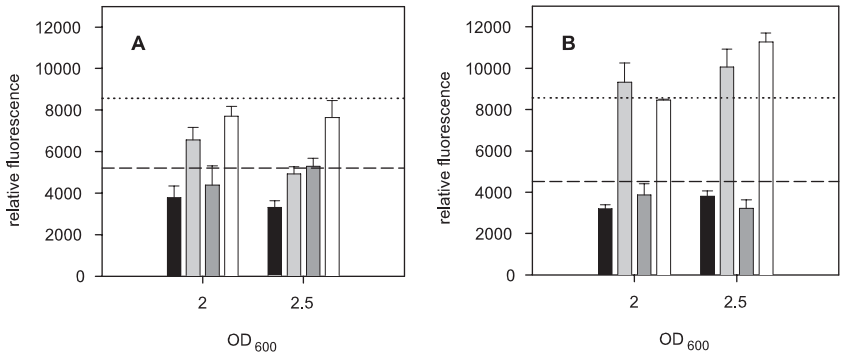

FIG. 3. Impact of the addition of 50 μM L-epinephrine to stationary-phase supernatants of *L. reuteri* on *ler* expression. (A) Relative fluorescence obtained with *L. reuteri* ATCC 55730 (wild-type) supernatants without epinephrine (black bar) and with epinephrine (light gray bar) and with *L. reuteri* LTH6560 (*luxS* mutant) supernatants without epinephrine (dark gray bar) and with epinephrine (white bar). (B) Relative fluorescence obtained with *L. reuteri* 100-23C (wild-type) supernatants without epinephrine (black bar) and with epinephrine (light gray bar), and with *L. reuteri* 100-23C *luxS* mutant supernatants without epinephrine (dark gray bar) and with epinephrine (white bar). Dashed lines indicate the mean relative fluorescence of the negative control mMRS medium, and dotted lines mean relative fluorescence of mMRS medium supplemented with epinephrine used as positive control. Error bars denote standard deviations of two independent experiments performed in duplicate.

spiked 100-23C wild-type and *luxS* mutant supernatants compared to that of the positive control mMRS medium with epinephrine (Fig. 3B) but reduced fluorescence caused by spiked ATCC 55730 wild-type supernatant (Fig. 3A). Evidently, the full extent of epinephrine-mediated *ler* induction was constrained by secreted substances of *L. reuteri* ATCC 55730 but not by those of its isogenic *luxS* mutant nor by those of the *L. reuteri* 100-23C wild type and the *luxS* mutant.

On the basis of the results obtained, we propose that LuxS of *L. reuteri* ATCC 55730 is responsible, either directly or indirectly, for the production and/or secretion of molecules that negatively regulate *ler* transcription in the stationary phase (OD_{600}, 2.5). Since LuxS affects the central metabolism, a mutation consequently leads to pleiotropic effects; thus no conclusion can be drawn about the nature of these molecules. Interestingly, the LuxS activity differs among strains of the same species, leading to opposite effects on EHEC virulence gene transcription. A possible explanation of the mode of action would be that the secreted molecules of *L. reuteri* ATCC 55730 share structural homology with AI-3 or epinephrine and bind to the sensor kinase QseE or QseC, blocking the phosphorylation of the cognate response regulators QseF and QseB. This could abolish the signal transduction cascades analogous to alpha- and beta-adrenergic antagonists (25). However, a negative regulation of *ler* expression via these two-component systems has not been described to date. The facts that *ler* expression is significantly reduced by *L. reuteri* ATCC 55730 compared to that of medium and of the *luxS* mutant supernatant and that the epinephrine stimulus does not result in a full induction of *ler* transcription support the hypothesis of competing antagonistic molecules.

The in vivo relevance of EHEC virulence gene repression by *L. reuteri* ATCC 55730 and the underlying principles remain to be investigated. Nevertheless, it seems a promising approach for anti-QS-based therapeutic strategies for the treatment of infectious diseases that recently have gained considerable research interest (2, 7), for example, the inhibition of *Pseudomonas aeruginosa* or *Staphylococcus aureus* infections by QS interference (17, 19). Furthermore, the virulence gene repression was shown for the two strains *L. reuteri* ATCC 55730 and *L. acidophilus* La-5, which are proven probiotics, whereas the nonprobiotic *L. reuteri* 100-23C does not display this ability. Whether the observed repression is a characteristic of probiotic lactobacilli should be the subject of further investigation.

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