Plants produce a wide variety of secondary metabolites with a broad range of functionalities that are of industrial interest, including antimicrobial, antifungal, antioxidant, flavor-enhancing, or health-promoting properties (33). One obvious approach for harvesting these compounds is their isolation from plants. In many cases, however, productivities of target compounds are low and they may have to be isolated from complex raw materials requiring advanced downstream processing procedures. In recent years, there has been growing interest in using recombinant microbial systems as alternative production platforms for the efficient production of specific bioactive plant compounds. Microbial production systems offer the possibility for production of target compounds in a clean and simple metabolic background that minimizes the risk of formation of unwanted side products. Moreover, additional metabolic engineering strategies aimed at increasing the availability of precursors or the addition of functional groups that increase bioactivity, as for instance through the addition of glycosyl groups, may be applied. Various groups have described the construction of Escherichia coli strains producing carotenoids, terpenoids, flavonoids, and flavanones after the introduction of the respective regulatory genes integrated into the chromosomal DNA. This has been facilitated by the development of efficient expression systems such as the nisin-induced expression system. The specific activity of SAAT can be improved threefold (up to 564 pmol octyl acetate h⁻¹ mg protein⁻¹) by increasing the concentration of tRNA¹⁰₅₆, which is a rare tRNA molecule in Lactococcus lactis and actively expressed using the nisin-induced expression system. The expression can be efficiently controlled through the addition of nisin (34).

This system has several interesting properties, including the use of a food grade inducer molecule, a linear dose-response curve, and the absence of formation of inclusion bodies and endospores (46). Moreover, the relatively simple metabolism of Lactococcus lactis allows efficient rerouting of metabolic fluxes, enabling the rational increase of production levels of desired products. Finally, its food grade status favors its application as a host for the production of plant metabolites that are used as food ingredients. Recently, Martinez-Cuesta et al. (42) reported the first example of the functional expression of a plant protein, coumarate:coenzyme A (CoA) ligase from Arabidopsis thaliana, in Lactococcus lactis.

In the current paper, we report on the expression of genes from strawberry (Fragaria x ananassa) in Lactococcus lactis. We focus on enzymes involved in the production of two major classes of fruit flavor metabolites: terpenoids and esters. Terpenoids are an important class of biologically active molecules, including flavor and fragrance compounds, pheromones, medically active compounds, growth regulators, and vitamins (2, 44). Terpenoid classification is based on the number of C₅ moieties (isopentyl diphosphate and dimethylallyl diphosphate) used to build the carbon skeleton of the molecule. Monoterpenes are the simplest terpenes, composed of two of these moieties, whereas compounds containing three moieties are referred to as sesquiterpenes. Linool is a monoterpene directly derived from geranyl diphosphate (GPP) and a key flavor compound in strawberry and other fruits (1, 5, 38). This acyclic monoterpenoid has a sweet, floral, and citrus-like odor, and recently, various linool synthases have been characterized (1, 17, 29, 40) and used for the enhancement of volatile production in transgenic plants (38). Another component of strawberry flavor is nerolidol, a sesquiterpene with a floral and woody odor that plays a role in the defense response of plants against arthropod pests.

**Expression of Plant Flavor Genes in Lactococcus lactis**

Igor Hernández,¹ Douwe Molenaar,¹ Jules Beekwilder,² Harro Bouwmeester,² and Johan E. T. van Hylckama Vlieg¹†

NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands,¹ and Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands²

Received 7 August 2006/Accepted 21 December 2006

Lactic acid bacteria, such as Lactococcus lactis, are attractive hosts for the production of plant-bioactive compounds because of their food grade status, efficient expression, and metabolic engineering tools. Two genes from strawberry (Fragaria x ananassa), encoding an alcohol acyltransferase (SAAT) and a linalool/nerolidol synthase (FaNES), were cloned in L. lactis and actively expressed using the nisin-induced expression system. The results illustrate the potential of the application of L. lactis as a food grade expression platform for the recombinant production of proteins and bioactive compounds from plants.
Acetyl-CoA

Mevalonate pathway

Isopentyl-PP

Geranyl-PP

Farnesyl-PP

Geranylgeranyl-PP

Methylallyl-PP

FaNES

Undecaprenyl-PP

Linalool

Nerolidol

Bacterial cell wall

Lipid II route

FIG. 1. Partial view of the undecaprenyl diphosphate acid pathway in *L. lactis*, focused on the formation of GPP and FPP, and the reaction catalyzed by FaNES leading to the production of the monoterpene linalool, the major terpene produced in *L. lactis*, and the sesquiterpene nerolidol (30).

The production of linalool and nerolidol occurs via the same pathway, where the last biosynthetic step(s) is catalyzed by the *Fragaria x ananassa* linalool/nerolidol synthase (FaNES) (9) (Fig. 1).

Esters are key components of the flavors of strawberry, apple, mango, and other fruits and vegetables. In plants they are typically produced by the enzyme-mediated transfer of the acyl chain from an acyl-CoA ester to an alcohol, and a wide variety of such enzymes in many plant species have been characterized (3, 9, 18, 57, 59).

Here we report the cloning and expression of a linalool/nerolidol synthase and an alcohol acyltransferase from strawberry in *L. lactis* as examples of the suitability of *L. lactis* as an expression platform for plant genes. Functional expression was analyzed, and the production of monoterpene, sesquiterpenes, and long-chain alcohol esters during *L. lactis* fermentation is reported.

MATERIALS AND METHODS

**L. lactis** strains and growth conditions. Strains and plasmids used are listed in Table 1. *L. lactis* strain NZ9000, an MG1363-derived strain with the *nisR* and *nisK* genes integrated into the chromosome, was used for cloning and expression purposes. Strain NZ9000 was grown in M17 medium (61) supplemented with 1% glucose (GM17) at 30°C unless indicated otherwise. The following antibiotics were added when appropriate for the selection of plasmid-containing clones: chloramphenicol (10 mg ml⁻¹) and erythromycin (10 μg ml⁻¹). Growth experiments with milk were carried out using skim milk after sterilization (10 min at 110°C) supplemented with Casitone (0.5%) and glucose (1%) prior to inoculation as a protein and carbon source for the nonproteolytic and Lac⁺ strain *L. lactis* NZ9000. Milk fermentation tests were carried out at 30°C, without the addition of antibiotics to the medium.

DNA and plasmids. The genes expressed in *L. lactis* were originally isolated from *Fragaria x ananassa* (strawberry). The *SAAT* gene encodes an alcohol acyltransferase (SAAT) that was previously described by Aharoni et al. (3) (GenBank accession number AF193789). The FaNES gene encodes the *Fragassa x ananassa* enzyme linalool/nerolidol synthase (FaNES), a terpenesesquiterpene synthase that was initially described by Aharoni and O’Connell (4) and subsequently characterized by Aharoni et al. (1, 4) (GenBank accession number AX592905).

SAAT was previously cloned into the pRSSET-B vector (3), designed for expression in *E. coli*. It was amplified with Pwo DNA polymerase (30 cycles of 15 s at 94°C, 30 s at 47°C, and 90 s at 72°C), using the SAAT forward primer 5’-ATTTGAGAAATATTGAGTCG-3’ and SAAT reverse primer 5’-CGCC GCAATGCGACCATACTTTCTTAAC-3’. The PCR product was digested with SphI and the resulting fragment was introduced into the pNZ8150 vector using ScaI and SpHl sites, producing a 4,581-bp plasmid designated pNZ7601.

For the pNZ7610 insert, the nucleotide sequence described in the supplemental material was purchased in the pPCR-Script vector from Geneart (Regensburg, Germany). This vector was digested with DraI and KpnI, and the resulting 1,339-bp fragment was cloned into pL253 using the HaeIII and KpnI sites. Hence, this construct was a fusion between the *nisF* promoter (15), a spacer region usually preceding *L. lactis* tRNA operons (49), and the sequence predicted for tRNA^18^ in *L. lactis* strain IL-1403 (GenBank locus L200111). In this cloning step, the ColEI origin of replication for *E. coli* was introduced with the synthetic sequence into the pL253 vector, producing pNZ7610, an *E. coli* and *L. lactis* shuttle vector. The synthetic codon-optimized variant of the *SAAT* gene (see the nucleotide sequence described in the supplemental material) was purchased from Geneart. For cloning purposes, a PstI site was introduced between the *nisF* promoter and the ATG start codon of *SAAT*. The insert was received in the pPCR-script vector and was introduced into pNZ8150 using the PstI and KpnI sites in the vector and in the insert. The resulting vector was designated pNZ7630 and maintains the main characteristics of pNZ8150.

FaNES was amplified using Pwo DNA polymerase, and the resulting fragment was digested with XbaI and introduced into pNZ8150 using ScaI and XbaI sites. The resulting vector carries the FaNES gene under the control of the *nisA* promoter and was designated pNZ7640. All the plasmids were sequenced to confirm that the sequences obtained were correct. Standard molecular biology techniques and protocols were carried out as described by Sambrook et al. (56).

**Enzymatic assays.** For determination of enzyme activities, an aliquot from an overnight culture was used to inoculate (5%, vol/vol) fresh medium, and subsequently, growth was monitored until the optical density at 600 nm (OD₆₀₀) reached between 0.4 and 0.5 (early exponential phase). The culture was split in two equal subsamples, and nisin (final concentration, 1 ng ml⁻¹) was added to one of these, whereas the other subsample was used as a noninduced control. Aliquots were taken every hour to monitor bacterial growth. At sampling times, 40- to 50-ml aliquots were taken and centrifuged (6,000 rpm, 15 min, 4°C). The pellet was resuspended in 1 ml of cold reaction buffer (described below for each enzyme assay).

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
</table>
| **Strain**
| *L. lactis* subsp. cremoris NZ9000 | MG1363 pepN::nisRK; host strain for the NICE system | 36 |
| **Plasmids**
| pIL253 | Ery<sup>a</sup> | 58 |
| pNZ8150 | Cm<sup>a</sup>; *nis4* transcriptional fusion vector | 46 |
| pNZ7601 | Cm<sup>a</sup>; pNZ8150 derivative carrying the *SAAT* gene | This work |
| pNZ7610 | Ery<sup>a</sup>; pIL253 derivative carrying the *nisF* promoter fused to the tRNA<sup>18</sup> gene of *L. lactis* strain IL-1403 | This work |
| pNZ7630 | Cm<sup>a</sup>; pNZ8150 derivative carrying the codon-optimized *SAAT* gene | This work |
| pNZ7640 | Cm<sup>a</sup>; pNZ8150 derivative carrying the FaNES gene | This work |

<sup>a</sup> Cm<sup>a</sup> and Ery<sup>a</sup>, resistance to chloramphenicol and erythromycin, respectively.
determination), and crude extracts (CE) were prepared by disrupting cells by bead beating (FastprepTM FP120 beater; twice for 30 s each time) using 1 mg of silica sand. An amount of 0.5 ml of buffer was added to wash the sand, and the liquid phase was transferred to an Eppendorf vial, which was centrifuged again (14,000 rpm, 10 min, 4°C). The soluble fractions (cell extracts [CFE]) were used immediately for enzymatic activity assays or stored (−20°C) until the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Acyltransferase activities were determined essentially as described by Aharoni et al. (3). Just before the reaction was started, 70 µl of 1-octanol stock solution (160 mM in reaction buffer) and 50 µl of acetyl-CoA stock solution (4 mM in water) were mixed with 50 µl of the reaction buffer (50 mM Tris-HCl, 1 mM dithiothreitol, pH 8.0) and 30 µl of the sample (CE or CFE) to a final volume of 200 µl in 10-ml vials, and each vial was closed with a Teflon septum. Reactions were carried out at 35°C with vigorous shaking for 5, 10, 15, 20, or 30 min, after which the reaction was stopped by injecting 200 µl CaCl₂ (4 M in reaction buffer). Samples were stored at 4°C until injection into a gas chromatograph-mass spectrometer (GC-MS).

Linalool synthase activity was determined as described previously (1). Briefly, in a 10-ml vial, 500 µl of the CFE in reaction buffer (15 mM 3-morpholinol-2-hydroxypropanesulfonic acid, pH 7.0, 10% [vol/vol] glycerol, 10 mM MgCl₂, 1 mM MnCl₂, 1 mM sodium ascorbate; and 2 mM dithiothreitol) was mixed with 480 µl reaction buffer and with 20 µl GPP (from a 2.1 mM solution). The vial was closed, placed in a Teflon septum, and incubated with shaking for 60 min at 30°C. Then, 1 ml CaCl₂ (4 M in reaction buffer) was added to stop the reaction. Samples were stored at 4°C until injection into the GC-MS.

Reaction products were detected with a GC-MS (ThermoFinigan) operating in selective ion mode (m/z = 61 for octyl acetate and m/z = 93 for linalool, with an ionization potential of 70 eV). Injection was done by an automatic injector (PAL system) equipped with a solid-phase dynamic extraction fiber (polydiem-silica sand). An amount of 0.5 ml of buffer was added to wash the sand, and the GC oven conditions were from 40°C (1-min hold) to 250°C (2-min hold) with a 35°C increment per min. At sampling time, chlorohexane was added as an internal standard. Retention times and spectra were compared with those of authentic standards.

Monitoring of product formation during fermentation. Fermentation conditions were similar to those used for enzymatic assay sampling. In this case, every 60 min, 2 ml of medium was transferred to a 10-ml vial. Samples were quenched by adding 250 µl of a solution consisting of HgSO₄ (2.97 mM) and NaCl (6.8 M) in water. The vial was closed at 4°C until injection into the GC-MS.

Stability of SAAT- and FaNES-recombinant strains. The standard plasmid pNZ8150 was used for the cloning of SAAT and FaNES, resulting in pNZ7601 and pNZ7640, respectively. In order to demonstrate that pNZ7601 and pNZ7640 were stable in L. lactis NZ9000, plasmid-containing L. lactis NZ9000 clones were incubated for 100 generations without antibiotic in the medium and the retention of the plasmid was confirmed by comparing numbers of CFU per milliliter on M17 medium with or without chloramphenicol for plasmid selection. Moreover, the intactness of the plasmids in 10 colonies isolated from plates without chloramphenicol every 20 generations was confirmed by PCR and restriction analysis (Scal and Spbl).

Expression of FaNES in L. lactis NZ9000(pNZ7640). Protein production was evaluated by SDS-PAGE analysis. In crude extracts of induced cultures of L. lactis NZ9000(pNZ7640), an extra band with an apparent molecular mass of 54 kDa was detected, similar to the predicted molecular mass of FaNES (59.2 kDa) (1). This band was observed in samples after 2 h of induction and did not appear in samples of L. lactis NZ9000 with pNZ8150 or in L. lactis NZ9000(pNZ7640) cultures that were not induced with nisin (Fig. 2). According to the densitometry analysis, this protein represented approximately 10% of the total cellular protein. Linalool synthase activity was measured with CFE of nisin-induced cultures of L. lactis NZ9000(pNZ7640). The activity was 0.4 ± 0.1 pmol linalool h⁻¹ μg total protein⁻¹, and no linalool production was detected in the noninduced samples or in the control strain [L. lactis NZ9000(pNZ8150)]. Using purified protein, the specific activity reported by Aharoni et al. (1) was 2.3 nmol h⁻¹ μg protein⁻¹. Hence, it can be calculated that recombinant extracts contained 0.017% of active soluble FaNES. Considering that SDS-PAGE analysis showed that that enzyme accounted for approximately 10% of the soluble protein, these results indicate that the majority of the soluble FaNES in L. lactis is inactive or that other compounds in CFE negatively affected protein activity.

Expression of SAAT in L. lactis NZ9000(pNZ7601). When CE or CFE of nisin-induced L. lactis NZ9000(pNZ7601) were analyzed by SDS-PAGE gels and stained with Coomassie brilliant blue, no extra bands were detected. The specific SAAT activity was determined as described by Fuglsang (21) for the same culture conditions in SAAT and FaNES with the codon usage values (RSCU) were calculated as described by Fuglsang (21) to compare codon usage in SAAT and FaNES with the codon usage in the entire genome of L. lactis IL-1403 and in highly expressed proteins in L. lactis IL-1403. Total codon usage data were collected from the KEGG database (30), and most of the codon usage of highly expressed proteins was derived from the work of Fuglsang (21).
activity detected in these extracts was 192 ± 1 pmol of octyl acetate h⁻¹ mg⁻¹ of total protein⁻¹ (Table 2). No detectable octyl acetate production was observed in extracts of L. lactis NZ9000(pNZ8150) or in extracts of noninduced L. lactis NZ9000(pNZ7601) cultures, demonstrating that ester production was due to expression of the SAAT gene (Table 2). Using purified SAAT protein carrying a His tag isolated from E. coli, Aharoni et al. reported a specific SAAT activity of 257 ± 49 pmol h⁻¹ mg⁻¹ protein⁻¹ (3). According to these data, SAAT protein represents 0.004% of total soluble protein in CFE of L. lactis NZ9000(pNZ7601). This amount of SAAT protein in our L. lactis is, indeed, too low to be detected on a protein gel.

Transcription analysis. Because the SAAT protein levels detected by SDS-PAGE were much lower than FaNES levels, further research focused on increasing the expression of SAAT protein. As described above, plasmid instability due to the toxicity of the induced protein was not observed. Hence, we hypothesized that limited protein production may be due to low mRNA levels as a result of improper transcription or mRNA instability. Therefore, SAAT mRNA levels in nisin-induced L. lactis NZ9000(pNZ7601) cultures were analyzed and compared with FaNES mRNA concentrations in nisin-induced L. lactis NZ9000(pNZ7640) cultures. Northern blots of mRNA samples form L. lactis NZ9000(pNZ7601) and L. lactis NZ9000(pNZ7640) showed the presence of two clear bands that were around 1.5 kb in induced samples, with similar intensities in both samples (Fig. 3). This size is in agreement with the predicted mRNA size (1,357 bp for SAAT and 1,559 bp for FaNES). No signal was detected with strain NZ9000 (pNZ8150) or in noninduced samples, demonstrating that mRNA was produced exclusively in induced recombinant strains carrying plant genes. Therefore, a reduced transcription level or mRNA instability was unlikely to be the major cause of the lower protein level of SAAT produced in recombinant L. lactis NZ9000 strains.

Codon usage analysis. It is well documented that codon usage is one of the main factors interfering with the efficient production of eukaryotic proteins in microorganisms (46). There are many examples of low translation efficiencies caused by the accumulation of rare codons in bacteria (25, 55) or yeast (8). Accumulations of rare Arg codons can inhibit bacterial growth (23, 51), and in other cases where a protein is highly expressed, aberrant incorporation of amino acids resulted in a high fraction of inactive protein (11).

RSCU were calculated for SAAT and FaNES and plotted in Fig. 4. Frequently used codons in wild-type SAAT or FaNES are rarely used in the highly expressed gene set in L. lactis. These appear as high values in Fig. 4, and the results show that several “rare” codons occur in the SAAT and FaNES (Fig. 4B) sequences. In the SAAT sequence, 35 “rare” codons occur and major “rare” codon accumulations occur at positions 108 to 109 (TGA CGT), 241 to 242 (GAA TCA), 309 to 312 (ATT GTA GAA TTA), and 411 to 412 (ATC ATT). The FaNES gene contains only six rare codons in a total of 520 codons, and there are no rare-codon accumulations in this well-expressed protein. Therefore, we hypothesized that frequencies and the distribution of rare codons are responsible for low protein expression levels due to inefficient translation.

Optimization of protein expression through codon usage optimization. Different solutions have been proposed to improve protein expression when there are codon usage prob-

<table>
<thead>
<tr>
<th>Plasmid(s) in the clone</th>
<th>Description</th>
<th>Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNZ7601</td>
<td>“Wild-type” SAAT in pNZ8150</td>
<td>192 ± 1^A</td>
</tr>
<tr>
<td>pNZ7630</td>
<td>“Codon-optimized” SAAT in pNZ8150</td>
<td>225 ± 7^A</td>
</tr>
<tr>
<td>pNZ7601 + pIL253</td>
<td>pNZ7601 with the empty pIL253 vector</td>
<td>257 ± 49^A</td>
</tr>
<tr>
<td>pNZ7601 + pNZ7610</td>
<td>pNZ7601 and pIL253 with tRNA^AGC</td>
<td>564 ± 14^B</td>
</tr>
<tr>
<td>pNZ7630 + pNZ7610</td>
<td>“Codon-optimized” gene and pIL253 with tRNA^AGC</td>
<td>Not detectable^C</td>
</tr>
<tr>
<td>pNZ7610</td>
<td>Empty vector</td>
<td>Not detectable^D</td>
</tr>
</tbody>
</table>

a Activity was measured in picomoles of octyl acetate per hour per milligram of protein. Superior letters (A to D) placed next to enzymatic activities indicate values that were significantly different (P < 0.05) from values with a different letter.
lems. In some cases, gene synthesis with optimal codon usage of the complete gene (60) or in the initial part of the gene (8, 63) proved to be an effective means of increasing protein production. Alternatively, rare-codon tRNA may be coexpressed to increase the availability of rare-codon tRNAs (24). To the best of our knowledge, no examples of the last strategy for improving protein production in *L. lactis* have been reported.

Initially, we tried to improve SAAT protein production by introducing a codon usage-optimized gene containing only 32 of a possible 64 codons, potentially solving in a single strategy the translation initiation and translation blockage problems derived by rare-codon accumulation. The codon usage of the resulting gene was similar to that for the set of highly expressed genes in *L. lactis* (Fig. 4A). The codon-optimized gene was introduced into the vector pNZ8150; the resulting plasmid was designated pNZ7630 and used to transform *L. lactis* NZ9000. Protein production and enzyme activity were analyzed in induced cultures of *L. lactis* NZ9000(pNZ7630) as described above. However, no protein band was observed by SDS-PAGE analysis, and enzyme activities in both CE (data not shown) and CFE (Table 2) were comparable to the activities measured with “wild-type” SAAT in extracts of induced *L. lactis* NZ9000(pNZ7601). From these results, we concluded that codon optimization did not improve protein production. Similar observations have been made by others working on the optimization of protein expression in *E. coli* (20) and in *L. lactis* (Igor Mierau, unpublished results).

Optimization of protein expression through tRNA supplementation. As a next step, we decided to supplement a potentially rare tRNA. Three primary candidates can be recognized based on the rare-codon analysis of SAAT (Fig. 4A). These are tRNA<sub>Leu</sub> <sup>CUA</sup>, tRNA<sub>Arg</sub> <sup>AGG</sup>, and tRNA<sub>Arg</sub> <sup>AGA</sup>, and they are frequently reported to interfere with the efficient expression of eukaryotic proteins in bacteria. To our knowledge, no tRNA concentrations have been reported for *L. lactis*, so we decided to base our selection on the genetic organization above.
of tRNA loci in the genome of strain IL-1403 (10). In this genome, tRNA$^{34}_{34}$ is in a cluster with other tRNA and tRNA genes, and this cluster is under the control of a predicted promoter. The tRNA$^{34}_{34}$ and tRNA$^{34}_{34}$ genes, however, are located between two terminators and may not be efficiently expressed. We decided to supplement the tRNA$^{34}_{34}$ (AGG) gene because it has the lowest RSCU and may also allow the incorporation of arginine at positions corresponding to the AGA codon (21). In the SAAT sequence, AGG appears five times and AGA six times, which represents 58% of Arg codons in the protein.

Rare codons are used mostly in proteins that are expressed in non-exponential growth phases (22), and in theory, tRNA supplementation should be done carefully to avoid any toxic effect. Therefore, we designed a strategy in which this rare tRNA was added as an extra sequence in an independent plasmid under the control of an inducible promoter, analogously to the strategy successfully applied for E. coli (60). The nisF promoter was selected because it is inducible by nisin, is strictly controlled, and is compatible with the NICE system (15). By this strategy, the pIL253 vector was used to insert a copy of the L. lactis tRNA$^{34}_{34}$ gene under the control of the nisF promoter, generating the plasmid pNZ7610. This plasmid was transformed into L. lactis NZ9000, resulting in L. lactis NZ9000(pNZ7610). Also, the control strain L. lactis NZ9000 (pIL253), with unmodified pIL253, was created. Strains with pIL253 or pNZ7610 are erythromycin resistant. The pIL253-derived vectors are compatible with plasmids derived from pNZ8150 carrying a chloramphenicol resistance marker (45). Cotransformation of L. lactis NZ9000 with pNZ7601 and pNZ7610 results in a strain carrying both the SAAT and tRNA$^{34}_{34}$ genes under the control of a nisin-inducible promoter. Control strains were constructed by replacing either pNZ7601 or pNZ7610 with the corresponding empty vector pNZ8150 or pIL252, respectively. All of these control strains were able to grow on GM17 supplemented with erythromycin and chloramphenicol.

**SAAT expression in tRNA$^{34}_{34}$ gene-supplemented clones.** The supplementation with the tRNA$^{34}_{34}$ gene was a successful strategy. The coexpression of SAAT and the tRNA$^{34}_{34}$ gene resulted in a threefold increase in specific SAAT activity in L. lactis NZ9000(pNZ7630pNZ7610) CFE, compared to activities in "wild-type" and "codon-optimized" SAAT clones (Table 2). The specific octyl acetate production rate with L. lactis NZ9000 (pNZ7601pIL253) was similar to that in L. lactis NZ9000 (pNZ7601), demonstrating that the increased acyl-transf erase activity is due the expression of the tRNA$^{34}_{34}$ gene (Table 2). No octyl acetate production was detected with L. lactis NZ9000(pNZ7610), demonstrating that the SAAT gene is necessary for the enzymatic activity. Finally, when the tRNA$^{34}_{34}$ gene was coexpressed with the codon-optimized gene in strain L. lactis NZ9000(pNZ7630pNZ7610), acyltransferase activity was reduced dramatically to below the detection limit (Table 2).

This codon-optimized gene no longer contained the rare codon AGG or AGC, and hence the overexpression of tRNA$^{34}_{34}$ may lead to the accumulation of Arg linked to tRNA$^{34}_{34}$, which cannot be used for protein synthesis and ultimately may result in reduced levels of usable tRNA$^{34}_{34}$ molecules. Analysis of the noninduced clones resulted in no octyl acetate production, which is in agreement with the tight control of the NICE system. A sample of all CFE was subjected to SDS-PAGE analysis. No protein band with the expected molecular mass could be visualized in extracts of nisin-induced clones.

**Production of terpenes by FaNES-producing L. lactis NZ9000 clones during fermentation in GM17 or milk.** L. lactis NZ9000 (pNZ7640) carrying the FaNES gene was grown in GM17 medium and induced with nisin at early exponential phase (OD$_{600}$ = 0.4). Linalool synthase uses GPP as a substrate for linalool production. GPP is an intermediate in the production of lipid II in L. lactis (Fig. 1). In this pathway, GPP is converted to farnesyl-PP (FPP), which is also accepted as a substrate by FaNES and is converted to the sesquiterpene nerolidol. We hypothesized that FaNES-producing L. lactis NZ9000 strains may be able to produce both linalool and nerolidol, and therefore samples were removed from the medium to monitor terpene production.

Initially, we used purge-and-trap analysis to concentrate the volatile compounds formed during L. lactis NZ9000(pNZ7640) fermentation in GM17. Chromatographic conditions were similar to those used by Aharoni et al. (1). Comparative analysis of induced and uninduced samples showed that FaNES expression indeed resulted in the production of two compounds that were identified as linalool and nerolidol, respectively, by comparing their retention times and mass spectra with those of authentic standards. In all samples, the amount of linalool produced was four times higher than the amount of nerolidol, and therefore we focused subsequent experiments on linalool production.

In L. lactis NZ9000(pNZ7640), linalool was rapidly produced upon induction with nisin, and after 3 h, a linalool concentration of 85 ± 26 nM was detected (Fig. 5A). Subsequently, the linalool concentration remained constant during the stationary phase. The results show that there is a clear correlation between bacterial growth and linalool production, and it can be calculated that approximately 1.3 pmol of linalool was produced per mg of cells (with 30 mg ml$^{-1}$ being produced per OD$_{600}$ unit) (52). When L. lactis NZ9000(pNZ7640) cell suspensions or CFE were incubated with linalool, no degradation of the monoterpene was observed (data not shown), indicating that the constant linalool levels are not caused by an equilibrium between linalool formation and degradation. When fresh GM17 medium was added to a fully grown culture, linalool levels increased in proportion to the bacterial growth (data not shown). The correlation between linalool and growth may be related to GPP availability. In nongrowing cells, the lipid II synthesis rates and GPP levels may be low. Hence, nongrowing cells may no longer produce linalool due to limiting levels of GPP. Alternatively, FaNES may be sensitive to low intracellular pH levels in the later stages of the fermentation process.

FaNES productivity during fermentation was high compared with activities determined in CFE. The production rate in the first 3 h of the fermentation process was 146 pmol linalool h$^{-1}$ mg total protein$^{-1}$. This corresponds to approximately 40% of the production rate measured in vitro with CFE (400 pmol linalool h$^{-1}$ mg total protein$^{-1}$). Differences in the substrate concentrations or intracellular pH values that are suboptimal for FaNES could explain these differences.

Finally, FaNES was expressed during growth in skim milk supplemented with Casitone and glucose, required for proper growth of the expression host L. lactis NZ9000. Linalool was produced to
a concentration of 65.0 ± 7.8 nM after 4 h of incubation, and subsequently, the concentration remained constant (Fig. 5B). There was no detectable linalool production in noninduced samples or in the clones with the empty vector (pNZ8150) in all fermentation tests. These results are in agreement with the results obtained with GM17 medium and clearly indicate that L. lactis NZ9000(pNZ7640) can be used for the in situ production of the plant flavor compound linalool in fermented milk.

Octyl acetate production during fermentation in GM17 and milk. L. lactis NZ9000(pNZ7601) carrying the wild-type SAAT gene was grown in GM17 and skim milk to study ester production. In initial fermentations in GM17, we did not observe significant ester production (data not shown). SAAT catalyzes the production of ethyl esters from acetyl-CoA and terminal alcohols, and we postulated that levels of alcohols in GM17 are too low to allow ester production. Various alcohols can be used as substrates by SAAT with 1-octanol as the preferred substrate (3). Therefore, 1-octanol was added to the medium to a concentration of 100 μM as the alcohol substrate. No growth inhibition of L. lactis NZ9000 occurred at this concentration of 1-octanol (data not shown). For monitoring ester production in the SAAT-producing clone, cultures were grown in GM17 medium until the OD600 was 0.4. The cells were harvested by centrifugation and resuspended in the same volume of fresh GM17 supplemented with 100 μM 1-octanol.

The results clearly demonstrated that alcohol addition was necessary for measurable ester production and that octyl acetate production was linear with time for at least 4 h after induction and transfer to the fresh medium (data not shown). The octyl acetate production rate with an induced culture of L. lactis NZ9000(pNZ7601) was 326 pmol octyl acetate h⁻¹ mg of total protein⁻¹, which is nine times higher than the activity observed with a noninduced culture and with a clone carrying the pNZ8150 vector (Table 3). This background octyl acetate production activity may be caused by the indigenous L. lactis esterase EstA, which is capable of catalyzing ester production through the condensation of acid and alcohols or through transacylation reactions (39, 47). We also included the L. lactis NZ9000(pNZ7601pNZ7610) strain producing tRNA1550HERNÁNDEZ ET AL. APPL. ENVIRON. MICROBIOL.

<table>
<thead>
<tr>
<th>Fermentation medium and plasmid(s) used</th>
<th>Activity (pmol h⁻¹ mg protein⁻¹)²</th>
<th>Induced</th>
<th>Noninduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNZ7601</td>
<td>326 ± 87A</td>
<td>65 ± 33B</td>
<td></td>
</tr>
<tr>
<td>pNZ7601 + pNZ7610</td>
<td>371 ± 2A</td>
<td>73 ± 38B</td>
<td></td>
</tr>
<tr>
<td>pNZ7630</td>
<td>355 ± 57A</td>
<td>40 ± 1B</td>
<td></td>
</tr>
<tr>
<td>pNZ8150</td>
<td>40 ± 9B</td>
<td>55 ± 7B</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNZ7601</td>
<td>776 ± 66C</td>
<td>80 ± 8D</td>
<td></td>
</tr>
<tr>
<td>pNZ8150</td>
<td>74 ± 13D</td>
<td>68 ± 9D</td>
<td></td>
</tr>
</tbody>
</table>

² Superior letters (A to D) placed next to enzymatic activities indicate values that were significantly different (P < 0.05) from other values with a different letter.
sion could be improved almost threefold using a rare-tRNA supplementation approach. Rare-tRNA supplementation has been successfully applied in *E. coli* and other microbial cell factories (6, 7, 11, 14). To our knowledge, the current study is the first successful example of rare-tRNA supplementation in *L. lactis*. It is important to notice that low yields, or even the complete absence, of functional expression levels are frequently observed when plant enzymes are expressed in *E. coli* or other microbial expression platforms. For instance, melon alcohol acyltransferase, an enzyme highly similar to SAAT, could not be functionally expressed in *E. coli*, whereas in this case, active enzyme could be produced in recombinant yeast (64). Our results and those presented by Martinez-Cuesta (42) represent three successful examples of very different plant proteins that were actively produced in *L. lactis* and justify a broader exploration of the suitability of this organism as a complementary screening host for elucidating protein function and screening of plant cDNA libraries in a food grade bacterial expression platform.

FaNES-expressing *L. lactis* NZ9000 was able to produce linalool and nerolidol without substrate addition. FaNES catalyzes the synthesis of these compounds from GPP and FPP, respectively (Fig. 1), and *L. lactis* has the enzymatic machinery necessary to produce GPP and FPP as intermediates in the undecaprenyl diphosphate acid pathway (30). Using the linalool synthetase activity measured in CFE, it can be calculated that the productivity of growing cells was approximately 40% of the maximal productivity. This indicates that indigenous GPP levels were not severely limiting for linalool production in growing cells, especially when the fact that intracellular conditions may be suboptimal for the enzyme is taken into account. To our knowledge there are no reports of GPP or FPP concentrations in *L. lactis*, but this example demonstrates the potential of using *L. lactis* for the characterization of enzymes for the biosynthesis of monoterpenes and sesquiterpenes, classes of compounds containing a wide range of high-value bioactive compounds. Several groups have reported the construction of microbial cell factories for these compounds, as exemplified by the introduction in yeast or in *E. coli* of the biosynthetic pathways for monoterpenes, sesquiterpenes, and carotenoids (12, 19, 41, 65). The efficient metabolic engineering strategies that are available for *L. lactis* could enable the construction of efficient cell factories for the production of target terpenes (26).

SAAT-expressing *L. lactis* NZ9000 required the addition of long-chain alcohols for the production of ethyl esters. These results show that acetyl-CoA levels were sufficient to sustain a base-level ester production, but the equal levels of productivity of *L. lactis* NZ9000 clones producing different SAAT levels indicates that acetyl-CoA levels may limit ester formation. Glucose, which was used as a carbon source in our experiments, is converted mainly to 1-lactate, whereas approximately 2% is converted to acetyl-CoA; ultimately, ethanol, acetate, or biomass formation occurs (16, 50). In our clones, at least three enzymes may compete for acetyl-CoA pools (phosphotransacetylase, acetaldelyde/alcohol dehydrogenase, and SAAT), and the relative amount of acetyl-CoA used for ester production depends on the amounts and kinetic parameters of these enzymes. Lactococcal primary metabolism can be efficiently rerouted, and this offers the potential of increasing acetyl-CoA levels (48) and hence ester productivity.

The octyl acetate and linalool concentrations produced in fermentation tests are higher than the odor threshold values for these molecules. The octyl acetate concentration in GM17 of 1.9 μM is 27 times higher than the reported odor threshold for octyl acetate in water (FlavorBase, version 2004, Leffinger & Associates, Canton, GA). Analogously, linalool levels of 13 ppb (85 nM) are twofold higher than its odor threshold (6 ppb) in water (37). In a preliminary sensorial analysis done with GM17 cultures, however, neither octyl acetate nor linalool could be detected, probably because of the strong background odor of this medium. The expression plasmids that were used can be easily transferred into a food grade expression system by exchanging the chloramphenical marker with lacF as the selective marker (45, 53). Hence, *L. lactis* may be a valuable production host for plant-derived bioactive compounds for food applications.

ACKNOWLEDGMENTS

Igor Hernández acknowledges Marke Beertuynen, Iris van Swam, and Jilbert Bruinsma for excellent assistance. Anders Fuglsang is acknowledged for assistance with the codon usage analysis. Igor Hernández acknowledges his postdoctoral fellowship from the Department of Education, Universities, and Research of the Basque Government.

REFERENCES


35. 34.
25.
24.
23.
22.
21.
Fuglsang, A.
20. 17.
15.
14.
13.
12.
11.
10.
9.
8.
7.
de Vos, W. M., and J. Hugenholtz.
1203 HERNA Ñ ED ET AL. APPL. ENVIRON. MICROBIOL.
10.
9.
8.
7.
6.
5.
4.
3.
2.
1.
143–149.
297–304.
270–285.