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Identification of an Enzyme System for Daidzein-to-Equol Conversion in *Slackia* sp. Strain NATTS

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An *Escherichia coli* library comprising 8,424 strains incorporating gene fragments of the equol-producing bacterium *Slackia* sp. strain NATTS was constructed and screened for *E. coli* strains having daidzein- and dihydrodaidzein (DHD)-metabolizing activity. We obtained 3 clones that functioned to convert daidzein to DHD and 2 clones that converted DHD to equol. We then sequenced the gene fragments inserted into plasmids contained by these 5 clones. All of the gene fragments were contiguous, encoding three open reading frames (ORF-1, -2, and -3). Analysis of *E. coli* strains containing an expression vector incorporating one of the *orf-1*, *-2*, or *-3* genes revealed that (i) the protein encoded by *orf-1* was involved in the conversion of *cis/trans*-tetrahydrodaidzein (*cis/trans*-THD) to equol, (ii) the protein encoded by *orf-2* was involved in the conversion of DHD to *cis/trans*-THD, and (iii) the protein encoded by *orf-3* was involved in the conversion of daidzein to DHD. ORF-1 had a primary amino acid structure similar to that of succinate dehydrogenase. ORF-2 was presumed to be an enzyme belonging to the short-chain dehydrogenase/reductase superfamily. ORF-3 was predicted to have 42% identity to the daidzein reductase of *Lactococcus* strain 20-92 and belonged to the NADH:flavin oxidoreductase family. These findings showed that the daidzein-to-equol conversion reaction in the *Slackia* sp. NATTS strain proceeds by the action of these three enzymes.

Soybean isoflavones and their derivatives have been reported to prevent sex hormone-dependent diseases, such as prostate cancer, breast cancer, menopausal disorders, premenstrual syndrome, and osteoporosis (3, 9, 10, 16, 21, 30). The isoflavone equol is expected to prevent hormone-dependent diseases, such as prostate cancer, because of its ability to bind to dihydrotestosterone and its high capacity to bind to estrogen receptor β; moreover, it is the most potent antioxidant of all the isoflavones (1, 2, 5, 17, 23).

To date, several bacteria capable of producing equol have been isolated from human or animal feces (18–20, 29, 31). Many of these strains are suggested to first metabolize daidzein as a substrate to dihydrodaidzein (DHD) and to then metabolize DHD to equol. Recently, daidzein reductase, which converts daidzein to DHD, has been purified from the equol-producing *Lactococcus* strain 20-92 (25). On the other hand, it has been suggested that, in the *Eggerthella* strain Julong 732, DHD is converted to equol by the production of *cis/trans*-tetrahydrodaidzein (*cis/trans*-THD) as an intermediate metabolite (13, 14). These studies have therefore suggested that daidzein is converted to equol via DHD and *cis/trans*-THD. However, the details of the enzymes involved in the production of equol from daidzein, and of the genes encoding them, remain largely unknown.

We have recently isolated *Slackia* sp. strain NATTS, which has potent daidzein-to-equol conversion ability, from healthy human feces (26). This strain has a more potent daidzein-equol conversion activity than the other equol-producing strains previously reported (26). This paper identifies the genes in *Slackia* sp. strain NATTS responsible for the daidzein-to-equol conversion reaction and examines the function of the enzymes encoded by such genes.

MATERIALS AND METHODS

Bacteria, culture medium, and plasmid. *Slackia* sp. strain NATTS was cultured on modified Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% (wt/vol) glucose. *Escherichia coli* JM109 (TaKaRa Bio, Osaka, Japan) was cultured on Luria-Bertani (LB) medium. For construction of the genomic library of *Slackia* sp. strain NATTS, the plasmids pUC19 (TaKaRa Bio) and pQE30Xa (Qiagen, Valencia, CA) as the expression vectors for the recombination enzymes were used. The amount of ampicillin added to the *E. coli* culture was 100 μg/ml.

Construction of genomic library. Chromosomal DNA was purified from *Slackia* sp. strain NATTS as previously reported (26). Purified chromosomal DNA was partially digested with MboI (Toyobo, Osaka, Japan), and the resulting partially digested DNA and pUC19 completely digested with MboI were ligated by using a ligation convenience kit (NIPPON GENE Co., Ltd., Tokyo, Japan). pUC19 into which the genome fragment had been inserted was transformed into *E. coli* JM109 to yield recombinants.

Screening of daidzein metabolism-related genes. A total of 8,424 recombinants into which the genome fragment had been inserted were inoculated onto 1 ml of GAM broth containing 100 μg/ml ampicillin and 100 μM daidzein (Fujicco Co., Ltd., Osaka, Japan) or DHD (Toronto Research Chemicals Inc., Ontario, Canada); the broth was then cultured at 37°C for 24 h under anaerobic conditions. Isoflavone was extracted from each culture medium and quantified by high-performance liquid chromatography (HPLC). Extraction and quantification of isoflavone were performed as previously described (26). Briefly, 100 μl diethyl ether was added to 200 μl medium, and the mixture was centrifuged at 1,000 $\times$ *g* for 10 min. Then, the upper layer was dehydrated thoroughly at 40°C under a stream of nitrogen gas, and the precipitate was dissolved in 100 μl of 80% (vol/vol) methanol. After filtration, the filtrate was analyzed by

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TABLE 1 Daidzein- or DHD-metabolizing properties in *Escherichia coli* A10, C11, E5, 15, and 203

Clone and plasmid	Substrate (100 μM)	Isoflavone concn (μM)[a]			Daidzein-to-DHD or DHD-to-equol % conversion
		Daidzein	DHD	Equol	
A10	Daidzein	5.4 ± 0.4	95.9 ± 4.5	ND	94.7
	DHD	NT	NT	NT	
C11	Daidzein	3.9 ± 1.0	95.6 ± 1.3	ND	96.1
	DHD	NT	NT	NT	
E5	Daidzein	3.9 ± 1.0	95.6 ± 1.3	ND	96.1
	DHD	NT	NT	NT	
15	Daidzein	92.4 ± 1.0	ND	ND	
	DHD	ND	53.7 ± 0.7	40.5 ± 1.4	43.0
203	Daidzein	90.8 ± 2.0	ND	ND	
	DHD	ND	63.0 ± 0.4	21.3 ± 0.7	25.2
pUC19	Daidzein	106.1 ± 1.9	ND	ND	
	DHD	ND	107.6 ± 1.6	ND	

[a] Data are expressed as means and standard deviations. NT, not tested; ND, not detected.

HPLC under the following conditions: apparatus, LC Module 1 (Waters Corp., Milford, MA); column, YMC-Pack CN (Y.M.C. Co., Kyoto, Japan). Known amounts of daidzein (Fujicco Co.), DHD (Toronto Research Chemicals Inc.), THD (Apin Chemicals Limited, Abingdon, United Kingdom), and equol (Extrasynthése S.A., Genay, France) were used as isoflavone standards.

Determination and analysis of DNA sequences. For cycle sequencing PCR, an ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) was used. The 20-μl reaction mixture contained 1 μl of purified plasmid (30 ng) extracted from *E. coli*, 1.6 μl BigDye Terminator premix, and 8.0 pmol M13R or M13F primer (TaKaRa Bio). Cycle sequencing PCR was performed at an initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The cycle sequencing PCR products were purified by ethanol precipitation, and the precipitate was dissolved in 15 μl of Hi-Di formamide (Applied Biosystems) and sequenced by using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were analyzed with the bio-informatics software program Genetyx version 9 (Genetix Co., Ltd., Tokyo, Japan). For analysis of nucleotide sequences and amino acid sequences, DDBJ-BLAST (http://www.ddbj.nig.ac.jp/), BPROM (SoftBerry), GeneMark version 2.5 (http://opal.biology.gatech.edu/GeneMark/), FindTerm (SoftBerry), and PSORT (http://psort.ims.u-tokyo.ac.jp/) were used.

Analysis of expression of open reading frame 1 (ORF-1), ORF-2, and ORF-3. Plasmids pQESL-1, -2, and -3, into which the *orf-1*, *orf-2*, and *orf-3* genes had been inserted, were generated by using the following procedures. Using as a template genomic DNA extracted from strain NATTS, full-length *orf-1*, *orf-2*, and *orf-3* genes were amplified by PCR with the following primer sets containing a BamHI digestion site: *orf-1* gene, 5′-ATGGCCGAATTCGATGTTG-3′ (ORF1-F) and 5′-GGGGGATCCTAGTATGGGCGAAACCGTT-3′ (ORF1-R-BamHI); *orf-2* gene, 5′-ATGACTACCATTCCTAAGCTCAAGG-3′ (ORF2-F) and 5′-GGGGGATCCTACTCAATTTCGCCCTGCATAG-3′ (ORF2-R-BamHI); and *orf-3* gene, 5′-ATGCAGCACGCGAAATACCC-3′ (ORF3-F) and 5′-GGGGGATCCTAGATCATGCGCGCAACC-3′ (ORF3-R-BamHI). Each of the amplified products completely digested with BamHI was ligated to the StuI and BamHI sites of pQE30Xa (Qiagen) to generate plasmids pQESL-1, -2,

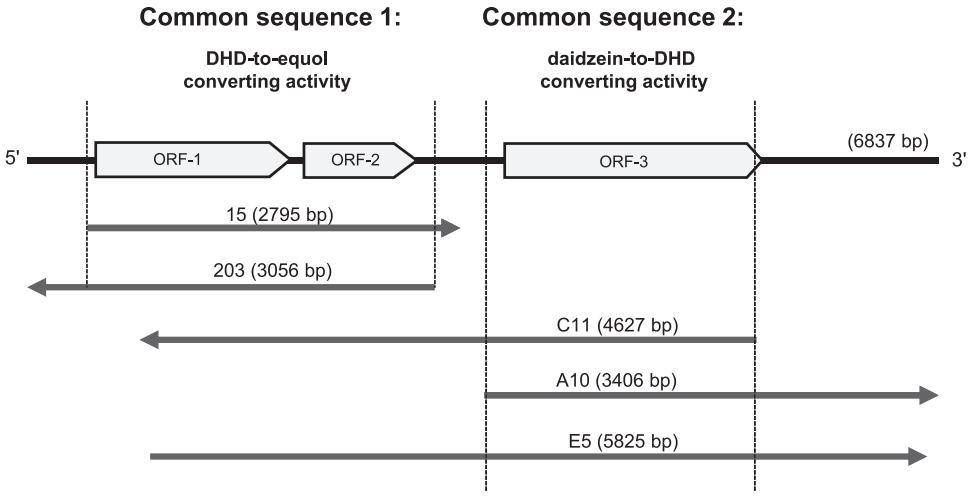

FIG 1 Organization of the genetic fragment in clones expressing daidzein-to-DHD- and DHD-to-equol-converting activity, and cloning of fragments involved in the deduced open reading frames.

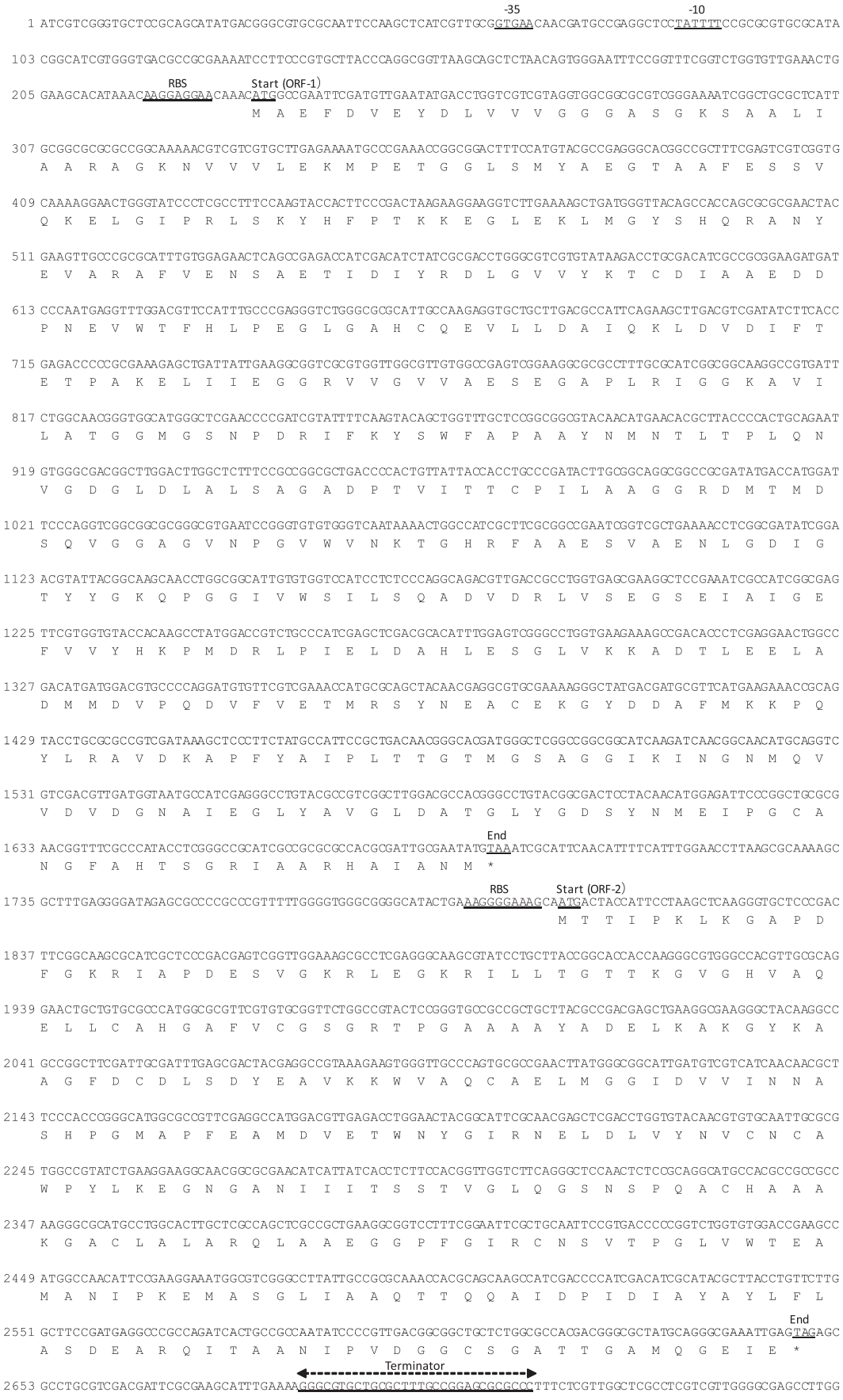

FIG 2 Nucleotide sequences and deduced amino acids of components of DHD-to-equol-converting enzymes (ORF-1 and -2). The putative promoter, ribosome-binding site (RBS), terminator site, and start and stop codons are shown as underlined letters.

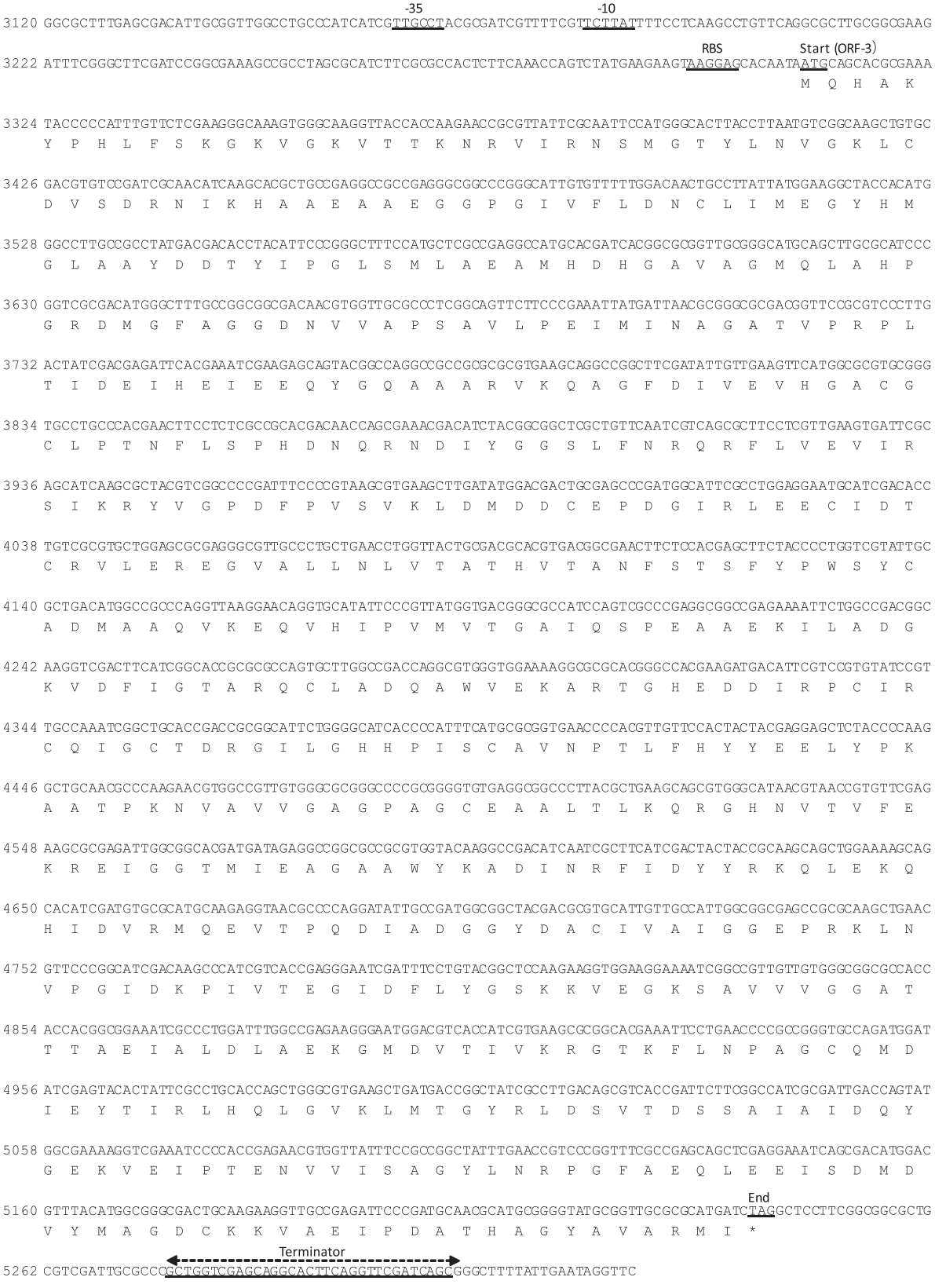

FIG 3 Nucleotide sequences and deduced amino acids of the daidzein-to-DHD-converting enzyme (ORF-3). The putative promoter, ribosome-binding site (RBS), terminator site, and start and stop codons are shown as underlined letters.

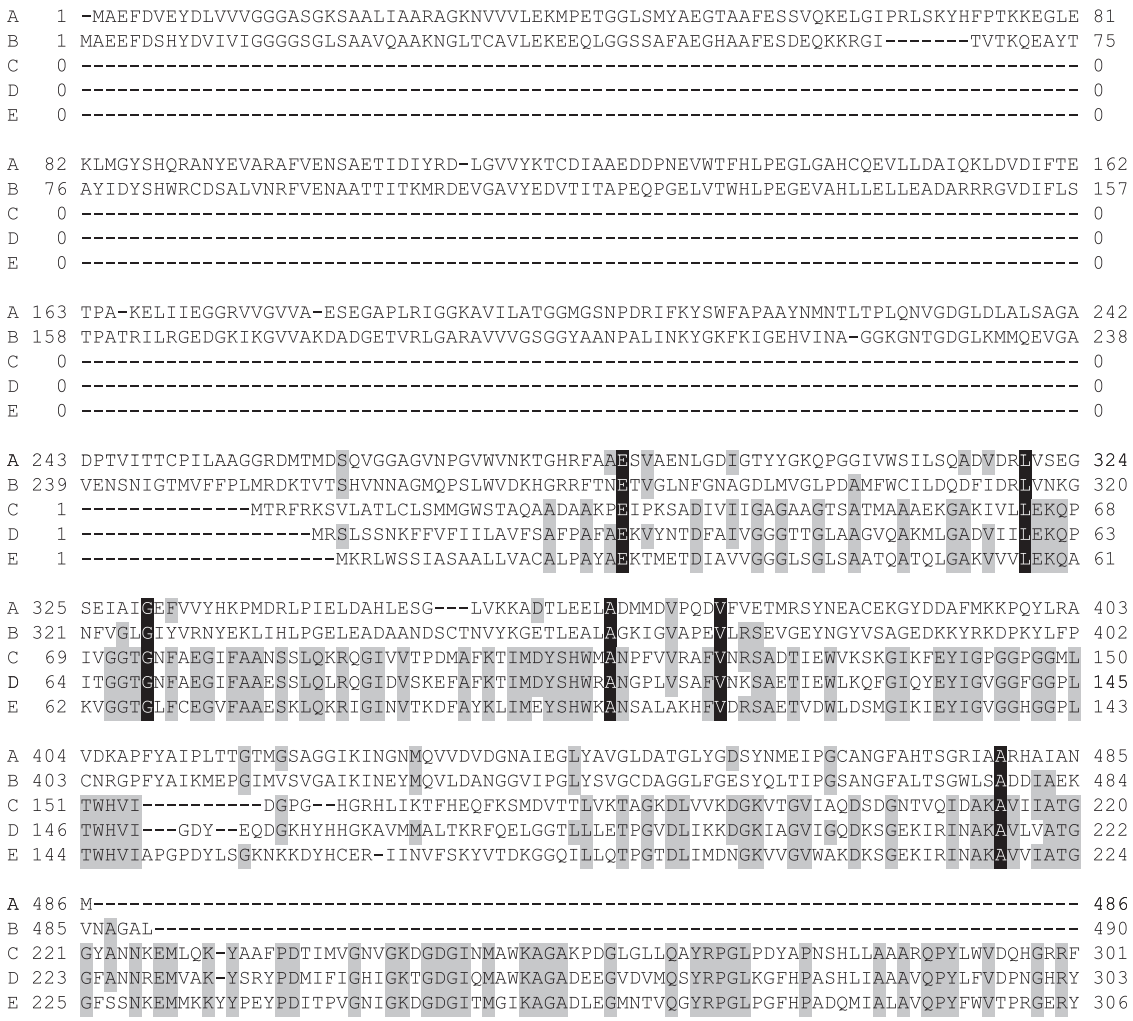

FIG 4 Sequence alignment of ORF-1 (A; accession no. AB646272) from *Slackia* sp. strain NATTS, succinate dehydrogenase (B; accession no. ZP_07333219) from *Desulfovibrio fructosovorans*, putative flavoprotein subunit of a reductase (C; accession no. ZP_03828868) from *Pectobacterium carotovorum* subsp. *brasiliensis* PBR1692, fumarate reductase/succinate dehydrogenase flavoprotein domain protein (D; accession no. YP_003505338) from *Denitrovibrio acetiphilus* DSM 12809, and succinate dehydrogenase (E; accession no. YP_001951188) from *Geobacter lovleyi* SZ. Identical amino acid residues are indicated in black boxes, and three-quarter-matched amino acid residues are in gray boxes.

and -3, each of which was transformed into *E. coli* JM109 to yield recombinants. Each recombinant was subjected to shaking culture at 37°C for 2 h on 3 ml of LB medium; the medium was then supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and subjected to another shaking culture at 37°C. The culture solution was centrifuged at 10,000 × *g* at 4°C for 10 min, and the resulting recombinant cells were suspended in 500 μl of 50 mM phosphate buffer (pH 7.0). The suspension and 0.3 g of glass beads (diameter, 0.1 mm; BioSpec Products, Inc., Bartlesville, OK) were added to a 2-ml tube, which was shaken violently with Shake-Master AUTO (Biomedical Science, Tokyo, Japan) for 15 min. The resulting homogenate was centrifuged at 12,000 × *g* at 4°C for 10 min, and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Identification of daidzein-metabolizing enzyme genes in *Slackia* sp. strain NATTS. The *E. coli* library comprising 8,424 strains incorporating *Slackia* sp. strain NATTS genomic fragments was screened for *in vivo* daidzein-to-DHD conversion activity and DHD-to-equol conversion activity. Three clones with daidzein-to-DHD conversion activity (clones A-10, C-11, and E-5) and two clones (clones 15 and 203) with DHD-to-equol conversion activity were identified (Table 1). These enzyme activities were not observed in *E. coli* strains harboring only pUC19. The gene sequences of the *Slackia* sp. strain NATTS gene fragments inserted into the five *E. coli* strains obtained were decoded. Each strain carried an independent gene fragment, and clones 15 and 203 had a common sequence (common sequence 1) (Fig. 1). Also, clones A10, C11, and E5 had a common sequence (common sequence 2) (Fig. 1). Furthermore, clones 15, 203, C11, and E5 had a common domain. This suggested that the daidzein-metabolizing enzyme genes (daidzein-to-DHD- and DHD-to-equol-converting enzyme genes) were present as a series of clusters on the *Slackia* sp. strain NATTS genome.

Characteristics of genes and amino acid sequences of daidzein-metabolizing enzymes. The *orf-1* gene consisted of 1,458 nucleotides, with a promoter (−35 and −10), and a

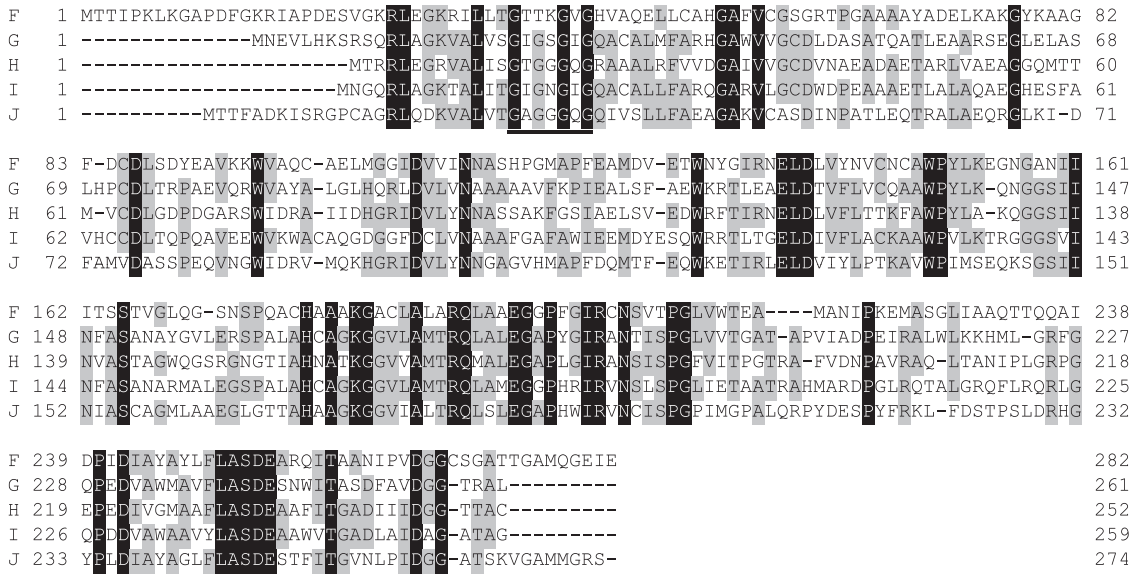

FIG 5 Sequence alignment of ORF-2 (F; accession no. AB646272) from *Slackia* sp. strain NATTS; short-chain dehydrogenase/reductase (G; accession no. YP_003508312) from *Meiothermus ruber* DSM 1279; short-chain dehydrogenase/reductase (H; accession no. YP_001683547) from *Caulobacter* sp. K31; putative dehydrogenase (I; accession no. YP_002871585) from *Pseudomonas fluorescens* SBW25; and short-chain dehydrogenase/reductase (J; accession no. YP_003607655) from *Burkholderia* sp. CCGE1002. Identical amino acid residues are indicated in black boxes, and three-quarter-matched amino acid residues are in gray boxes. Consensus amino acid residues of the putative NADH/NADPH binding motif are indicated by underlining.

ribosome-binding site being located upstream and the terminator downstream (Fig. 2). The *orf-1* gene encoded a polypeptide of 486 amino acids and a calculated molecular mass of 51.8 kDa. The *orf-2* gene consisted of 846 nucleotides, with a ribosome-binding site being located upstream and the terminator downstream (Fig. 2). The *orf-2* gene encoded a polypeptide of 282 amino acids and a calculated molecular mass of 29.4 kDa. Because no promoter region was found in the region between the *orf-1* and *orf-2* genes (108 bp), it was inferred that *orf-1* and *orf-2* were transcribed as polycistronic mRNA. The *orf-3* gene consisted of 1,935 nucleotides, with −35, −10, and a ribosome-binding site located upstream and the terminator downstream. The *orf-3* gene was therefore inferred to be transcribed as monocistronic mRNA. The *orf-3* gene encoded a polypeptide of 644 amino acids and a calculated molecular mass of 70.1 kDa (Fig. 3). A homology search of the *orf-1* gene products showed that these products were similar (32% to 35% identical) to the ORF annotated as a succinate dehydrogenase derived from several bacteria (Fig. 4). Analysis of the secondary structure of the amino acids suggested that ORF-1 was hydrophilic and was localized in the cytoplasmic compartment.

A homology search of the *orf-2* gene products showed that these products were similar (33% to 36% identical) to the ORF annotated as short-chain dehydrogenase/reductase derived from several bacteria (Fig. 5). Analysis of the primary structure of the amino acids showed that amino acid regions 36 to 42 contained an NADH/NADPH binding motif (GXXXGXG). Analysis of the secondary structure of amino acids showed that ORF-2 was hydrophilic and localized in the cytoplasmic compartment. The *orf-3* gene products were, as amino acid sequences, 42% identical to daidzein reductase derived from *Lactococcus* strain 20-92. Furthermore, the *orf-3* gene products were similar (32% to 34% identical) to the ORF annotated as NADH/NADPH oxidoreductase derived from several bacteria (Fig. 6). Analysis of the primary structure of amino acids revealed the following in ORF-3: a putative 4Fe-4S iron-sulfur cluster motif ($CXXCX_3CX_{12}C$) containing cysteine at residue 4 in amino acid domains 343 to 363 and an NADH/NADPH binding motif (GXGXXG) in amino acid domains 390 to 395. In addition, an old yellow enzyme (OYE)-like flavin mononucleotide (FMN) binding domain sequence was found at the N-terminal domain of this protein. Analysis of the secondary structure of the amino acids showed that ORF-3 was hydrophilic and was localized in the cytoplasmic compartment.

Analysis of expression of recombinant daidzein-metabolizing enzymes. In the *E. coli* strains into which pQESL-1, pQESL-2, and pQESL-3 had been introduced, SDS-PAGE confirmed that a protein with a molecular mass corresponding to the size of each gene was expressed (Fig. 7). The recombinants in which protein expression was induced with IPTG were cultured at 37°C for 18 h in the presence of 100 μM daidzein or DHD under anaerobic conditions. Only the *E. coli* strains harboring pQELS-3 had daidzein-to-DHD conversion activity; DHD-to-equol conversion activity was observed in the copresence of the *E. coli* strain incorporating pQESL-1 and the *E. coli* strain incorporating pQESL-2 (Table 2). *cis/trans*-THD was detected both in the culture of the *E. coli* strain incorporating pQESL-2 and in the coculture of the *E. coli* strain incorporating pQESL-1 and the *E. coli* strain incorporating pQESL-2.

DISCUSSION

We showed that, in *Slackia* sp. NATTS strain, the daidzein-to-equol conversion reaction proceeded by the action of a series of three enzymes: ORF-3 of the *Slackia* sp. NATTS strain was responsible for daidzein-to-DHD conversion activity, and ORF-1 and ORF-2 were responsible for *cis/trans*-THD-to-equol and DHD-to-*cis/trans*-THD conversion activity, respectively. Furthermore, the genes encoding these three enzymes

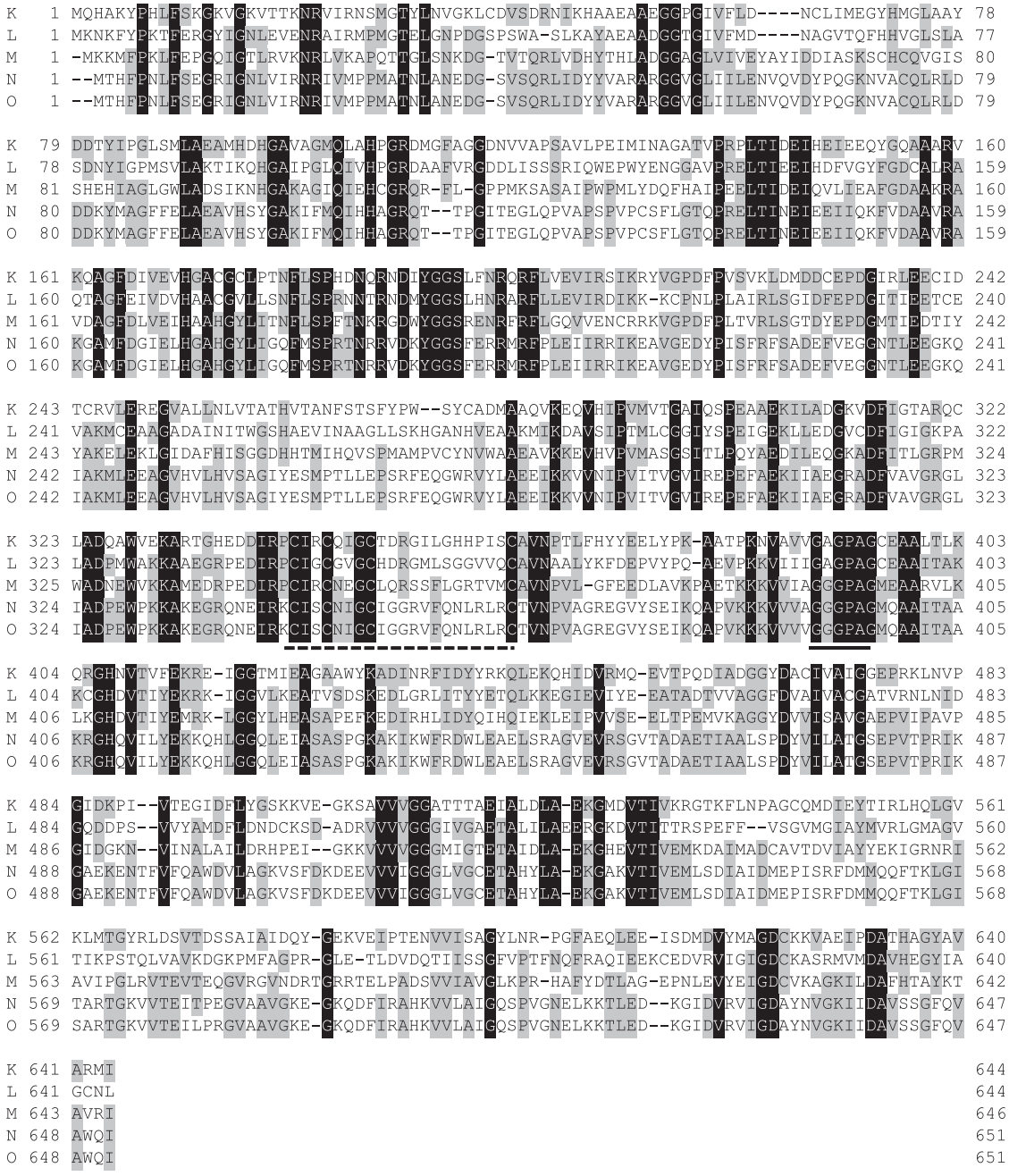

FIG 6 Sequence alignment of ORF-3 (K; accession no. AB646272) from *Slackia* sp. strain NATTS, daidzein reductase (L; accession no. BAJ22678) from *Lactococcus garvieae* strain 20-92, NADH oxidase (M; accession no. ZP_06113274) from *Clostridium hathewayi* DSM 13479, NADH:flavin oxidoreductase/NADH oxidase (N; accession no. ZP_07547992) from *Thermoanaerobacter wiegelii* Rt8.B1, and NADH oxidase (O; accession no. P32382) from *Thermoanaerobacter brockii*. Identical amino acid residues are indicated in black boxes, and three-quarter-matched amino acid residues are in gray boxes. Consensus amino acids of the putative NADH/NADPH binding motif and the 4Fe-4S iron-sulfur cluster motif are indicated by underlining and broken underlining, respectively.

were present collectively in a specific region of the genome. As far as we are aware, this is the first evidence showing that this series of enzymes involved in daidzein-to-equol conversion reaction, as well as the genes encoding them, have been identified in a single strain. On an amino acid sequence level, ORF-3, which was responsible for the daidzein-to-DHD conversion reaction in *Slackia* sp. NATTS strain, was highly homologous to the daidzein reductase (25) derived from *Lactococcus* strain 20-92 (Fig. 6). ORF-3, like daidzein reductase, contains a 4Fe-4S iron-sulfur cluster motif comprising 4 cysteine residues, an NADH/NADPH binding motif, and an OYE-like FMN binding domain, suggesting that both of these enzymes belong to the NADH:flavin oxidoreductase family (4, 6), with similar reaction mechanisms.

However, whereas daidzein reductase derived from *Lactococcus* is presumed to be a membrane protein, ORF-3 was pre-

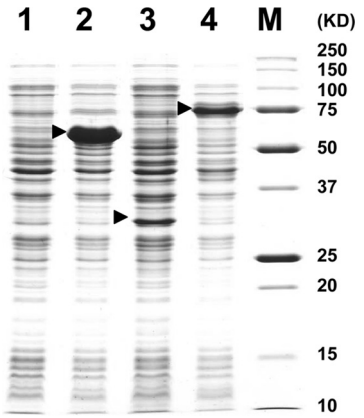

FIG 7 SDS-PAGE (12.5%) analysis of cell extracts from recombinant enzymes. 1, cell extract from pQE30Xa/*E. coli* JM109; 2, cell extract from pQESL-1/*E. coli* JM109; 3, cell extract from pQESL-2/*E. coli* JM109; 4, cell extract from pQESL-3/*E. coli* JM109; M, Precision Plus protein standard (Bio-Rad, Richmond, CA). Recombinant enzymes are indicated by arrows.

sumed to be a cytoplasmic protein, suggesting that their locations are different. *Lactococcus* and *Slackia* are bacteria belonging to different phyla; the former is found mainly in fish and animal milk and is rarely isolated from human (7, 24, 27, 28), whereas the latter inhabits the human intestines (11, 19, 22, 26). At present, although the phyletic evolution of these enzyme genes is unknown, the interbacterium propagation of the genes and their physiological roles in the bacteria are of interest.

This is the first report of the identification of 2 enzymes (ORF-1 and ORF-2) that catalyze the *cis/trans*-THD-to-equol and DHD-to-*cis/trans*-THD conversion reactions and their genes. Through a homology search of amino acid sequences, ORF-1 of *Slackia* sp. strain NATTS was shown to have a primary structure similar to that of the succinate dehydrogenase and fumarate reductase/succinate dehydrogenase flavoprotein domain protein derived from several bacteria, such as *Desulfovibrio fructosovorans* JJ and *Geobacter lovleyi* SZ (Fig. 4) However, these previously reported enzymes were among those found in a recent genomic sequencing, and only their functions have so far been inferred (8, 15). In addition, since the alignment analyses of the amino acid sequences of these enzymes and ORF-1 identified no consensus sequences, it is likely that the ORF-1 identified is a novel one involved in the metabolism of *cis*/*trans*-THD. On the other hand, our homology search and analysis of the primary structure of amino acids suggested that ORF-2 belongs to the short-chain dehydrogenase/reductase (SDR) superfamily (Fig. 5). The SDR family is a group of enzymes that catalyzes the oxidation-reduction reactions of steroids, cofactors, carbohydrates, lipids, aromatic compounds, and amino acids, using NADPH as an electron donor or acceptor (12). Until now, it was not known that the enzymes belonging to this family are involved in *cis/trans*-THD production; we have therefore suggested for the first time that this family is responsible for *cis/trans*-THD production.

Our analysis of the genes in *Slackia* sp. NATTS strain encoding the daidzein-to-equol conversion enzymes, and their genes, showed that the daidzein-to-equol conversion reaction proceeds by the action of three independent enzymes. These find-

TABLE 2 Daidzein- or DHD-metabolizing properties of clones

Plasmid	Substrate (100 μM)	Isoflavone concn (μM)[a]				Daidzein-to-DHD or DHD-to-equol % conversion
		Daidzein	DHD	*cis/trans*-THD	Equol	
pQESL-1	DHD	ND	97.4 ± 4.1	ND	ND	
pQESL-2	DHD	ND	43.2 ± 5.7	53.6 ± 1.4	ND	53.5
pQESL-1 and pQESL-2	DHD	ND	38.7 ± 1.6	7.3 ± 1.9	53.0 ± 1.2	
pQESL-3	Daidzein	1.4 ± 2.4	94.5 ± 1.0	ND	ND	98.6
pQE30Xa	Daidzein	100.5 ± 3.6	ND	ND	ND	
	DHD	ND	122 ± 3.3	ND	ND	

[a] Data are expressed as means and standard deviations. ND, not detected.

ings will lead to progress in enzymological studies of the daidzein-to-equol conversion reaction in enteric bacteria and in research on the ecology of equol-producing bacteria.

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