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Research Letter

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Heterologous expression of equol biosynthesis genes from *Adlercreutzia equolifaciens*

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One sentence summary: Heterologous equol production from synthetic genes based on those of *Adlercreutzia equolifaciens*.

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ABSTRACT

Equol is the isoflavone-derived metabolite with the greatest estrogenic and antioxidant activity. It is produced from daidzein by fastidious and oxygen-susceptible intestinal bacteria, which hinders their use at an industrial scale. Therefore, expressing the equol production machinery into easily-cultivable hosts would expedite the heterologous production of this compound. In this work, four genes (*racemase, tdr, ddr* and *dzr*) coding for key enzymes involved in equol production in *Adlercreutzia equolifaciens* DSM19450^T were synthesized and cloned in a pUC-derived vector (pUC57-equol) that was introduced in *Escherichia coli*. Recombinant clones of *E. coli* produced equol in cultures supplemented with daidzein (equol precursor) and dihydrodaidzein (intermediate compound). To check whether equol genes were expressed in Gram-positive bacteria, the pUC57-equol construct was cloned into the low-copy-number vector pIL252, and the new construct (pIL252-pUC57-equol) introduced into model strains of *Lacticaseibacillus casei* and *Lactococcus lactis*. *L. casei* clones carrying pIL252-pUC57-equol produced a small amount of equol from dihydrodaidzein but not from daidzein, while *L. lactis* recombinant clones produced no equol from either of the substrates. This is the first time that *A. equolifaciens* equol genes have been cloned and expressed in heterologous hosts. *E. coli* clones harboring pUC57-equol could be used for biotechnological production of equol.

Keywords: soy isoflavones; daidzein; equol; *Adlercreutzia equolifaciens*; daidzein reductase; dihydrodaidzein reductase; tetrahydrodaidzein reductase; gene cloning; gene expression

INTRODUCTION

Epidemiological and interventional studies suggest that consumption of soy and soy products helps to prevent, and can be used to treat, postmenopausal symptoms, cardiovascular and neurological diseases, osteoporosis and hormone-dependent cancers (Smeriglio *et al.* 2019; Zaheer and Humayoun Akhtar 2017). The beneficial effects of soy are attributed to the isoflavones and their bacteria-derived metabolites. Chemically, some of these compounds resemble the endogenous 17-β-estradiol and possess hormone-like activity (Vitale *et al.* 2013; Franke, Lai and Halm 2014). Among the isoflavone-derived metabolites, equol, formed from daidzein in the human intestine by a few bacterial species, is the isoflavone metabolite

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with the strongest estrogenic and antioxidant activity (Mayo, Vázquez and Flórez 2019). However, it is produced in just 25–50% of people, depending on the human population to which they belong and their dietary habits. The persons carrying in their gut microbiota equol-producing species might be the only ones to fully benefit from isoflavone consumption (Birru *et al.* 2016).

Several equol-producing bacterial strains from the human gut have been identified and characterized over the last decades (Mayo, Vázquez and Flórez 2019). Most belong to minority populations of strict anaerobic species within the family *Eggerthellaceae* (phylum Actinobacteria) (Salam *et al.* 2020), including *Adlercreutzia equolifaciens*, *Slackia isoflavoniconvertens* and *Slackia equolifaciens* (Vázquez *et al.* 2017; Braune and Blaut 2018). Equol biosynthesis in these bacteria proceeds via dihydrodaidzein and tetrahydrodaidzein intermediates in a process involving a racemase plus daidzein reductase, dihydrodaidzein reductase and tetrahydrodaidzein reductase (Tsuji *et al.* 2012; Schröder *et al.* 2013). Indeed, equivalent and very similar racemase and reductase enzymes have been found in every equol-producing strain characterized so far, always encoded by the correponding genes organized in a 10 kb operon-like structure (Shimada *et al.* 2011; Schröder *et al.* 2013; Flórez *et al.* 2019).

Equol-producing bacteria are fastidious and extremely oxygen-susceptible, which hinders their use in the biotechnological production of equol at the industrial scale (Clavel, Lepage and Charrier 2014). Cloning the genetic machinery of equol producers into model organisms might, however, overcome this problem. The genes of a *Lactococcus garvieae* equol-producing strain coding for the above three reductases (*dzr, ddr* and *tdr*) have already been cloned and expressed in *Escherichia coli* (Shimada *et al.* 2010, 2011, 2012), as have those of *S. isoflavoniconvertens* and *Eggerthella* sp. YY7918, with all the resulting recombinant bacteria producing this compound (Schröder *et al.* 2013; Kawada *et al.* 2016; Lee *et al.* 2017; Peirotén, Gaya and Landete 2020). Large-scale production would allow for a greater number of human trials to evaluate the health benefits of equol, and help extend them to individuals beyond those that harbor equol-producing microbes in their gut. In particular, lactic acid bacteria (LAB) species and strains have qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel 2020). They, therefore, offer a potentially safe vehicle for producing equol. Genetically modified LAB capable of developing in soybean extracts (Delgado *et al.* 2019) might even be used to produce fermented soybean products enriched in equol.

The present work reports the cloning in suitable vectors of a synthetic DNA fragment based on sequences of the equol operon from *A. equolifaciens* DSM19450^T and its subsequent expression in *E. coli*, *Lacticaseibacillus casei* and *Lactococcus lactis* (Maruo *et al.* 2008). This fragment contains the genes *dzr, ddr* and *tdr* under their native expression signals, preceded by the *racemase* gene under the control of a strong constitutive promoter from *L. lactis*. The conversion of daidzein into equol was seen in the recombinant *E. coli*, and small amounts were produced by *L. casei* when dihydrodaidzein was used as a substrate. No equol was ever produced by recombinant cells of *L. lactis*.

MATERIALS AND METHODS

Plasmids, bacteria and culture conditions

The bacterial strains and plasmid vectors used in the present work are summarized in Table 1. *Escherichia coli* DH10B was grown in Luria Bertani (LB) or 2xTY broth media with shaking at 37°C. *Lactococcus lactis* NZ9000 and *Lacticaseibacillus casei* BL23 were grown statically at 32°C in M17 medium (Biokar, Beauvais, France) supplemented with 1% (w/v) glucose (GM17) and MRS (Merck, Darmstadt, Germany), respectively. Agar (2% w/v) was added to the media when required. Media were also supplemented with antibiotics for the selection of transformants and plasmid maintenance (100 μg/mL ampicillin and 300 μg/mL erythromycin for *E. coli*, and 2.5 or 5 μg/mL of erythromycin, respectively, for *L. lactis* and *L. casei*).

Design of synthetic DNA

The sequence of the equol biosynthesis gene cluster from *Adlercreutzia equolifaciens* DSM19450^T was retrieved from GenBank (accession number NC_022567.1). The sequence of four open reading frames (ORFs) thought to be involved in the synthesis of equol by their homology to equol sequences from other species, including the *racemase* gene (AEQU_2234), and those encoding the downstream reductases tetrahydrodaidzein reductase (*tdr* , AEQU_2231), dihydrodaidzein reductase (*ddr* , AEQU_2230) and daidzein reductase (*dzr* , AEQU_2228; Fig. 1), were codon-optimized with the NG Codon System (Synbio Technologies; Monmouth Junction, NJ) using default settings for *E. coli*. The native intergenic expression signals were left in place, but the first gene (the *racemase*) was located under the control of the constitutive promoter P59 from *L. lactis* (van der Vossen, van der Lelie and Venema 1987). To facilitate cloning, some restriction enzyme sites were removed from or added to the sequence, and 20 bp flanking sequences identical to those flanking the multiple cloning site of pUC57 were annexed. A final synthetic DNA consisting of 5206 bp (Figure S1, Supporting Information) was synthesized at Synbio Technologies (Monmouth Junction, NJ).

DNA manipulation and cloning

General procedures for *in vitro* DNA manipulation were followed essentially as described by Sambrook and Russell (2001). Restriction endonucleases (Takara, Otsu, Shiga, Japan) and T4 DNA ligase (Invitrogen, Carlsbad, CA) were used as recommended by their manufacturers. DNA from agarose gels was purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare Biosciences, Buckinghamshire, UK). The In-Fusion cloning kit (Clontech, Mountain View, CA) was used according to the manufacturer's instructions to join the synthetic DNA and the linearized pUC57.

Electrocompetent cells of *E. coli* DH10B were prepared as reported by Sambrook and Russell (2001). Electrocompetent *L. casei* and *L. lactis* cells were prepared according to the procedure by Holo and Nes (1989). Electrotransformation (electroporation) was performed using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) following standard protocols for Gram-negative and Gram-positive bacteria. White/blue screening for pUC57 in *E. coli* was performed on LB plates supplemented with appropriate antibiotics, and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal; 20 mg/mL; Sigma-Aldrich, St. Louis, CA) and isopropyl-ß-D-thiogalactopyranoside (0.5 M; Sigma-Aldrich). Transformants of *L. lactis* and *L. casei* were selected on GM17 or MRS agar plates with erythromycin.

Plasmid DNA from *E. coli* was isolated and purified as described by Sambrook and Russell (2001). Plasmids from *L. casei* and *L. lactis* were isolated using a modified version of the procedure described by O'Sullivan and Klaenhammer (1993), involving the addition of 4 μL of mutanolysin (5 U μL) and 20 μL

Table 1. Bacterial strains, synthetic DNA and plasmids utilized in the present study.

Strain, synthetic DNA, plasmid	Relevant genotype, description or properties	Reference or source
Strains		
Escherichia coli DH10B	F⁻, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, recA1, endA1, araD139, Δ(ara-leu)7697, galU, galK, λ⁻, rpsL(StrR), nupG	ThermoFisher Scientific
Lactococcus lactis NZ9000	*Lc. lactis* subsp. *cremoris* MG1363 derivative *pepN::nisRK*; plasmid-free	Kuipers *et al.* (1998)
Lacticaseibacillus casei BL23	Plasmid free strain	Acedo-Félix and Pérez-Martínez (2003)
E. coli pUC57	*E. coli* DH10B carrying pUC57; Am^r	This study
L. lactis pIL252	*L. lactis* NZ9000 carrying pIL252; Em^r	This study
E. coli DH10B-pUC57-equol	*E. coli* DH10B carrying pUC57-equol; Am^r	This study
E. coli DH10B-pIL252-pUC57-equol	*E. coli* DH10B carrying pIL252-pUC57-equol; Am^r	This study
L. lactis NZ9000-pIL252-pUC57-equol	*L. lactis* NZ9000 carrying pIL252-pUC57-equol; Em^r	This study
L. casei BL23-pIL252-pUC57-equol	*L. casei* BL23 carrying pIL252-pUC57-equol; Em^r	This study
Plasmids		
pUC57	pUC19-derived general cloning vector; Am^r	ThermoFisher Scientific
pUC57-equol	Recombinant plasmid containing the synthetic DNA carrying the equol genes *racemase*, *tdr*, *ddr* and *dzr* preceded by P59 promoter cloned in pUC57; Am^r	This study
pIL252	Low copy-number cloning vector for Gram-positives, based on the replicon of pAMβ1 from *Enterococcus faecalis*; Em^r	Simon and Chopin (1988)
pIL252-pUC57-equol	Recombinant plasmid containing pUC57-equol cloned in pIL252; Am^r, Em^r	This study
Synthetic DNA		
-equol	A DNA segment of 5206 nucleotides long (Figure S1, Supporting Information), including four ORFs based on the genome sequence of *Adlercreutzia equolifaciens* DSM19450^T: *racemase* (AEQU_2234[a]), tetrahydrodaidzein reductase (*tdr*, AEQU_2231), dihydrodaidzein reductase (*ddr*, AEQU_2230) and daidzein reductase (*dzr*, AEQU_2228).	This study

Am^r, resistance to ampicillin; Em^r, resistance to erythromycin.
[a]Numbering of ORFs as in the *A. equolifaciens* DSM19450^T genome accession number NC_022567.1.

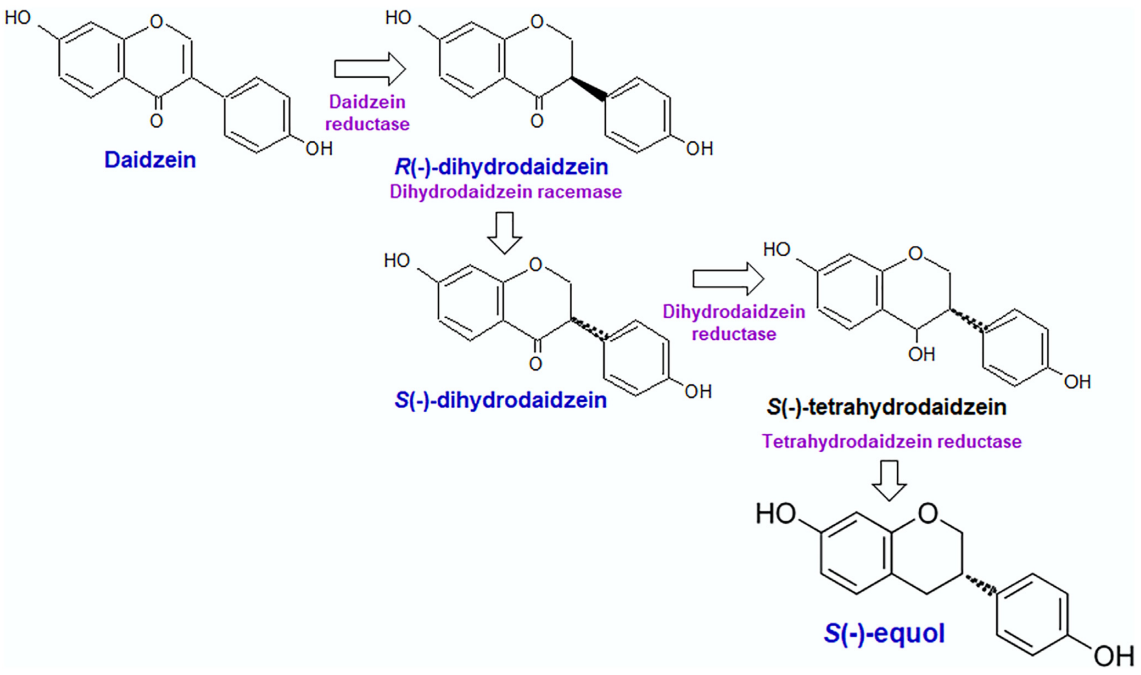

Figure 1. Substrates and enzymes of the equol biosynthesis pathway from daidzein. In color, enzymes (purple) involved in equol production and metabolites analysed (blue).

of proteinase K (20 mg/mL) to the lysis buffer before phenol-chloroform extraction. Constructs were verified by digestion with restriction enzymes, sequencing and sequence analysis.

Identification and quantification of isoflavones

Single colonies of each clone were selected and grown in 5 mL of appropriate liquid medium and incubated overnight under the species-specific conditions stated above. These cultures were then used for inoculating into 1% fresh medium supplemented with 200 μM of daidzein or dihydrodaidzein (both from LC Laboratories, Woburn, MA). As negative controls, plasmid-free bacterial hosts and strains carrying empty pUC57 and pIL252 plasmids were cultured under the same conditions. Isoflavones and their metabolites were extracted from the supernatant of triplicate cultures (except for the controls, for which only a supernatant was analysed) after filtering through a 0.2 μm PTFE membrane (VWR, Radnor, PA) according to the procedure by Guadamuro *et al.* (2015). Metabolite separation and detection were achieved by high-performance liquid chromatography (HPLC). Metabolite quantification was determined against calibration curves prepared using commercial standards (all from LC Laboratories).

RESULTS

To express the *A. equolifaciens*-based genes in *E. coli*, the synthetic DNA was first cloned *in vitro* in pUC57 using the In-Fusion technique and the cloning mixture transformed into electrocompetent *E. coli* cells. Purified colonies of several transformants were selected at random and verified by restriction analysis. Further, one clone carrying an insert of the right size was verified by sequencing. Recombination between the identical 20 bp sequences at the two ends of the synthetic DNA and the linearized pUC57 extremities, produced a new molecule (pUC57-equol) in which the whole multi-cloning site of the vector was replaced by 5166 bp of the synthetic DNA (Fig. 2). The pUC57-equol plasmid was transformed again into *E. coli*. The novel clones carrying this construct were inoculated in a culture medium supplemented with 200 μM of daidzein or dihydrodaidzein, the substrate precursor and an intermediate of equol, respectively (Fig. 1), and incubated at 37°C. Supernatants from the cultures were sampled at 0, 8 and 24 h of incubation and analysed for daidzein, dihydrodaidzein and equol. No differences in bacterial growth in the presence of daidzein or dihydrodaidzein were observed among controls and clones (data not shown). As expected, the supplemented daidzein or dihydrodaidzein were recovered untransformed from control cultures, while the different *E. coli* clones converted these substrates into equol to different extents (Table 2 and Figure S2, Supporting Information). Cultures of pUC57-equol converted around 90% of the daidzein into equol at 24 h. However, and surprisingly, equol production by *E. coli* via this construct was only partial when dihydrodaidzein was used as a substrate (≈15%). Despite these variations, the results showed that the synthetic *A. equolifaciens*-based equol sequences were functional in *E. coli* and allowed for the synthesis of equol from both daidzein and dihydrodaidzein.

The pUC57-equol construct and pIL252 vector were independently digested with EcoRI, ligated with T4 ligase and the ligation mixture transformed into electrocompetent *E. coli* cells. Cloning of pUC57-equol in pIL252 resulted in the formation of pIL252-pUC57-equol (Fig. 2). This construct could have been created in either *L. lactis* or *L. casei*, but this procedure is straightforward in *E. coli*. After verification, *E. coli* transformants carrying the pIL252-pUC57-equol were cultured in the presence of daidzein and dihydrodaidzein and the supernatants analysed as before. At 24 h, equol was recovered from *E. coli* cultures, indicating again that the biochemical pathway for equol production supplied by pIL252-pUC57-equol was functional, although the amount of equol produced with this construct was much lower than with pUC57-equol (Table 2).

The plasmid pIL252-pUC57-equol was then electrotransformed into *L. casei* and *L. lactis*. Transformants from the two strains carrying the construct were cultured in MRS or GM17, respectively, with daidzein or dihydrodaidzein and the supernatants analysed as before. Equol was detected in supernatants at 24 h of incubation for *L. casei* clones carrying pIL252-pUC57-equol only when dihydrodaidzein was used as a substrate (Table 2). No *L. lactis* clone producing equol from either daidzein or dihydrodaidzein was ever detected. To check whether these results derived from the non-functionality of the genes or mutations, the pIL252-pUC57-equol construct from *L. casei* and *L. lactis* was purified and transformed back into *E. coli*. The new *E. coli* transformants produced equol in amounts equal to that initially measured (10–12 μM).

DISCUSSION

The evidence that isoflavone-rich diets help reduce the risk of different syndromes and chronic diseases obtained in many intervention studies and via meta-analyses is far from conclusive (Fang *et al.* 2016; Akhlaghi, Zare and Nouripour 2017; Zhou and Yuan 2015; Liu *et al.* 2014; He and Cheng 2013; Harland and Haffner 2008; Bolaños, Del Castillo and Francia 2010; Wei *et al.* 2012). The discrepancies in the results have been attributed to a large part of the human population possessing a non-equol-producing phenotype, due to the absence of equol-producing microbes in their intestine (Daily *et al.* 2019). Since equol has the strongest hormonal activity and the highest antioxidant action of all isoflavone metabolites (Setchell and Cole 2006), variation in the results of an intervention might thus be expected depending on the human population sampled. To be more certain about its health benefits, large-scale biotechnological production is necessary; this would overcome equol shortages, enabling more trials to be conducted (Selvaraj *et al.* 2004). However, equol is produced from the plant isoflavone daidzein by strict anaerobes with many nutritional needs (Salam *et al.* 2020), hampering their use in the industrial-scale of equol production, as a result of the requirement of high investment inexpensive ingredients and equipment (Clavel, Lepage and Charrier 2014). The 'aerobic domestication' of equol-producing strains has been suggested as a means of overcoming these difficulties (Zhao *et al.* 2011), as has the cloning of the equol production machinery into easily cultivable heterologous hosts (Tsuji *et al.* 2012; Schröder *et al.* 2013).

In the present work, the four synthetic genes based on sequences from the *A. equolifaciens* equol operon, drove equol production in *E. coli* when either daidzein or dihydrodaidzein was present as a substrate. This is not surprising; the expression of equol genes from other equol-producing species in *E. coli* has been reported before (Kawada *et al.* 2016; Shröder *et al.* 2013; Shimada *et al.* 2010, 2011, 2012). In agreement with the present results, equol production by recombinant clones at the μmolar level has also been reported in other works (Lee *et al.* 2016; Li *et al.* 2018). This is attributed to the poor solubility of isoflavones (and thus daidzein) in aqueous systems (del Rio *et al.* 2013), which restrains using larger amounts of daidzein. This problem has recently been overcome by adding hydrophilic polymers to the culture medium (Lee *et al.* 2018). Nonetheless, the daidzein to

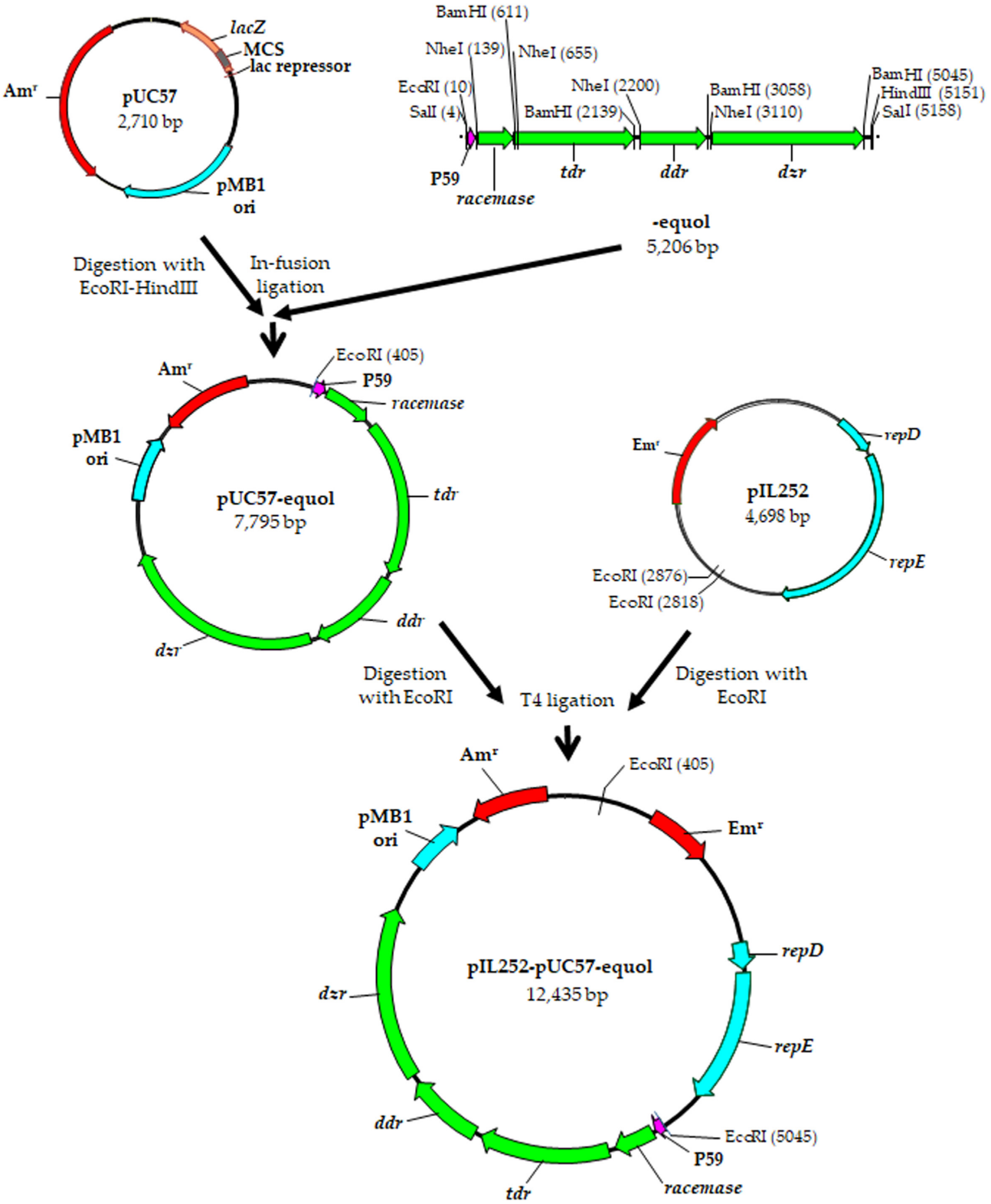

Figure 2. Physical map of the plasmid constructs obtained in this work. The synthetic DNA cloned in pUC57 carries four equol-related genes from *A. equolifaciens* DSM19450T: the genes *racemase*, *tdr*, *ddr* and *dzr*, which encode a racemase, and the tetrahydrodaidzein, dihydrodaidzein and daidzein reductase, respectively. The genes are preceded by the P59 promoter from *L. lactis* subsp. *cremoris* Wg2. Color key: in red, antibiotic resistance genes (Am^r, ampicillin resistance; Em^r, erythromycin resistance); in light blue, the origin of replication; in orange, the gene encoding the β-galactosidase-complementing peptide, including the multiple cloning site (MCS) in brown; in green, genes involved in equol production; in purple, P59 promoter. Relevant restriction enzyme sites are also indicated. Molecules are not drawn to scale.

Table 2. Daidzein and its derived metabolites (in μM) from daidzein- or dihydrodaidzein-supplemented cultures of recombinant *E. coli* DH10B, *L. casei* BL23 and *L. lactis* NZ9000 cells harboring equol-associated genes from *A. equolifaciens* DSM19450^T.

Substrate/strain/construct	Daidzein	Dihydrodaidzein Sampling at 0 h	Equol	Daidzein	Dihydrodaidzein Sampling at 8 h	Equol	Daidzein	Dihydrodaidzein Sampling at 24 h	Equol
Daidzein (200 μM)									
E. coli pUC57-equol	34.1 ± 1.1	–	–	8.2 ± 0.9	105.8 ± 2.3	0.2 ± 0.1	–	–	179.6 ± 38.6
E. coli pIL252-pUC57-equol	24.9*	–	–	10.4 ± 1.0	114.2 ± 14.2	2.5 ± 0.4	9.4 ± 2.6	132.1 ± 11.3	11.1 ± 2.1
L. casei pIL252-pUC57-equol	70.9 ± 1.7	–	–	30.9 ± 5.1	–	–	38.7 ± 9.6	–	–
L. lactis pIL252-pUC57-equol	23.6 ± 2.4	–	–	23.4 ± 2.6	–	–	29.0 ± 0.6	–	–
E. coli	25.8*	–	–	9.3*	–	–	44.5 ± 6.9	–	–
E. coli pUC57	nd	nd	nd	nd	nd	nd	147.4*	–	–
L. casei	67.9*	–	–	23.5*	–	–	35.4*	–	–
L. lactis	30.7*	–	–	31.9*	–	–	26.8*	–	–
L. lactis pIL252	nd	nd	nd	nd	nd	nd	255.7*	–	–
Dihydrodaidzein (200 μM)									
E. coli pUC57-equol	–	181.0 ± 2.4	–	12.9*	146.2 ± 32.3	0.6*	–	84.1 ± 5.9	34.9 ± 2.1
E. coli pIL252-pUC57-equol	–	98.8 ± 25.8	–	4.7 ± 0.2	155.4 ± 4.9	2.3 ± 0.3	–	149.4 ± 8.2	12.0 ± 2.7
L. casei pIL252-pUC57-equol	–	136.5 ± 3.1	–	–	126.1 ± 6.4	–	–	48.6 ± 2.9	4.8 ± 2.2
L. lactis pIL252-pUC57-equol	–	233.7 ± 37.7	–	–	201.7 ± 12.6	–	–	182.5 ± 1.0	–
E. coli	–	193.6*	–	2.7*	187.2*	–	–	119.9*	–
E. coli pUC57	nd	nd	nd	nd	nd	nd	–	175.2*	–
L. casei	–	191.2*	–	–	190.4*	–	–	176.6*	–
L. lactis	–	188.6*	–	–	191.3*	–	–	204.2*	–
L. lactis pIL252	nd	nd	nd	nd	nd	nd	–	148.6*	–

nd, not determined; -, not detected or below the limit of quantification; ∗data from a single experiment.

equol conversion ratio of *E. coli* clones carrying pUC57-equol at 24 h (≈90%), higher than that obtained with the original strain *A. equolifaciens* DSM19450^T (Flórez *et al.* 2019), is considered a promising result for industrial scale-up implementation.

The fact that the recombinant *E. coli* cultures carrying pUC57-equol produced more equol than those carrying pIL252-pUC57-equol might be explained by the larger size of the latter construct, which may lead to a reduced copy number. However, it cannot be ruled out that the larger construct is less stable. Indeed, the pUC57-equol was occasionally seen to be somewhat unstable, lacking some clones the ability to produce equol. This instability might also account for the large variation in equol production seen between different cultures of replicates of the same clone, giving rise to high standard deviations. Certainly, instability has also been reported for equol genes from *S. isoflavoniconvertens* in *E. coli*. As such, Lee and co-workers have shown that mutations in the *ddr* gene (encoding the dihydrodaidzein reductase) helped to stabilize the cloned genes, leading to greater equol production (Lee *et al.* 2016). Mutations in *E. coli* housekeeping genes have also been shown to promote equol formation in cultures. In that sense, a mutation in *ydiS*, which codes for a putative electron-transfer flavoprotein-quinone oxidoreductase, has been shown to increase equol production by overcoming the latter compound's inhibition of *E. coli* growth (Li *et al.* 2018).

Given that the equol operon of *L. garvieae* seems to have been acquired by horizontal transfer from an *Eggerthellaceae* species (Shimada *et al.* 2010, 2011), the expression of equol genes in other LAB species ought to be feasible. However, neither the present recombinant *L. casei* nor *L. lactis* strains produced equol from daidzein; indeed, daidzein was recovered (largely) untransformed from the LAB cultures at all sampling points. The fact that a small amount of equol was formed by *L. casei* from dihydrodaidzein, however, indicates that the dihydrodaidzein reductase and the tetrahydrodaidzein reductase enzymes were active, implying that the genes were correctly transcribed and translated. In contrast, no equol was ever detected in *L. lactis* cultures, irrespective of the substrate provided. The P59 promoter of *L. lactis* drives the expression of homologous and heterologous proteins in strains of *E. coli* (van der Vossen, van der Lelie and Venema 1987), *L. lactis* (Que *et al.* 2000; Quistián-Martínez *et al.* 2010) and *L. casei* (Gold *et al.* 1996), suggesting the trouble does not lie at this point. Perhaps, then, some of the intergenic regions containing native *A. equolifaciens* expression signals were not properly identified by the LAB transcription or translation machinery. Moreover, the optimization of codon usage for *E. coli* could have caused the poor (or lack of) expression of some genes in LAB (the GC content of *L. lactis* [34%] is rather low compared to *E. coli* and *L. casei* [≈50%]). Despite similarity in GC content, gene expression in *L. casei*, as compared to *E. coli*, might require specific signals (Bintsis 2018). Alternatively, gene-disrupting or inactivating mutations may have appeared. However, the fact that the transformation of the recombinant DNA from *L. casei* and *L. lactis* back into *E. coli* enabled the latter host to produce equol, indicates the constructs were still functional. Finally, all three reductases are thought to be intracellular enzymes, and transport systems for isoflavone and equol have yet to be identified (Maruo *et al.* 2008; Shimada *et al.* 2011; Schröder *et al.* 2013). As daidzein and its derivatives were analysed in culture supernatants, differences between *E. coli* and LAB species in the import of substrates and/or the secretion of the metabolic end products could also account for the divergences.

In conclusion, synthetic genes coding for equol production, based on sequences from *A. equolifaciens*, were cloned and successfully expressed in *E. coli* and LAB species. To our knowledge, this is the first time that equol genes from *A. equolifaciens* have been cloned and expressed in heterologous hosts. The recombinant *E. coli* clones produced equol from daidzein, while those of LAB strains did not, although recombinant *L. casei* did produce small amounts from dihydrodaidzein. *Escherichia coli* clones harboring pUC57-equol could already be used for the large-scale biotechnological production of equol. Attempts are currently being made to express new synthetic genes in LAB based on those of *A. equolifaciens* but with LAB-specific transcription and translation signals.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

Conflicts of Interest. None declared.

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