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Meeting Report

Fifth Joint Meeting of J-CaP and CaPSURE: Advancing the Global Understanding of Prostate Cancer and Its Management

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This report summarizes the presentations and discussions that took place at the Fifth Joint Meeting of J-CaP and CaPSURE held in Tokyo, Japan, in July 2011. The J-CaP and CaPSURE Joint Initiative was established in 2007 with the objective of analyzing, reviewing, comparing and contrasting data on prostate cancer patients from Japan and the USA within the two important large-scale, longitudinal, observational databases—J-CaP and CaPSURE. Since its inception, the initiative has reviewed a wide range of topics and generated valuable data on the patterns of prostate cancer treatment and patient outcomes in the two geographical regions. The objectives of this 5th Joint Meeting were to provide an update on the current status of the J-CaP and CaPSURE databases, and also to discuss perspectives from a range of other Asian countries—Japan, China, Indonesia and Korea—on the use of androgen deprivation therapy for prostate cancer. The collaborators recognize that large databases, such as J-CaP and CaPSURE, provide valuable 'real-world' information, to complement data from clinical trials, which can help to advance the clinical management of prostate cancer patients worldwide. It is anticipated that in the near future, the Joint Initiative will expand globally to include patient registries from other countries so that best practice can be shared and regional differences in patients, treatments and outcomes can be explored.

Key words: prostate cancer − androgen deprivation therapy − overall survival − risk stratification − J-CAPRA

OVERVIEW

The J-CaP and CaPSURE Joint Initiative was established in 2007 with the overall objective of analyzing, reviewing, comparing and contrasting data on prostate cancer patients from Japan and the USA within the two important large-scale, longitudinal, observational databases—J-CaP and CaPSURE. The aim of this ongoing collaboration is to identify trends within patient characteristics, treatment approaches and outcomes (clinical, economic and health-related quality of life) that might assist physicians, patients and others when selecting treatment options at different prostate cancer disease stages. The collaborators recognize that patient registries are an important source of data on therapeutic efficacy and patient outcomes in the 'real-world' situation and are an invaluable complement to data obtained from randomized clinical trials.

This report summarizes the presentations and discussions that took place at the Fifth Joint Meeting of J-CaP and CaPSURE held in Tokyo, Japan, in July 2011. At the four previous meetings of the Joint Initiative, a wide range of topics have been addressed and valuable data have been generated on the patterns of prostate cancer treatment and patient outcomes in the two geographical regions. One important tool that has been developed by the group is a modified CAPRA risk stratification method, named 'J-CAPRA'. This novel risk instrument is the first to be developed and validated for patients undergoing primary androgen deprivation therapy (PADT) and is applicable to those with both localized and advanced disease. It performs well in diverse populations more relevant than existing instruments to the high-risk patients that form a large proportion of the J-CaP database and (5).

In Japan, the J-CaP database was established in 2001 when the Japan Study Group of Prostate Cancer (J-CaP Study Group) supported by the Japan Kidney Foundation and authorized by the Japanese Urological Association commenced a study to gather information about hormone therapy administered to Japanese patients and to analyze the outcomes of treatment. This was prompted by the extensive use of the different forms PADT, including combined androgen blockade (CAB) therapy, for the treatment of prostate cancer within Japan. The result was the J-CaP registry, a large, multicenter, population-based database of men newly starting PADT for prostate cancer. The rationale for its development and an interim analysis of the registration status of the patients and their background variables was reported in 2003 (1), and more recently, treatment patterns with PADT have been reported along with an interim analysis of prognosis (12). As of 2005, J-CaP included data for 26 272 patients from 406 institutions comprising 77 University Hospitals (67% of those in Japan), 267 General Hospitals and 62 Private Hospitals. Around 50% of new prostate cancer patients treated with hormone therapy in Japan were registered with J-CaP at that time.

In the USA, the CaPSURE database was founded in 1995 and currently contains data on around 14 000 prostate cancer patients treated with all forms of therapy. The objectives of the CaPSURE initiative are to collect longitudinal data on a large cohort of patients with prostate cancer, to record existing and developing patterns in these data and in patient outcomes and to identify any variables that might predict outcome. Data on socio-demographics, clinical characteristics, resource use and health-related quality of life are collected prospectively from patients, community-based urologists and hospital records at centers across the USA.

The objectives of this Fifth Joint Meeting of J-CaP and CaPSURE were to provide an update on the current status of the J-CaP and CaPSURE databases and to discuss perspectives from a range of Asian countries—Japan, China, Indonesia and Korea—on the use of androgen deprivation therapy (ADT) for prostate cancer. The latest comparative data from the J-CaP and CaPSURE databases were also

reviewed. The meeting was co-chaired by Professor Hideyuki Akaza (The University of Tokyo, Japan) and Professor Peter Carroll (University of California, San Francisco, CA, USA).

PRESENTATION 1: WHAT CONCLUSIONS CAN BE DRAWN USING INFORMATION FROM LARGE-SCALE DATABASES? REGISTRY DATA VERSUS RANDOMIZED CONTROLLED TRIALS

S.H. (Kyoto University, Kyoto, Japan) discussed the advantages and disadvantages of the types of information that could be drawn from large-scale databases, such as J-CaP and CaPSURE, in contrast to that which could be concluded from randomized, controlled trials (RCTs).

A range of different designs of clinical study can contribute to the different levels of evidence specified in treatment guidelines, such as those of the European Association of Urology (11). These include meta-analysis, randomized, case-controlled, observational, cohort, cross-sectional, plus retrospective medical record reviews. When comparing between RCTs and observational studies, each has its own particular advantages: RCTs eliminate confounding and minimize bias while observational studies have the advantage of greater generalizability, studies can be conducted more rapidly, expense is minimized and they address a broader range of questions.

Confounding factors are recognized as an inherent problem in observational studies—these are factors associated with the risk factor being observed and are causally related to the outcome. Methods to eliminate confounding include randomization (as in RCTs), matching (as in case-controlled trials) and exclusion of certain patients. In addition, in observational studies, the statistical methodology employs stratification and multivariate analyses.

Generalizability of study data refers to the ability to apply the results of a study to patient populations other than the study sample. In general, the results of RCTs only apply to a selected patient population as identified in the study protocol, and many patients are specifically excluded from a trial. As an example, the results of a study performed in patients with good renal function might not be generalized to patients with renal dysfunction. In observational studies, no such selection is performed and as a result their generalizability is increased and they are more likely to reflect treatment effects in the real world.

Results from RCTs and observational studies, including data from large-scale databases such as J-CaP and CaPSURE, should therefore be considered as complementary: information from both is of value and should be considered to help build a full clinical picture and inform clinical decision making.

S.H. cited the example of the results of an RCT evaluating CAB with bicalutamide for advanced prostate cancer (2).

The study reported survival outcomes after long-term follow-up of a Phase III RCT. Five-year survival with CAB was 75.3% and with luteinizing hormone-releasing hormone (LHRH) alone was 63.4% ($P = 0.043$). These findings are supported by results from the J-CaP database of overall survival in high-risk patients (J-CAPRA score 8+) which also found a significant difference in favor of CAB compared with non-CAB hormonal therapy ($P < 0.0001$).

PRESENTATION 2: CONFOUNDING FACTORS: AN INHERENT PROBLEM WHEN COMPARING DIFFERENT DATABASES

P.C. [University of California, San Francisco (UCSF), CA, USA] provided the meeting participants with some insight into an inherent problem often encountered when analyzing and comparing information from observational databases—confounding factors. A confounder can affect the relationship between the predictor and outcome leading to bias, and it is therefore important to control for confounders in order to get closer to the true relationship between the predictor and outcome. In observational research, confounding is commonly addressed by adjusting or controlling for these potentially confounding factors. However, databases differ in the demographic and clinical factors they capture and the outcomes they assess and therefore some confounding factors might not be recorded in the database. As a result, these cannot be adjusted for, leading to potential bias.

P.C. went on to describe how a range of studies investigating an association between ADT and cardiovascular (CV) mortality had shown mixed results in observational studies, while RCTs had failed to show any such association. An analysis was initially undertaken on the CaPSURE database to assess mortality outcomes by treatment type with a particular focus on the effects of ADT and CV mortality. The results suggested that there was an increase in CV mortality in some men receiving ADT (27). Because of the conflicting data in the literature, this subject was addressed in the CaPSURE database more recently. The results showed an almost 2-fold increase in CV mortality in the ADT group compared with those receiving local treatment only. However, there was also a greater than 2-fold increased CV mortality in the watchful waiting/active surveillance group compared with local treatment alone. It was therefore considered that perhaps factors affecting treatment selection, e.g. age or co-morbidity, might be contributing to CV risk. These data were also analyzed using propensity scoring—a measure of the patient's probability of receiving treatment (ADT), conditional on important clinical and socio-demographic covariates (21). No differences in CV mortality were noted between those who did and did not receive ADT inferring that unmeasured variables—which influence treatment decision-making but are not recorded in CaPSURE—might have confounded the association between ADT and CV mortality in many previous studies.

He concluded that it could be challenging to properly adjust risk for outcomes like CV mortality. Although CaPSURE assessed the presence or absence of important co-morbidities like cardiac disease and diabetes, it did not record HbA1c levels, heart failure risk classifications and other important risk factors for cardiac death. Therefore, it was possible that these variables might be confounding the association between ADT use and CV mortality. Matching patients on their propensity to receive ADT was an effective method of attempting to control for confounding in observational studies, although it could not eliminate it completely.

PRESENTATION 3: OBSERVED DIFFERENCES BETWEEN THE J-CaP AND CaPSURE DATABASES IN THE SURVIVAL OF PATIENTS RECEIVING PADT

H.A. (Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan) reminded participants that any hormonal therapy for prostate cancer should take into account not only testosterone of testicular origin but also the locally produced androgens: testosterone and dihydrotestosterone derived from dehydroepiandrosterone of adrenal origin. CAB—either medical or surgical castration plus an antiandrogen—is often the therapy of choice when the tumor is localized; however, resistance to the disease can develop if the tumor becomes metastatic. This is thought to be due to either elevated levels of the androgen receptor or to local biosynthesis of androgens.

New agents for the management of prostate cancer, in particular more potent antiandrogens, are continually being sought and H.A reviewed some recent examples that have shown promising results in patients with castration-resistant prostate cancer (CRPC).

Abiraterone acetate, an inhibitor of androgen biosynthesis, has recently received approval from the FDA for the treatment of metastatic CRPC. The results of a Phase III RCT have been published (10) which demonstrated that abiraterone acetate was able to prolong the overall survival among patients with metastatic CRPC who had previously received chemotherapy.

MDV3100 is an androgen receptor antagonist which also induces tumor cell apoptosis. Results of a Phase I–II trial have shown that MDV3100 has promising antitumor activity in patients with CRPC and also provides evidence that sustained androgen receptor signaling is a key driver in this disease (22).

XL184 (cabozantinib) is an inhibitor of tumor growth, metastasis and angiogenesis, which simultaneously targets MET and VEGFR2, key kinases involved in the development and progression of many cancers. Interim results of a Phase II clinical trial of cabozantinib for the treatment of men with metastatic CRPC have shown encouraging clinical activity in these patients (24).

The initial concept of the benefit of CAB therapy over LHRH monotherapy was proposed in 2002 (14); however, several other studies going back over 20 years have also demonstrated the benefit of this regimen (15): Crawford et al. (9) concluded that in patients with advanced prostate cancer, CAB treatment with leuprolide plus flutamide was superior to treatment with leuprolide alone, and more recently, Akaza et al. (2) showed that CAB with bicalutamide 80 mg offered a significant overall survival benefit compared with LHRH agonist monotherapy without reducing tolerability in patients with locally advanced or metastatic prostate cancer.

Despite such evidence, the National Comprehensive Cancer Network (17) Clinical Practice Guidelines in Oncology do not currently recommend primary hormone therapy for localized prostate cancer. However, the NCCN Clinical Practice Guidelines in Oncology: Asia Consensus Statement (18) focuses on the specific situation for Asian patients and takes into account the differences between Asian and Caucasian patients in their response to therapy. Primary ADT is recommended in these guidelines as an additional option in low-, intermediate- or high-risk Asian patients with a life expectancy of <10 years.

Published data on the benefits of CAB from RCTs are now supported by observational data from the J-CaP database, which although not randomized does include a large number of patients. H.A. presented data showing that for low-risk patients (J-CAPRA score 0–2), there is no observed difference in overall survival between those receiving CAB therapy or non-CAB therapy and by 10 years overall survival does not drop below 50% in either group. Overall survival of intermediate-risk (J-CAPRA score 3–7) and high-risk (J-CAPRA score 8+) patients in the J-CaP database is significantly higher for those receiving CAB therapy compared with non-CAB therapy ($P < 0.0001$). In the intermediate-risk group, overall survival only drops below 50% after 8 years and for high-risk patients after 4 years in each treatment group (Fig. 1a).

He then compared the J-CaP data with those analyzed by the J-CAPRA score from the CaPSURE database (Fig. 1b). Interestingly, in the low-risk group in the CaPSURE cohort, 50% overall survival is reached by 6 years, much shorter than that seen in the low-risk J-CaP cohort. Notably, in the CaPSURE database, the overall survival of low- and intermediate-risk patients is better for non-CAB treated patients than for CAB-treated patients—the opposite of that seen in the J-CaP database. Only high-risk patients in the CaPSURE database show improved survival with CAB compared with non-CAB therapy.

He concluded by saying that to explain these observed differences between the two databases, it is important to consider carefully the influence of possible confounding factors. It is possible that even within a single category, such as the intermediate risk group with J-CAPRA scores of 3–7, there were patients who responded differently to therapy.

PRESENTATION 4: ASIAN PERSPECTIVES ON THE USE OF ADT FOR PROSTATE CANCER

KOREA

B.-H.C. (Yonsei University College of Medicine, Seoul, Korea) reported that data from the National Statistics Office in Korea showed that the number of patients diagnosed with prostate cancer had quadrupled between 2002 and 2008 (16,19). A survey of 50 urologists demonstrated an increase in the average number of prostate cancer patients seen each month, which had risen to an average of 70 per month in 2009 in the outpatient setting (unpublished data). These patients have predominantly localized disease (37%) with 23% having locally advanced disease, 29% advanced (metastatic) disease and 12% hormone-refractory disease. There appears to be a stage migration to a greater proportion of patients with localized disease and a reduction in the numbers with advanced disease.

In terms of treatment modalities used in Korea, hormone therapy alone is administered to 13% of the patients with localized disease, 17% with locally advanced disease, 88% with advanced disease and 37% with hormone-refractory disease. In the case of localized or locally advanced disease, often it is used in combination with radical prostatectomy or radiotherapy. He went on to compare these survey data with those from his own institution, the Gangnam Severance Hospital, where a total of 157 patients were newly diagnosed with prostate cancer between 2008 and 2010. Hormone therapy alone is administrated as a primary therapy to 12% of the patients with localized disease, 25% of those with locally advanced disease and 76% of those with advanced disease.

The available ADT for prostate cancer in Korea includes leuprolide acetate, leuprorelin acetate, goserelin acetate, bicalutamide (50 mg and 150 mg), cyproterone acetate (50 mg) and flutamide (250 mg).

The Korean Urological Oncology Society issued guidelines in 2008 for the use of ADT for prostate cancer. In contrast to the situation in Japan where ADT is used as a primary therapy in around 80% of low-risk patients, primary ADT is not commonly used in Korea for this patient group. Again, in contrast to the recommendations for high-risk patients within the NCCN Clinical Practice Guidelines in Oncology: Asia Consensus Statement, CAB with bicalutamide (80 mg daily) is not used in Korea as this dose was not available; bicalutamide 50 or 150 mg daily was used instead, according to efficacy and the patient's quality of life. B.-H.C. concluded that Korean treatment patterns for prostate cancer patients are similar to those seen in the USA and Europe, rather than in Japan, and primary ADT is not the preferred choice for T1c–T3N0M0 patients (26).

CHINA

X.Z. (Peking University, China) reported that the overall incidence of prostate cancer in Asia is relatively low at around
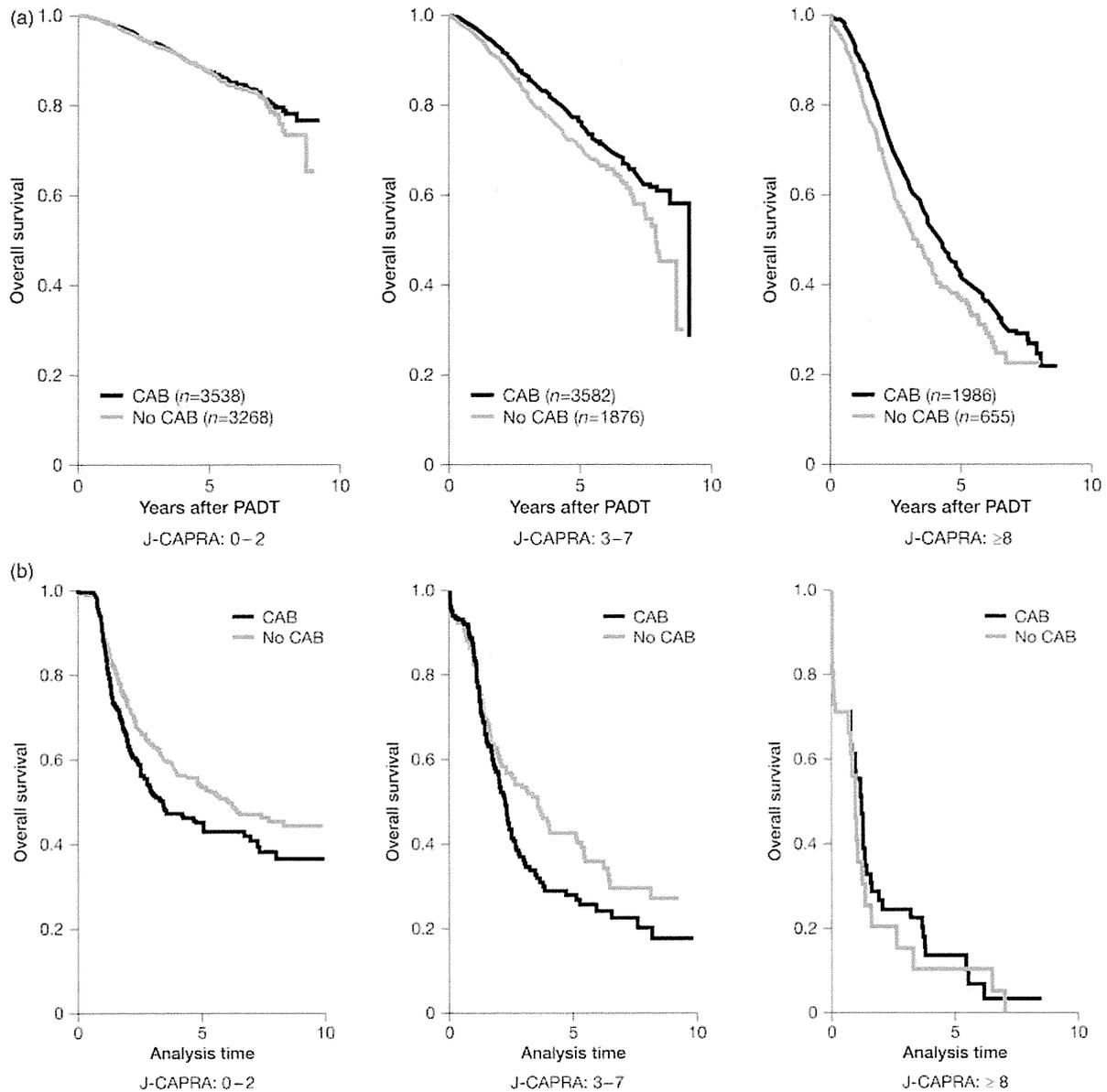
Figure 1. Overall survival by type of hormone therapy according to the J-CAPRA score in (a) the J-CaP database and (b) the CaPSURE database.

90 per 100 000 men, but there are variations in the incidence across different Asian regions. In the city of Shanghai, China, the incidence is 2–3 per 100 000 men. Ten-year data from 1990 to 2000 show a sharp 3-fold increase from a standardized incidence of 2.7 to 7.7.

When data for the incidence of different forms of cancer in various cities in China from the period 1993–97 are compared with that for the period 1998–2002, it can be seen that prostate cancer was the 10th most common form of cancer in Shanghai in the period 1993–97 but has risen to become the eighth most common in the period from 1998–2002.

Hormonal therapy, particularly CAB treatment, is widely used in China, based on guidelines for prostate cancer treatment issued by the Chinese Urological Association. X.Z. advised that the types of patients who are selected for hormone therapy generally fall into four categories:

metastatic prostate cancer (clinical stage M), those given it prior to radical prostatectomy (clinical stage T3a), those given it following radical prostatectomy (pathological stage T1–T3) or those receiving radiation therapy (RT) (clinical stage T3–T4, M0). The usual treatment modality is CAB in the majority of patients, while antiandrogen alone, medical castration alone or surgical castration alone are only administered to around 10% of patients each.

He concluded by noting that the incidence of prostate cancer in China was likely to increase significantly in future years, particularly in major cities.

INDONESIA

R.U. (University of Indonesia, Depok, Indonesia) reported that prostate cancer had accounted for 40% of all the

urological malignancies diagnosed between 1995 and 2010 at two hospitals in Jakarta, Indonesia—RSCM and RSKD. The incidence of prostate cancer at these centers had increased around 3-fold from the period 1995–99 (135 cases) to 2005–09 (404 cases). In many cases, patients presented with advanced, metastatic disease; only about 38% had non-metastatic disease.

He went on to describe the treatment options administered to patients with T1–T4,N0,M0 prostate cancer from 1995 to 2010 ($n = 262$ patients) at these two institutions. Hormonal therapy had been given to 22% of the patients with T1 disease, 29% with T2, 57% with T3 and 94% with T4. The type of hormonal therapy given was either orchiectomy (about one-third of the patients), LHRH monotherapy or intermittent androgen blockade (IAB) using CAB [6 months on treatment and 6 months off, depending on prostate specific antigen (PSA) levels]. R.U. had compared the patients treated with IAB and orchiectomy at these institutions but found no statistically significant difference in survival between the two treatment groups.

Data collected for the period 2004–08 from four cities in Indonesia (Jakarta, Bandung, Surabaya, Yogyakarta; $n = 851$ cases) confirmed that ADT is the most commonly used prostate cancer treatment (around 60% of the cases) in Indonesia.

JAPAN

Y.H. (Nara Medical University, Kashihara City, Nara, Japan) reported results of a study evaluating the distribution of primary therapy and clinical characteristics of over 2000 prostate cancer patients at Nara Medical University and its 23 affiliated hospitals (25). The study had also investigated the differences between the preferential primary therapy conceived by the primary doctors and the primary therapy actually administered for prostate cancer patients.

Data from 2004 to 2006 showed that of the 2371 patients included in the analysis, 30% had undergone radical prostatectomy (RP), 13.8% RT and 50.1% PADT. However, when Nara Medical University (NMU) was compared with its affiliated hospitals (non-NMU), differences were observed in the treatment modalities used. Non-NMU hospitals were found to treat a much higher proportion of patients with PADT (55.7%) compared with NMU (24.3%) and a lower proportion with RT (7.4%) compared with NMU (42.8%). The proportion of patients treated with RP was similar between NMU and non-NMU (around 30%). The likely reason is that RT (IM-RT and brachytherapy) is more easily available at the NMU than at affiliate hospitals.

Data from NMU comparing the two different time periods (2004–06 and 2007–09) showed that the trends in patients' ages, PSA levels, Gleason scores, clinical stage and risk classification of prostate cancer patients diagnosed between 2007 and 2009 were significantly lower than those diagnosed between 2004 and 2006 ($P < 0.001$). In addition, the proportion of patients treated with PADT from 2007 to 2009 was

significantly lower than that seen from 2004 to 2006 (40 vs. 50%). The proportion undergoing RP was similar in both time periods (30%); however, the proportion receiving radiotherapy from 2007 to 2009 was significantly higher than in 2004–06 (24 vs. 14%, $P < 0.001$).

The trend in primary therapy was significantly different between university hospital and affiliated hospitals in both time periods (2004–06 and 2007–09). Within NMU, over the two time periods, there was a trend to increasing use of RT with a decrease in RP and the use of PADT. Non-NMU hospitals did not show much change in the use of primary therapy over this time other than a slight decrease in the use of PADT.

Y.H. then compared data for NMU (2004–06 and 2007–09) with that from the CaPSURE database (1989–2002) according to risk: T-stage, Gleason score, PSA and D'Amico risk score. The results showed that Japan appeared to be 15–20 years behind the USA in terms of the pattern of prostate cancer risk, having a greater proportion of high-risk patients. When comparing primary treatment used at NMU and recorded in CaPSURE, substantial differences were observed. In NMU, a much smaller proportion of patients underwent watchful waiting (around 3%) compared with CaPSURE (around 23%) while a much higher proportion were treated with PADT (40%) compared with CaPSURE (17%).

He summarized by noting that while the number of high-risk patients at NMU had decreased significantly from 2004–06 to 2007–09, as had the proportion of patients treated with PADT, the proportion of high-risk patients was still greater than that seen in the CaPSURE database.

PRESENTATION 5: EVALUATION OF THE OUTCOMES OF PATIENTS RECEIVING CAB THERAPY USING THE J-CAPRA RISK SCORE

RESULTS FROM THE J-CAP DATABASE

S.H. (Kyoto University, Kyoto, Japan) noted that a major difference between the J-CaP and CaPSURE databases related to age distribution: in the J-CaP database, almost 50% of the patients were aged 75 years or over and around 90% were aged 66 years or over. In contrast, in CaPSURE only around 15% were aged 75 years or over and almost 50% were 65 years of age or younger. Analysis of age distribution and risk according to the J-CAPRA score showed that a higher proportion of younger patients had high-risk disease compared with older patients; this was the opposite of the trend seen in CaPSURE. When the trend in the type of PADT employed is analyzed for the period 2001–03, it is apparent that there were no significant changes in treatment trends over this time with the majority of patients (about 60%) receiving PADT.

An important factor potentially affecting treatment outcomes in any patient is the presence of co-morbidities. Within the J-CaP database, a range of co-morbidity factors is

recorded; however, it is not possible to specify the severity of the co-morbidity or assign any importance or degree of risk. S.H. described how using Charlson's comorbidity index co-morbid conditions can be 'weighted' depending on the risk of dying associated with this condition to give a score that predicts mortality. Unfortunately, this could not be applied accurately to patients in the J-CaP database since certain data were lacking—the database does not record all conditions, e.g. dementia, leukemia, lymphoma, AIDS, plus it did not record the severity of liver or renal disease. In view of this, an alternative method of analysis was employed: patients were assigned as 'cardiovascular disease (CVD)+' if they had either heart disease or stroke. Then, the combination of number of different co-morbidities plus the presence or absence of CVD was assigned to four categories: no co-morbidities (6248 patients), ≤2 co-morbidities but CVD- (7341 patients), ≤2 co-morbidities but CVD+ (2990 patients) and >2 co-morbidities (2458 patients). An analysis was then undertaken of co-morbidity distribution according to the J-CAPRA score (low, intermediate and high), age range (≤65, 66–75 and >75 years) and the type of hormonal therapy selected (CAB or non-CAB). The results showed that the distribution of the number of co-morbidities was comparable between CAB and non-CAB treatment regimens.

S.H. went on to describe the results of the survival analysis of J-CaP patients. Both cause-specific and overall survival showed similar separations of the curves for patients with low, intermediate and high J-CAPRA scores. In terms of overall survival, for low-risk patients, there was no difference in survival between CAB and non-CAB therapy; however, in both intermediate- and high-risk patients, the prognosis was better with CAB than with non-CAB therapy.

In order to evaluate these differences, PADT treatment of patients in the J-CaP database was reclassified according to the following three categories: (i) orchiectomy, (ii) LHRH monotherapy ($n = 2991$) or LHRH plus short antiandrogen ($n = 1657$) or (iii) CAB ($n = 10\,369$). In terms of cause-specific survival, CAB and LHRH monotherapy resulted in better prognosis than orchiectomy in patients with an intermediate J-CAPRA score (3–7). CAB therapy resulted in a better prognosis than LHRH monotherapy or orchiectomy in patients with a high J-CAPRA score (>8). No significant differences were observed between CAB and LHRH monotherapy according to the J-CAPRA score in terms of background factors, such as age, PSA at diagnosis and Gleason score at diagnosis.

RESULTS FROM THE CaPSURE DATABASE

M.C. (University of California, San Francisco, CA, USA) reported that the prostate cancer mortality rate in the USA has fallen by 40% in the PSA screening era at a time when men were generally living longer and should in fact be at greater risk from prostate cancer mortality (13). However, he

noted that the observed treatment patterns in the USA give rise to concerns about both overtreatment and undertreatment of many patients. Few men with low-risk disease undergo watchful waiting/active surveillance and, the improved mortality rates have come at the cost of many other men being treated unnecessarily with surgery or radiation for tumors that would ultimately have not been fatal.

He noted that PADT is commonly used in the USA, particularly for higher risk patients (6). This raises the issue of undertreatment of men with high-risk disease who might benefit from local therapy. The reason for this appears to in part reflect an age bias, as reported in a recent publication from the CaPSURE team (4). The results had shown that older patients were more likely to have high-risk prostate cancer at diagnosis but less likely to receive local therapy and that underuse of potentially curative local therapy among older men with high-risk disease might in part explain observed differences in cancer-specific survival across age groups.

When comparing risk groupings and age between J-CaP and CaPSURE, he noted that the patterns were quite different: in J-CaP the oldest patients were more likely to be diagnosed with high-risk disease, and the opposite was true for CaPSURE (5), possibly due to the different screening practices in Japan and the USA. Interestingly, patients in CaPSURE treated with PADT showed similar relationship between age and risk as the J-CaP cohort (4).

Thus, in CaPSURE at least, age appears to be a stronger driver of clinical decision-making than disease risk. Younger patients tend to receive surgical therapy regardless of their disease risk, whereas in the older age group (>75 years), a much smaller proportion receive local therapy and many high-risk patients only receive ADT monotherapy. Primary treatment trends in CaPSURE from 1999 to 2007 showed that the use of ADT has been relatively consistent over this time period. Two other large databases—Medicare and i3—show similar trends to CaPSURE across all forms of therapy (7). In 2005, however, reimbursement arrangements for ADT in the USA changed significantly and there is now less incentive to prescribe ADT, which might influence treatment patterns.

Results of a study into factors that drive selection of ADT for prostate cancer treatment suggest that the particular urologist a patient sees might be more important in determining whether they receive ADT than tumor or patient characteristics (23). Treatment patterns in CaPSURE also vary markedly across clinical sites, and this variation is not explained by case-mix variability or known patient factors (6).

M.C. advised that there were currently13 893 patients in the CaPSURE database. Follow-up as of September 2010 showed an all-cause mortality (ACM) of 3752 patients (27.0%) at a median of 7.0 years and a prostate cancer-specific mortality (PCM) of 714 patients at a median of 6.2 years—this represented 5.1% of overall cohort and 19% of all deaths. The overall median follow-up was 4.6 years.

Both ACM and PCM by treatment type in CaPSURE show similar trends, with surgery patients having a better

prognosis than those treated with radiation, followed by watchful waiting/active surveillance and ADT; however, it should be noted that these are unadjusted figures and reflect the particular case mix.

Looking at treatment according to the J-CAPRA risk group over time, it is apparent that in recent years, there has been less use of ADT monotherapy for low-risk patients and an increase in its use for higher risk patients (J-CAPRA score ≥8). For patients treated with ADT, PCM according to the J-CAPRA score shows a similar separation of the curves (low-, intermediate- and high-risk patients) as seen in J-CaP except that they are shifted to the left—i.e. for a given J-CAPRA category, survival is shorter in CaPSURE.

With the accumulation of data now in CaPSURE due to longer follow-up and a greater number of events, cancer-specific mortality can also be analyzed by the individual J-CAPRA score and shows good distinction between the curves with a discriminatory accuracy (C-index) of 0.81. Curves for ACM show a similar distribution pattern but slightly more overlap between the curves and a C-index of 0.61.

Of the CaPSURE cohort, 2030 men received ADT monotherapy, predominantly either LHRH monotherapy (916 patients) or CAB (864 patients). In general, the use of CAB seems to correlate with risk, with higher risk patients being more likely to receive it. However, if figures are adjusted according to the J-CAPRA score, this divergence disappears and there is no difference between LHRH monotherapy and CAB. A multivariate analysis (Cox's proportional hazards model) also confirms no increased mortality risk with CAB [hazard ratio (HR): 0.92] but an increased risk with orchiectomy (HR: 1.72) compared with LHRH monotherapy. Similarly, predicted 10-year survival curves using this model show no difference between LHRH monotherapy and CAB. A competing risks analysis (Fine–Gray) also supports these results.

M.C. summarized by saying that while US guidelines for prostate cancer, such as NCCN(17) and the American Urological Association (3), do not currently endorse the use of PADT, it is commonly used in practice. J-CAPRA scoring provides excellent risk stratification for prediction of mortality for US men receiving PADT and reveals that CAB does not appear to result in improved survival over LHRH monotherapy. These observations are consistent with a meta-analysis undertaken by the Prostate Cancer Trialists' Collaborative Group (PCTCG) of 27 trials per 8275 patients (20). The underlying reasons for the different responses to hormonal therapy across populations remain to be elucidated.

PRESENTATION 6: SIGNIFICANCE OF J-CAPRA SCORE ON THE NEW PROGNOSTIC GROUPING WITHIN THE TNM CLASSIFICATION (7TH EDITION)

S.H. (Kyoto University, Kyoto, Japan) described how the American Joint Committee on Cancer (AJCC) Cancer

Staging Manual 7th edition had introduced new prognostic groupings for prostate cancer. He reviewed these new groupings in terms of the specified T-stage, N and M category, PSA level and Gleason score for Group I–IV disease and contrasted them with the J-CAPRA score which was calculated from a sum of points assigned to different variables (T, N, M, PSA and Gleason score) from 0–12 score, where low risk scored 0–2, intermediate risk 3–7 and high risk 8 or greater (5).

When TNM prognostic groupings were calculated using data from J-CaP, the results showed that about one-third of the patients fell into Group IV (advanced disease). He went on to examine the relationship between J-CAPRA risk and TNM prognostic grouping for the J-CaP data: all patients identified as high risk by J-CAPRA score (>8) were TNM Group IV, those identified as intermediate-risk by J-CAPRA score (3–7) comprised a mixture of Group IIA, II and IV, and those in the low-risk J-CAPRA group comprised a mixture of TNM groups from I to IV.

S.H. concluded by saying that all groups within the TNM prognostic grouping classification are represented within the J-CAPRA low-risk patient group in J-CaP, while about one-third of the patients are TNM Group IV. Analysis of progression-free and overall survival shows that patients in TNM Group IV have a poor prognosis. He therefore suggested that to achieve a balanced proportion and prognosis between TNM prognostic groupings, some modifications should be considered for patients in J-CaP database, for example, creating a subcategory in Group IV, which currently comprises the majority of the patients.

DISCUSSION

Participants agreed that due to the possibility of confounding factors, it was important to exercise caution when reporting and interpreting data from observational databases such as J-CaP and CaPSURE. The possible confounding factors that might contribute to the differences in the results seen in the two databases were discussed. M.C. commented that it depended whether the endpoint being considered was overall mortality or cancer-specific mortality. For cancer-specific mortality, it was important to confirm that the risk profiles were the same in the two arms, or whether the patients selected for CAB were actually higher risk patients in the first place, as was probably true of the CaPSURE cohort. In the case of overall mortality, it was important to consider what the effects of treatment might be, for example, on CV risk. Patients might have different severities of co-morbidities, such as diabetes, and these subtle differences in risk profile were not captured within the database information. P.C. considered that propensity analysis was one way to help address this.

M.N. noted that aside from any confounding factors, there were racial differences in lifetime CV risk profile between Asian and Caucasian patients, so it was difficult

to compare the effect of ADT on CV risk in these two populations. P.C. agreed and commented that conclusions about prostate cancer therapy were often based on the US experience; however, it was a worldwide disease and what was applicable in one population might not be applicable in another, due to environmental or dietary influences, for example. A larger, global registry, as proposed by H.A., would be very valuable in evaluating these differences.

It was noted that the effect of ADT on CV risk had also been analyzed in the J-CaP database and no adverse effects had been found, in line with the CaPSURE data presented by P.C.

P.C. considered that it would be interesting to evaluate the effectiveness and outcomes of different therapeutic modalities in the different populations. It was known from work at UCSF that lifestyle differences after radical prostatectomy could influence treatment outcomes.

It was recognized that patients were now being diagnosed with prostate cancer at a younger age and differences in the treatment of younger versus older patients were discussed. Baseline J-CaP data had been collected over 10 years ago and now new data were being collected on treatment patterns which would allow an analysis of the change in treatment modalities over time.

While the incidence of prostate cancer was increasing in Asia, the stage distribution seemed to be different from that in the USA. It was queried whether routine screening was performed in the countries represented at the meeting. It was noted that in Korea, screening was not done routinely; ~20% are screened but it varies between rural and urban areas. China does not have a routine PSA screening system; it is normally done if the patient presents with symptoms. The underlying reason for the rise in the incidence of prostate cancer in Asian countries was discussed. It was suggested that this could be due to an increase in PSA screening and education about testing, and also to a rise in living standards in certain areas which resulted in better access to health care.

The extensive use of PADT in Japan was possibly due to older urologists who were accustomed to using it to treat the high number of advanced cases seen in previous years and who continued to use it for lower-risk patients. It was considered that younger urologists might be more likely to choose other, newer treatment modalities.

It was noted that intermittent hormonal therapy was popular in some Asian countries. P.C. confirmed that it was also popular in the USA in patients with low-risk disease but cautioned that it may not be optimal for patients with higher risk disease. M.C. added that there were also many inconsistencies between intermittent therapy regimens in terms of PSA trigger, time on and off therapy etc., which made comparison between patients and studies difficult.

M.N. queried why the cause-specific survival of patients in J-CaP who underwent orchiectomy was inferior to that seen with LHRH monotherapy, and also why CAB had not been found to be superior to LHRH monotherapy. In the case of orchiectomy, he proposed it might be that patients did not receive a secondary therapy if orchiectomy failed. P.C. suggested that there might be a confounding factor and noted that the sample size in the case of orchiectomy was small. S.H. also commented that it could be due to the age distribution of the of the orchiectomy cohort: patients who received orchiectomy were older than other treatment groups, so the number at risk was smaller at an earlier time point in orchiectomy group than in the LHRH group, meaning that a single event would causes a large decrease in survival. It might also reflect the inferiority of the cause-specific survival of patients who underwent orchiectomy. M.C. added that there could be differences in biological treatment effects between LHRH and orchiectomy—*in vitro* studies had suggested a possible direct cytotoxic effect of LHRH therapy.

Secondary treatment effects were considered to be an important issue. S.H. confirmed that in the J-CaP database, only data on initial treatment were recorded routinely. The first 90 days of treatment were recorded as the initial therapy and survival data were calculated for this initial treatment.

The four PSA categories within the J-CAPRA risk scoring system were queried: 0–20 mg/ml = 0, 20–100 ng/ml = 1, 100–500 ng/ml = 2 and >500 ng/ml = 3. These differed from the levels used in some countries, e.g. 0–10, 10–20 and >20 ng/ml. S.H. explained that registration of patients to the J-CaP database had been undertaken for 3 years with 7-year follow-up. Ten years ago, the PSA distribution had been higher so the cut-points had been set relatively high. M.C. confirmed that the J-CAPRA scoring system had been developed for patients who were relatively high risk. He advised that another risk scoring system, CAPRA-S, which incorporated pathological data, had been developed for post-surgical patients (8).

K.N. queried how T-category within CaPSURE was assessed. M.C. confirmed that they recorded both digital rectal examination and ultrasound imaging results and then calculated the stage; few patients underwent magnetic resonance imaging for tumor staging. Stage did not appear to be a meaningful predictor of outcomes until the patient reached T-stage 3.

H.A. commented on the poor prognosis of patients undergoing PADT within CaPSURE compared with those who received local therapy and queried whether there were any underlying reasons for this, e.g. socio-economic factors. M.C. agreed that the survival benefit of ADT in US patients was indeed limited, but the reason for this was still unclear. Within CaPSURE, there was also a 2-fold benefit in survival of surgery over radiation and a 70% benefit of radiation over ADT, although mostly in higher risk patients. The durability of response to a particular therapy may be different in the US and Japanese populations, due to, for example, lifestyle or genetic factors, and these remained to be elucidated.

CONCLUSION AND FUTURE DIRECTION OF THE J-CaP and CaPSURE JOINT INITIATIVE

H.A. (Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan) acknowledged that the discussions at the Fifth Joint Meeting of J-CaP and CaPSURE had been extremely thought provoking; however, there were still many unanswered questions. He was convinced that large databases, such as J-CaP and CaPSURE, provided valuable 'real-world' information that would help advance clinical management of prostate cancer patients in the future.

P.C. (University of California, San Francisco, CA, USA) stressed the importance of the relationship between the J-CaP and CaPSURE teams and noted the contribution that the initiative had made over the last 5 years to the understanding of prostate cancer in these two distinct regions—the USA and Japan. He was pleased to see that the initiative had now expanded to include input from other Asian countries and hoped to see that the collaboration further expand globally as there was a lot more information about prostate cancer and its management to learn and share between countries.

It was noted that the Korean group was now planning to establish 'K-CaP' which would be a complementary database to J-CaP and CaPSURE. In addition, the Korean group had held the 1st Congress of the Asian Pacific Prostate Society (APPS) in March 2011. The President of the APPS was Professor Kim, the Vice-President was Professor Chung and the Secretary General was Professor Lee, all participants at this meeting. Future APPS meetings were already planned: the 2nd APPS Meeting would be held in Seoul, Korea, in 2012 and the 3rd APPS Meeting was to be held in Australia in 2013.

In closing the meeting, the Co-Chairmen thanked all the participants for attending and for their valuable contributions to the discussions, and also acknowledged Takeda Pharmaceutical Company Ltd for their continued support of these joint meetings.

Conflict of interest statement

None declared.

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Appendix

Participants at the 5th Joint Meeting of J-CaP and CaPSURE.

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

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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.

oybean 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 Phar-

maceutical 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 × *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

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′-A TGGCCGAATTCGATGTTG-3′ (ORF1-F) and 5′-GGGGGATCCTAGT ATGGGCGAAACCGTT-3′ (ORF1-R-BamHI); *orf-2* gene, 5′-ATGACT ACCATTCCTAAGCTCAAGG-3′ (ORF2-F) and 5′-GGGGGATCCTAC TCAATTTCGCCCTGCATAG-3′ (ORF2-R-BamHI); and *orf-3* gene, 5′-ATGCAGCACGCGAAATACCC-3′ (ORF3-F) and 5′-GGGGGATCCTA GATCATGCGCGCAACC-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,
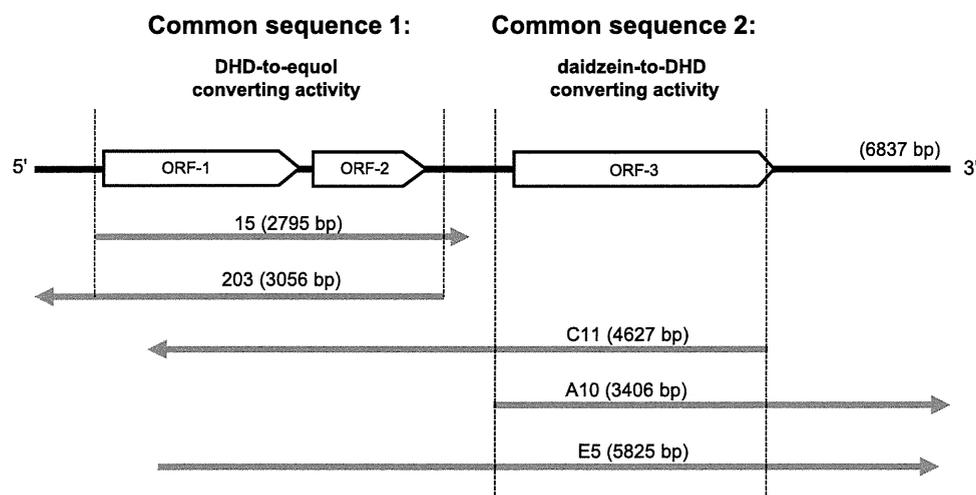

FIG 1 Organization of the genetic fragment in clones expressing daidzein-to-DHD- and DHD-to-equol-converting activity, and cloning of fragments involved in the deduced open reading frames.

— 156 —

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                                                          -35                    -10
  1 ATCGTCGGGTGCTCCGCAGCATATGACGGGCGTGCGCAATTCCAAGCTCATCGTTGCGGTGAACAACGATGCCGAGGCTCCTATTTTCCGCGCGTGCGCATA

103 CGGCATCGTGGGTGACGCCGCGAAAATCCTTCCCGTGCTTACCCAGGCGGTTAAGCAGCTCTAACAGTGGGAATTTCCGGTTTCGGTCTGGTGTTGAAACTG

                         RBS            Start (ORF-1)
205 GAAGCACATAAACAAGGAGGAACAAACATGGCCGAATTCGATGTTGAATATGACCTGGTCGTCGTAGGTGGCGGCGCGTCGGGAAAATCGGCTGCGCTCATT
                                        M  A  E  F  D  V  E  Y  D  L  V  V  V  G  G  A  S  G  K  S  A  A  L  I

307 GCGGCGCGCGCCGGCAAAAACGTCGTCGTGCTTGAGAAAATGCCCGAAACCGGCGGACTTTCCATGTACGCCGAGGGCACGGCCGCTTTCGAGTCGTCGGTG
      A  A  R  A  G  K  N  V  V  V  L  E  K  M  P  E  T  G  G  L  S  M  Y  A  E  G  T  A  A  F  E  S  S  V

409 CAAAAGGAACTGGGTATCCCTCGCCTTTCCAAGTACCACTTCCCGACTAAGAAGGAAGGTCTTGAAAAGCTGATGGGTTACAGCCACCAGCGCGCGAACTAC
      Q  K  E  L  G  I  P  R  L  S  K  Y  H  F  P  T  K  K  E  G  L  E  K  L  M  G  Y  S  H  Q  R  A  N  Y

511 GAAGTTGCCCGCGCATTTGTGGAGAACTCAGCCGAGACCATCGACATCTATCGCGACCTGGGCGTCGTGTATAAGACCTGCGACATCGCCGCGGAAGATGAT
      E  V  A  R  A  F  V  E  N  S  A  E  T  I  D  I  Y  R  D  L  G  V  V  Y  K  T  C  D  I  A  A  E  D  D

613 CCCAATGAGGTTTGGACGTTCCATTTGCCCGAGGGTCTGGGCGCGCATTGCCAAGAGGTGCTGCTTGACGCCATTCAGAAGCTTGACGTCGATATCTTCACC
      P  N  E  V  W  T  F  H  L  P  E  G  L  G  A  H  C  Q  E  V  L  L  D  A  I  Q  K  L  D  V  D  I  F  T

715 GAGACCCCCGCGAAAGAGCTGATTATTGAAGGCGGTCGCGTGGTTGGCGTTGTGGCCGAGTCGGAAGGCGCGCCTTTGCGCATCGGCGGCAAGGCCGTGATT
      E  T  P  A  K  E  L  I  I  E  G  G  R  V  V  G  V  V  A  E  S  E  G  A  P  L  R  I  G  G  K  A  V  I

817 CTGGCAACGGGTGGCATGGGCTCGAACCCCGATCGTATTTTCAAGTACAGCTGGTTTGCTCCGGCGGCGTACAACATGAACACGCTTACCCCACTGCAGAAT
      L  A  T  G  G  M  G  S  N  P  D  R  I  F  K  Y  S  W  F  A  P  A  A  Y  N  M  N  T  L  T  P  L  Q  N

919 GTGGGCGACGGCTTGGACTTGGCTCTTTCCGCCGGCGCTGACCCCACTGTTATTACCACCTGCCCGATACTTGCGGCAGGCGGCCGCGATATGACCATGGAT
      V  G  D  G  L  D  L  A  L  S  A  G  A  D  P  T  V  I  T  T  C  P  I  L  A  A  G  G  R  D  M  T  M  D

1021 TCCCAGGTCGGCGGCGCGGGCGTGAATCCGGGTGTGTGGGTCAATAAAAACTGGCCATCGCTTCGCGGCCGAATCGGTCGCTGAAAACCTCGGCGATATCGGA
       S  Q  V  G  G  A  G  V  N  P  G  V  W  V  N  K  T  G  H  R  F  A  A  E  S  V  A  E  N  L  G  D  I  G

1123 ACGTATTACGGCAAGCAACCTGGCGGCATTGTGTGGTCCATCCTCTCCCAGGCAGACGTTGACCGCCTGGTGAGCGAAGGCTCCGAAATCGCCATCGGCGAG
       T  Y  Y  G  K  Q  P  G  G  I  V  W  S  I  L  S  Q  A  D  V  D  R  L  V  S  E  G  S  E  I  A  I  G  E

1225 TTCGTGGTGTACCACAAGCCTATGGACCGTCTGCCCATCGAGCTCGACGCACATTTGGAGTCGGGGCCTGGTGAAGAAAGCCGACACCCTCGAGGAACTGGCC
       F  V  V  Y  H  K  P  M  D  R  L  P  I  E  L  D  A  H  L  E  S  G  L  V  K  K  A  D  T  L  E  E  L  A

1327 GACATGATGGACGTGCCCCAGGATGTGTTCGTCGAAACCATGCGCAGCTACAACGAGGCGTGCGAAAAGGGCTATGACGATGCGTTCATGAAGAAACCGCAG
       D  M  M  D  V  P  Q  D  V  F  V  E  T  M  R  S  Y  N  E  A  C  E  K  G  Y  D  D  A  F  M  K  K  P  Q

1429 TACCTGCGCGCCGTCGATAAAGCTCCCTTCTATGCCATTCCGCTGACAACGGGCACGATGGGCTCGGCCGGCGGCATCAAGATCAACGGCAACATGCAGGTC
       Y  L  R  A  V  D  K  A  P  F  Y  A  I  P  L  T  T  G  T  M  G  S  A  G  G  I  K  I  N  G  N  M  Q  V

1531 GTCGACGTTGATGGTAATGCCATCGAGGGCCTGTACGCCGTCGGCTTGGACGCCACGGGCCTGTACGGCGACTCCTACAACATGGAGATTCCCGGCTGCGCG
       V  D  V  D  G  N  A  I  E  G  L  Y  A  V  G  L  D  A  T  G  L  Y  G  D  S  Y  N  M  E  I  P  G  C  A

                                                                          End
1633 AACGGTTTCGCCCATACCTCGGGCCGCATCGCCGCGCGCCACGCGATTGCGAATATGTAAATCGCATTCAACATTTTCATTTGGAACCTTAAGCGCAAAAGC
       N  G  F  A  H  T  S  G  R  I  A  A  R  H  A  I  A  N  M  *

                                                            RBS          Start (ORF-2)
1735 GCTTTGAGGGGATAGAGCGCCCCGCCCGTTTTTGGGGTGGGCGGGGCATACTGAAAGGGGAAAGCAATGACTACCATTCCTAAGCTCAAGGGTGCTCCCGAC
                                                                           M  T  T  I  P  K  L  K  G  A  P  D

1837 TTCGGCAAGCGCATCGCTCCCGACGAGTCGGTTGGAAAGCGCCTCGAGGGCAAGCGTATCCTGCTTACCGGCACCACCAAGGGCGTGGGCCACGTTGCGCAG
       F  G  K  R  I  A  P  D  E  S  V  G  K  R  L  E  G  K  R  I  L  L  T  G  T  T  K  G  V  G  H  V  A  Q

1939 GAACTGCTGTGCGCCCATGGCGCGTTCGTGTGCGGTTCTGGCCGTACTCCGGGTGCCGCCGCTGCTTACGCCGACGAGCTGAAGGCGAAGGGCTACAAGGCC
       E  L  L  C  A  H  G  A  F  V  C  G  S  G  R  T  P  G  A  A  A  A  Y  A  D  E  L  K  A  K  G  Y  K  A

2041 GCCGGCTTCGATTGCGATTTGAGCGACTACGAGGCCGTAAAGAAGTGGGTTGCCCAGTGCGCCGAACTTATGGGCGGCATTGATGTCGTCATCAACAACGCT
       A  G  F  D  C  D  L  S  D  Y  E  A  V  K  K  W  V  A  Q  C  A  E  L  M  G  G  I  D  V  V  I  N  N  A

2143 TCCCACCCGGGCATGGCGCCGTTCGAGGCCATGGACGTTGAGACCTGGAACTACGGCATTCGCAACGAGCTCGACCTGGTGTACAACGTGTGCAATTGCGCG
       S  H  P  G  M  A  P  F  E  A  M  D  V  E  T  W  N  Y  G  I  R  N  E  L  D  L  V  Y  N  V  C  N  C  A

2245 TGGCCGTATCTGAAGGAAGGCAACGGCGCGAACATCATTATCACCTCTTCCACGGTTGGTCTTCAGGGCTCCAACTCTCCGCAGGCATGCCACGCCGCCGCC
       W  P  Y  L  K  E  G  N  G  A  N  I  I  I  T  S  S  T  V  G  L  Q  G  S  N  S  P  Q  A  C  H  A  A  A

2347 AAGGGCGCATGCCTGGCACTTGCTCGCCAGCTCGCCGCTGAAGGCGGTCCTTTCGGAATTCGCTGCAATTCCGTGACCCCCGGTCTGGTGTGGACCGAAGCC
       K  G  A  C  L  A  L  A  R  Q  L  A  A  E  G  G  P  F  G  I  R  C  N  S  V  T  P  G  L  V  W  T  E  A

2449 ATGGCCAACATTCCGAAGGAAATGGCGTCGGGCCTTATTGCCGCGCAAACCACGCAGCAAGCCATCGACCCCATCGACATCGCATACGCTTACCTGTTCTTG
       M  A  N  I  P  K  E  M  A  S  G  L  I  A  A  Q  T  T  Q  Q  A  I  D  P  I  D  I  A  Y  A  Y  L  F  L

                                                                                      End
2551 GCTTCCGATGAGGCCCGCCAGATCACTGCCGCCAATATCCCCGTTGACGGCGGCTGCTCTGGCGCCACGACGGGCGCTATGCAGGGCGAAATTGAGTAGAGC
       A  S  D  E  A  R  Q  I  T  A  A  N  I  P  V  D  G  G  C  S  G  A  T  T  G  A  M  Q  G  E  I  E  *

                                                     Terminator
2653 GCCTGCGTCGACGATTCGCGAAGCATTTGAAAAGGGCGTGCTGCGCTTTGCCGGAGCGCGCCCTTTCTCGTTGGCTCGCCTCGTCGTTGGGGCGAGCCTTGG
```

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.

— 157 —

```
                                                    -35                        -10
3120 GGCGCTTTGAGCGACATTGCGGTTGGCCTGCCCATCATCGTTGCCTACGCGATCGTTTTCGTTCTTATTTTCCTCAAGCCTGTTCAGGCGCTTGCGGCGAAG
```

```
                                                               RBS         Start (ORF-3)
3222 ATTTCGGGCTTCGATCCGGCGAAAGCCGCCTAGCGCATCTTCGCGCCACTCTTCAAACCAGTCTATGAAGAAGTAAGGAGCACAATAATGCAGCACGCGAAA
                                                                                         M  Q  H  A  K
```

```
3324 TACCCCCATTTGTTCTCGAAGGGCAAAGTGGGCAAGGTTACCACCAAGAACCGCGTTATTCGCAATTCCATGGGCACTTACCTTAATGTCGGCAAGCTGTGC
     Y  P  H  L  F  S  K  G  K  V  G  K  V  T  T  K  N  R  V  I  R  N  S  M  G  T  Y  L  N  V  G  K  L  C
```

```
3426 GACGTGTCCGATCGCAACATCAAGCACGCTGCCGAGGCCGCCGAGGGCGGCCCGGGCATTGTGTTTTGGACAACTGCCTTATTATGGAAGGCTACCACATG
     D  V  S  D  R  N  I  K  H  A  A  E  A  A  E  G  G  P  G  I  V  F  L  D  N  C  L  I  M  E  G  Y  H  M
```

```
3528 GGCCTTGCCGCCTATGACGACACCTACATTCCCGGGCTTTCCATGCTCGCCGAGGCCATGCACGATCACGGCGCGGTTGCGGGCATGCAGCTTGCGCATCCC
     G  L  A  A  Y  D  D  T  Y  I  P  G  L  S  M  L  A  E  A  M  H  D  H  G  A  V  A  G  M  Q  L  A  H  P
```

```
3630 GGTCGCGACATGGGCTTTGCCGGCGGCGACAACGTGGTTGCGCCCTCGGCAGTTCTTCCCGAAATTATGATTAACGCGGGCGCGACGGTTCCGCGTCCCTTG
     G  R  D  M  G  F  A  G  G  D  N  V  V  A  P  S  A  V  L  P  E  I  M  I  N  A  G  A  T  V  P  R  P  L
```

```
3732 ACTATCGACGAGATTCACGAAATCGAAGAGCAGTACGGCCAGGCCGCCGCGCGCGTGAAGCAGGCCGGCTTCGATATTGTTGAAGTTCATGGCGCGTGCGGG
     T  I  D  E  I  H  E  I  E  E  Q  Y  G  Q  A  A  A  R  V  K  Q  A  G  F  D  I  V  E  V  H  G  A  C  G
```

```
3834 TGCCTGCCCACGAACTTCCTCTCGCCGCACGACAACCAGCGAAACGACATCTACGGCGGCTCGCTGTTCAATCGTCAGCGCTTCCTCGTTGAAGTGATTCGC
     C  L  P  T  N  F  L  S  P  H  D  N  Q  R  N  D  I  Y  G  G  S  L  F  N  R  Q  R  F  L  V  E  V  I  R
```

```
3936 AGCATCAAGCGCTACGTCGGCCCCGATTTCCCCGTAAGCGTGAAGCTTGATATGGACGACTGCGAGCCCGATGGCATTCGCCTGGAGGAATGCATCGACACC
     S  I  K  R  Y  V  G  P  D  F  P  V  S  V  K  L  D  M  D  D  C  E  P  D  G  I  R  L  E  E  C  I  D  T
```

```
4038 TGTCGCGTGCTGGAGCGCGAGGGCGTTGCCCTGCTGAACCTGGTTACTGCGACGCACGTGACGGCGAACTTCTCCACGAGCTTCTACCCCTGGTCGTATTGC
     C  R  V  L  E  R  E  G  V  A  L  L  N  L  V  T  A  T  H  V  T  A  N  F  S  T  S  F  Y  P  W  S  Y  C
```

```
4140 GCTGACATGGCCGCCCAGGTTAAGGAACAGGTGCATATTCCCGTTATGGTGACGGGCGCCATCCAGTCGCCCGAGGCGGCCGAGAAAATTCTGGCCGACGGC
     A  D  M  A  A  Q  V  K  E  Q  V  H  I  P  V  M  V  T  G  A  I  Q  S  P  E  A  A  E  K  I  L  A  D  G
```

```
4242 AAGGTCGACTTCATCGGCACCGCGCGCCAGTGCTTGGCCGACCAGGCGTGGGTGGAAAAGGCGCGCACGGGCCACGAAGATGACATTCGTCCGTGTATCCGT
     K  V  D  F  I  G  T  A  R  Q  C  L  A  D  Q  A  W  V  E  K  A  R  T  G  H  E  D  D  I  R  P  C  I  R
```

```
4344 TGCCAAATCGGCTGCACCGACCGCGGCATTCTGGGGCATCACCCCATTTCATGCGCGGTGAACCCCACGTTGTTCCACTACTACGAGGAGCTCTACCCCAAG
     C  Q  I  G  C  T  D  R  G  I  L  G  H  H  P  I  S  C  A  V  N  P  T  L  F  H  Y  Y  E  E  L  Y  P  K
```

```
4446 GCTGCAACGCCCAAGAACGTGGCCGTTGTGGGCGCGGGCCCCGCGGGGTGTGAGGCGGCCCTTACGCTGAAGCAGCGTGGGCATAACGTAACCGTGTTCGAG
     A  A  T  P  K  N  V  A  V  V  G  A  G  P  A  G  C  E  A  A  L  T  L  K  Q  R  G  H  N  V  T  V  F  E
```

```
4548 AAGCGCGAGATTGGCGGCACGATGATAGAGGCCGGCGCCGCGTGGTACAAGGCCGACATCAATCGCTTCATCGACTACTACCGCAAGCAGCTGGAAAAGCAG
     K  R  E  I  G  G  T  M  I  E  A  G  A  A  W  Y  K  A  D  I  N  R  F  I  D  Y  Y  R  K  Q  L  E  K  Q
```

```
4650 CACATCGATGTGCGCATGCAAGAGGTAACGCCCCAGGATATTGCCGATGGCGGCTACGACGCGTGCATTGTTGCCATTGGCGGCGAGCCGCGCAAGCTGAAC
     H  I  D  V  R  M  Q  E  V  T  P  Q  D  I  A  D  G  G  Y  D  A  C  I  V  A  I  G  G  E  P  R  K  L  N
```

```
4752 GTTCCCGGCATCGACAAGCCCATCGTCACCGAGGGAATCGATTTCCTGTACGGCTCCAAGAAGGTGGAAGGAAAATCGGCCGTTGTTGTGGGCGGCGCCACC
     V  P  G  I  D  K  P  I  V  T  E  G  I  D  F  L  Y  G  S  K  K  V  E  G  K  S  A  V  V  V  G  G  A  T
```

```
4854 ACCACGGCGGAAATCGCCCTGGATTTGGCCGAGAAGGGAATGGACGTCACCATCGTGAAGCGCGGCACGAAATTCCTGAACCCCGCCGGGTGCCAGATGGAT
     T  T  A  E  I  A  L  D  L  A  E  K  G  M  D  V  T  I  V  K  R  G  T  K  F  L  N  P  A  G  C  Q  M  D
```

```
4956 ATCGAGTACACTATTCGCCTGCACCAGCTGGGCGTGAAGCTGATGACCGGCTATCGCCTTGACAGCGTCACCGATTCTTCGGCCATCGCGATTGACCAGTAT
     I  E  Y  T  I  R  L  H  Q  L  G  V  K  L  M  T  G  Y  R  L  D  S  V  T  D  S  S  A  I  A  I  D  Q  Y
```

```
5058 GGCGAAAAGGTCGAAATCCCCACCGAGAACGTGGTTATTTCCGCCGGCTATTTGAACCGTCCCGGTTTCGCCGAGCAGCTCGAGGAAATCAGCGACATGGAC
     G  E  K  V  E  I  P  T  E  N  V  V  I  S  A  G  Y  L  N  R  P  G  F  A  E  Q  L  E  E  I  S  D  M  D
```

```
                                                                                     End
5160 GTTTACATGGCGGGCGACTGCAAGAAGGTTGCCGAGATTCCCGATGCAACGCATGCGGGGTATGCGGTTGCGCGCATGATCTAGGCTCCTTCGGCGGCGCTG
     V  Y  M  A  G  D  C  K  K  V  A  E  I  P  D  A  T  H  A  G  Y  A  V  A  R  M  I  *
```

```
                            ◄┄┄┄┄┄┄┄┄┄┄┄┄ Terminator ┄┄┄┄┄┄┄┄┄┄┄┄┄►
5262 CGTCGATTGCGCCCGCTGGTCGAGCAGGCACTTCAGGTTCGATCAGCGGGCTTTTATTGAATAGGTTC
```

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.

— 158 —

```
A    1 -MAEFDVEYDLVVVGGGASGKSAALIAARAGKNVVVLEKMPETGGLSMYAEGTAAFESSVQKELGIPRLSKYHFPTKKEGLE  81
B    1 MAEEFDSHYDVIVIGGGGSGLSAAVQAAKNGLTCAVLEKEEQLGGSSAFAEGHAAFESDEQKKRGI------TVTKQEAYT  75
C    0 --------------------------------------------------------------------------------   0
D    0 --------------------------------------------------------------------------------   0
E    0 --------------------------------------------------------------------------------   0

A   82 KLMGYSHQRANYEVARAFVENSAETIDIYRD-LGVVYKTCDIAAEDDPNEVWTFHLPEGLGAHCQEVLLDAIQKLDVDIFTE 162
B   76 AYIDYSHWRCDSALVNRFVENAATTITKMRDEVGAVYEDVTITAPEQPGELVTWHLPEGEVAHLLELLEADARRRGVDIFLS 157
C    0 --------------------------------------------------------------------------------   0
D    0 --------------------------------------------------------------------------------   0
E    0 --------------------------------------------------------------------------------   0

A  163 TPA-KELIIEGGRVVGVVA-ESEGAPLRIGGKAVILATGGMGSNPDRIFKYSWFAPAAYNMNTLTPLQNVGDGLDLALSAGA 242
B  158 TPATRILRGEDGKIKGVVAKDADGETVRLGARAVVVGSGGYAANPALINKYGKFKIGEHVINA-GGKGNTGDGLKMMQEVGA 238
C    0 --------------------------------------------------------------------------------   0
D    0 --------------------------------------------------------------------------------   0
E    0 --------------------------------------------------------------------------------   0

A  243 DPTVITTCPILAAGGRDMTMDSQVGGAGVNPGVWVNKTGHRFAAESVAENLGDIGTYYGKQPGGIVWSILSQADVDRLVSEG 324
B  239 VENSNIGTMVFFPLMRDKTVTSHVNNAGMQPSLWVDKHGRRFTNETVGLNFGNAGDLMVGLPDAMFWCILDQDFIDRLVNKG 320
C    1 --------------MTRFRKSVLATLCLSMMGWSTAQAADAAKPEIPKSADIVIIGAGAAGTSATMAAAEKGAKIVLLEKQP  68
D    1 ------------------MRSLSSNKFFVFIILAVFSAFPAFAEKVYNTDFAIVGGGTTGLAAGVQAKMLGADVIILEKQP  63
E    1 --------------------MKRLWSSIASAALLVACALPAYAEKTMETDIAVVGGGLSGLSAATQATQLGAKVVVLEKQA  61

A  325 SEIAIGEFVVYHKPMDRLPIELDAHLESG---LVKKADTLEELADMMDVPQDVFVETMRSYNEACEKGYDDAFMKKPQYLRA 403
B  321 NFVGLGIYVRNYEKLIHLPGELEADAANDSCTNVYKGETLEALAGKIGVAPEVLRSEVGEYNGYVSAGEDKKYRKDPKYLFP 402
C   69 IVGGTGNFAEGIFAANSSLQKRQGIVVTPDMAFKTIMDYSHWMANPFVVRAFVNRSADTIEWVKSKGIKFEYIGPGGPGGML 150
D   64 ITGGTGNFAEGIFAAESSLQLRQGIDVSKEFAFKTIMDYSHWRANGPLVSAFVNKSAETIEWLKQFGIQYEYIGVGGFGGPL 145
E   62 KVGGTGLFCEGVFAAESKLQKRIGINVTKDFAYKLIMEYSHWKANSALAKHFVDRSAETVDWLDSMGIKIEYIGVGGHGGPL 143

A  404 VDKAPFYAIPLTTGTMGSAGGIKINGNMQVVDVDGNAIEGLYAVGLDATGLYGDSYNMEIPGCANGFAHTSGRIAARHAIAN 485
B  403 CNRGPFYAIKMEPGIMSVGAIKINEYMQVLDANGGVIPGLYSVGCDAGGLFGESYQLTIPGSANGFALTSGWLSADDIAEK 484
C  151 TWHVI---------DGPG--HGRHLIKTFHEQFKSMDVTTLVKTAGKDLVVKDGKVTGVIAQDSDGNTVQIDAKAVIIATG 220
D  146 TWHVI---GDY--EQDGKHYHHGKAVMMALTKRFQELGGTLLLETPGVDLIKKDGKIAGVIGQDKSGEKIRINAKAVLVATG 222
E  144 TWHVIAPGPDYLSGKNKKDYHCER-IINVFSKYVTDKGGQILLQTPGTDLIMDNGKVVGVWAKDKSGEKIRINAKAVVIATG 224

A  486 M--------------------------------------------------------------------------------- 486
B  485 VNAGAL-------------------------------------------------------------------------- 490
C  221 GYANNKEMLQK-YAAFPDTIMVGNVGKDGDGINMAWKAGAKPDGLGLLQAYRPGLPDYAPNSHLLAAARQPYLWVDQHGRRF 301
D  223 GFANNREMVAK-YSRYPDMIFIGHIGKTGDGIQMAWKAGADEEGVDVMQSYRPGLKGFHPASHLIAAAVQPYLFVDPNGHRY 303
E  225 GFSSNKEMMKKYYPEYPDITPVGNIGKDGDGITMGIKAGADLEGMNTVQGYRPGLPGFHPADQMIALAVQPYFWVTPRGERY 306
```

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 ac-

tivity 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

```
F    1  MTTIPKLKGAPDFGKRIAPDESVGKRLEGKRILLTGTTKGVGHVAQELLCAHGAFVCGSGRTPGAAAAYADELKAKGYKAAG  82
G    1  --------------MNEVLHKSRSQRLAGKVALVSGIGSGIGQACALMFARHGAWVVGCDLDASATQATLEAARSEGLELAS  68
H    1  --------------------MTRRLEGRVALISGTGGGQGRAAALRFVVDGAIVVGCDVNAEADAETARLVAEAGGQMTT  60
I    1  --------------------MNGQRLAGKTALITGIGNGIGQACALLFARQGARVLGCDWDPEAAAETLALAQAEGHESFA  61
J    1  ----------MTTFADKISRGPCAGRLQDKVALVTGAGGGQGQIVSLLFAEAGAKVCASDINPATLEQTRALAEQRGLKI-D  71
```

```
F   83  F-DCDLSDYEAVKKWVAQC-AELMGGIDVVINNASHPGMAPFEAMDV-ETWNYGIRNELDLVYNVCNCAWPYLKEGNGANII  161
G   69  LHPCDLTRPAEVQRWVAYA-LGLHQRLDVLVNAAAAAVFKPIEALSF-AEWKRTLEAELDTVFLVCQAAWPYLK-QNGGSII  147
H   61  M-VCDLGDPDGARSWIDRA-IIDHGRIDVLYNNASSAKFGSIAELSV-EDWRFTIRNELDLVFLTTKFAWPYLA-KQGGSII  138
I   62  VHCCDLTQPQAVEEWVKWACAQGDGGFDCLVNAAAFGAFAWIEEMDYESQWRRTLTGELDIVFLACKAAWPVLKTRGGGSVI  143
J   72  FAMVDASSPEQVNGWIDRV-MQKHGRIDVLYNNGAGVHMAPFDQMTF-EQWKETIRLELDVIYLPTKAVWPIMSEQKSGSII  151
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F  162  ITSSTVGLQG-SNSPQACHAAAKGACLALARQLAAEGGPFGIRCNSVTPGLVWTEA----MANIPKEMASGLIAAQTTQQAI  238
G  148  NFASANAYGVLERSPALAHCAGKGGVLAMTRQLALEGAPYGIRANTISPGLVVTGAT-APVIADPEIRALWLKKHML-GRFG  227
H  139  NVASTAGWQGSRGNGTIAHNATKCGVVAMTRQMALEGAPLGIRANSISPGFVITPGTRA-FVDNPAVRAQ-LTANIPLGRPG  218
I  144  NFASANARMALEGSPALAHCAGKGGVLAMTRQLAMEGGPHRIRVNSLSPGLIETAATRAHMARDPGLRQTALGRQFLRQRLG  225
J  152  NIASCAGMLAAEGLGTTAHAAGKCGVIALTRQLSLEGAPHWIRVNCISPGPIMGPALQRPYDESPYFRKL-FDSTPSLDRHG  232
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F  239  DPIDIAYAYLFLASDEARQITAANIPVDGGCSGATTGAMQGEIE              282
G  228  QPEDVAWMAVFLASDESNWITASDFAVDGG-TRAL---------              261
H  219  EPEDIVGMAAFLASDEAAFITGADIIIDGG-TTAC---------              252
I  226  QPDDVAWAAVYLASDEAAWVTGADLAIDAG-ATAG---------              259
J  233  YPLDIAYAGLFLASDESTFITGVNLPIDGG-ATSKVGAMMGRS-              274
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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

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K    1 MQHAKYPHLESKGKVGKVTTKNRVIRNSMGTYLNVGKLCDVSDRNIKHAAEAAEGGPGIVFLD----NCLIMEGYHMGLAAY 78
L    1 MKNKFYPKTFERGYIGNLEVENRAIRMPMGTELGNPDGSPSWA-SLKAYAEAADGGTGIVFMD----NAGVTQFHHVGLSLA 77
M    1 -MKKMFPKLFEPGQIGTLRVKNRLVKAPQTTGLSNKDG-TVTQRLVDHYTHLADGGAGLVIVEYAYIDDIASKSCHCQVGIS 80
N    1 --MTHFPNLFSEGRIGNLVIRNRIVMPPMATNLANEDG-SVSQRLIDYYVARARGGVGLIILENVQVDYPQGKNVACQLRLD 79
O    1 --MTHFPNLFSEGRIGNLVIRNRIVMPPMATNLANEDG-SVSQRLIDYYVARARGGVGLIILENVQVDYPQGKNVACQLRLD 79

K   79 DDTYIPGLSMLAEAMHDHGAVAGMQLAHPGRDMGFAGGDNVVAPSAVLPEIMINAGATVPRPLTIDEIHEIEEQYGQAAARV 160
L   78 SDNYIGPMSVLAKTIKQHGAIPGLQIVHPGRDAAFVRGDDLISSSRIQWEPWYENGGAVPRELTIEEIHDFVGYFGDCALRA 159
M   81 SHEHIAGLGWLADSIKNHGAKAGIQIEHCGRQR-FL-GPPMKSASAIPWPMLYDQFHAIPEELTIDEIQVLIEAFGDAAKRA 160
N   80 DDKYMAGFFELAEAVHSYGAKIFMQIHHAGRQT--TPGITEGLQPVAPSPVPCSFLGTQPRELTINEIEEIIQKFVDAAVRA 159
O   80 DDKYMAGFFELAEAVHSYGAKIFMQIHHAGRQT--TPGITEGLQPVAPSPVPCSFLGTQPRELTINEIEEIIQKFVDAAVRA 159

K  161 KQAGFDIVEVHGACGCLPTNFLSPHDNQRNDIYGGSLFNRQRFLVEVIRSIKRYVGPDFPVSVKLDMDDCEPDGIRLEECID 242
L  160 QTAGFEIVDVHAACGVLLSNFLSPRNNTRNDMYGGSLHNRARFLLEVIRDIKK-KCPNLPLAIRLSGIDFEPDGITIEETCE 240
M  161 VDAGFDLVEIHAAHGYLITNFLSPFTNKRGDWYGGSRENRFRFLGQVVENCRRKVGPDFPLTVRLSGTDYEPDGMTIEDTIY 242
N  160 KGAMFDGIELHGAHGYLIGQFMSPRTNRRVDKYGGSFERRMRFPLEIIRRIKEAVGEDYPISFRFSADEFVEGGNTLEEGKQ 241
O  160 KGAMFDGIELHGAHGYLIGQFMSPRTNRRVDKYGGSFERRMRFPLEIIRRIKEAVGEDYPISFRFSADEFVEGGNTLEEGKQ 241

K  243 TCRVLEREGVALLNLVTATHVTANFSTSFYPW--SYCADMAAQVKEQVHIPVMVTGAIQSPEAAEKILADGKVDFIGTARQC 322
L  241 VAKMCEAACADAINITWGSHAEVINAAGLLSKHGANHVEAAKMIKDAVSIPTMLCGGIYSPEIGEKLLEDGVCDFIGIGKPA 322
M  243 YAKELEKLCIDAFHISGGDHHTMIHQVSPMAMPVCYNVWAAEAVKKEVHVPVMASCSITLPQYAEDILEQGKADFITLGRPM 324
N  242 IAKMLEEACGVHVLHVSAGIYESMPTLLEPSRFEQGWRVYLAEEIKKVVNIPVITVGVIREPEFAEKIIAEGRADFVAVGRGL 323
O  242 IAKMLEEACGVHVLHVSAGIYESMPTLLEPSRFEQGWRVYLAEEIKKVVNIPVITVGVIREPEFAEKIIAEGRADFVAVGRGL 323

K  323 LADQAWVEKARTGHEDDIRPCIRCQIGCTDRGILGHHPISCAVNPTLFHYYEELYPK-AATPKNVAVVGAGPAGCEAALTLK 403
L  323 LADPMWAKKAAEGRPEDIRPCIGCGVGCHDRGMLSGGVVQCAVNAALYKFDEPVYPQ-AEVPKKVIIIGAGPAGCEAAITAK 403
M  325 WADNEWVKKAMEDRPEDIRPCIRCNEGCLQRSSFLGRTVMCAVNPVL-GFEEDLAVKPAETKKKVVIAGGGPAGMEAARVLK 405
N  324 IADPEWPKKAKEGRQNEIRKCISCNIGCIGGRVFQNLRLRCTVNPVAGREGVYSEIKQAPVKKKVVVAGGGPAGMQAAITAA 405
O  324 IADPEWPKKAKEGRQNEIRKCISCNIGCIGGRVFQNLRLRCTVNPVAGREGVYSEIKQAPVKKKVVVAGGGPAGMQAAITAA 405

K  404 QRGHNVTVFEKRE-IGGTMIEAGAAWYKADINRFIDYYRKQLEKQHIDVRMQ-EVTPQDIADGGYDACIVAIGGEPRKLNVP 483
L  404 KCGHDVTIYEKRK-IGGVLKEATVSDSKEDLGRLITYYETQLKKEGIEVIYE-EATADTVVAGGFDVAIVACGATVRNLNID 483
M  406 LKGHDVTIYEMRK-LGGYLHEASAPEFKEDIRHLIDYQIHQIEKLEIPVVSE-ELTPEMVKAGGYDVVISAVGAEPVIPAVP 485
N  406 KRGHQVILYEKKQHLGGQLEIASASPGKAKIKWFRDWLEAELSRAGVEVRSGVTADAETIAALSPDYVILATGSEPVTPRIK 487
O  406 KRGHQVILYEKKQHLGGQLEIASASPGKAKIKWFRDWLEAELSRAGVEVRSGVTADAETIAALSPDYVILATGSEPVTPRIK 487

K  484 GIDKPI---VTEGIDFLYGSKKVE-GKSAVVVGGATTTAEIALDLA-EKGMDVTIVKRGTKFLNPAGCQMDIEYTIRLHQLGV 561
L  484 CQDDPS---VVYAMDFLDNDCKSD-ADRVVVVGGGIVGAETALILAEERGKDVTITTRSPEFF--VSGVMGIAYMVRLGMAGV 560
M  486 GIDGKN--VINALAILDRHPEI--GKKVVVVGGGMIGTELAIDLA-EKGHEVTIVEMKDAIMADCAVTDVIAYYEKIGRNRI 562
N  488 GAEKENTFVFQAWDVLAGKVSFDKDEEVVVIGGGLVGCETAHYLA-EKGAKVTIVEMLSDIAIDMEPISRFDMMQQFTKLGI 568
O  488 GAEKENTFVFQAWDVLAGKVSFDKDEEVVVIGGGLVGCETAHYLA-EKGAKVTIVEMLSDIAIDMEPISRFDMMQQFTKLGI 568

K  562 KLMTGYRLDSVTDSSAIAIDQY-GEKVEIPTENVVISAGYLNR-PGFAEQLEE-ISDMDVYMAGDCKKVAEIPDATHAGYAV 640
L  561 TIKPSTQLVAVKDGKPMFAGPR-GLE-TLDVDQTIISSGFVPTFNQFRAQIEEKCEDVRVIGIGDCKASRMVMDAVHEGYIA 640
M  563 AVIPGLRVTEVTEQGVRGVNDRTGRRTELPADSVVIAVGLKPR-HAFYDTLAG-EPNLEVYEIGDCVKAGKILDAFHTAYKT 642
N  569 TARTGKVVTEITPEGVAAVGKE-GKQDFIRAHKVVLAIGQSPVGNELKKTLED--KGIDVRVIGDAYNVGKIIDAVSSGFQV 647
O  569 SARTGKVVTEILPRGVAAVGKE-GKQDFIRAHKVVLAIGQSPVGNELKKTLED--KGIDVRVIGDAYNVGKIIDAVSSGFQV 647

K  641 ARMI                                                                              644
L  641 GCNL                                                                              644
M  643 AVRI                                                                              646
N  648 AWQI                                                                              651
O  648 AWQI                                                                              651
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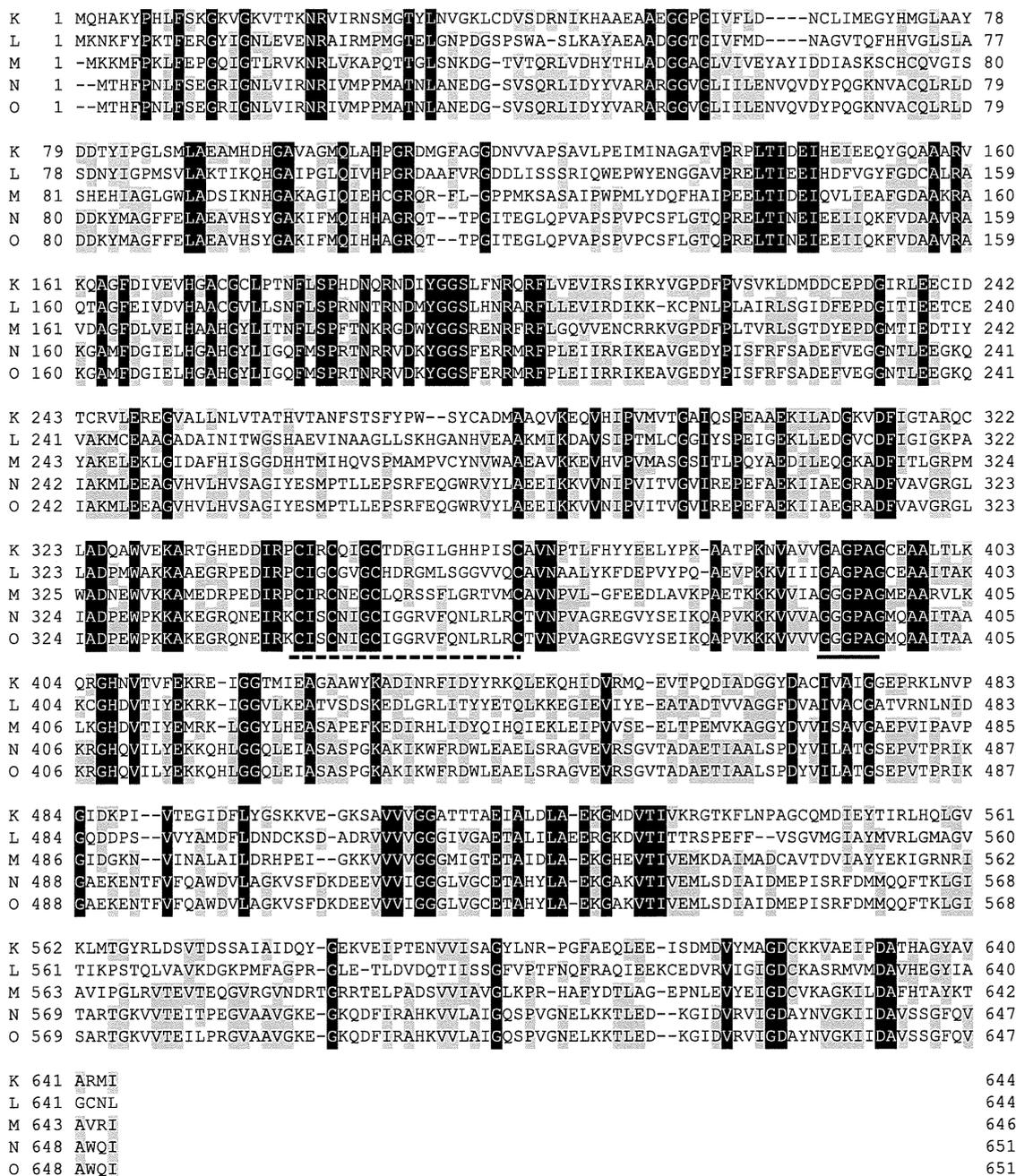
FIG 6 Sequence alignment of ORF-3 (K; accession no. AB646272) from *Slackia* sp. strain NATTS, daidzein reductase (L; accession no. BAJ22678) from *Lactococcus garvieae* strain 20-92, NADH oxidase (M; accession no. ZP_06113274) from *Clostridium hathewayi* DSM 13479, NADH:flavin oxidoreductase/NADH oxidase (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-

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