Lactococcus lactis as expression host for the biosynthetic incorporation of tryptophan analogues into recombinant proteins

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Incorporation of Trp (tryptophan) analogues into a protein may facilitate its structural analysis by spectroscopic techniques. Development of a biological system for the biosynthetic incorporation of such analogues into proteins is of considerable importance. The Gram-negative Escherichia coli is the only prokaryotic expression host regularly used for the incorporation of Trp analogues into recombinant proteins. Here, we present the use of the versatile Gram-positive expression host Lactococcus lactis for the incorporation of Trp analogues. The availability of a tightly regulated expression system for this organism, the potential to secrete modified proteins into the growth medium and the construction of the trp-synthetase deletion strain PA1002 of L. lactis rendered this organism potentially an efficient tool for the incorporation of Trp analogues into recombinant proteins. The Trp analogues 7-azatryptophan, 5-fluorotryptophan and 5-hydroxytryptophan were incorporated with efficiencies of > 97, > 97 and 89% respectively. Interestingly, 5-methylTrp (5-methyltryptophan) could be incorporated with 92% efficiency. Successful biosynthetical incorporation of 5-methylTrp into recombinant proteins has not been reported previously.

Key words: 3β-indoleacrylic acid, Lactococcus lactis, 5-methyltryptophan, N-phosphonomethylglycine (glyphosate), tryptophan analogue, tryptophan auxotroph.

INTRODUCTION

Trp (tryptophan) is an attractive intrinsic fluorescence probe in protein chemistry [1,2]. Its emission maximum (λem) and the quantum yield (Q) are highly sensitive parameters for monitoring changes in the structure and dynamics of a protein. In numerous studies, the intrinsic protein fluorescence was used to monitor processes like enzyme kinetics, protein–ligand interactions or protein (un)folding. Modern molecular biology techniques allow easy introduction of a Trp codon at a position sensitive to monitor the desired process, while native Trp residues can be replaced by a tyrosine or phenylalanine residue, often without a noticeable effect on protein function. The use of Trp analogues with altered optical properties can further broaden the intrinsic fluorescence approach. A number of Trp analogues like 7-AzaTrp (7-azatryptophan), 5-OHTrp (5-hydroxytryptophan) and 5-FTrp (5-fluorotryptophan) can be efficiently biosynthetically incorporated into recombinant proteins using an Escherichia coli Trp auxotroph [3,4].

The Gram-positive bacterium Lactococcus lactis has been shown to be an excellent host for the expression of different proteins. Both homologous and heterologous proteins are well expressed [5–8]. This high-level production of proteins has been obtained using constitutive as well as inducible promoters [9,10]. In the present study, we explored the possibility of using L. lactis as a production organism for the incorporation of Trp analogues. A suitable L. lactis Trp auxotroph was not available. Efficient incorporation of Trp analogues with Trp prototrophic E. coli strains has been achieved in some cases by adding inhibitors that block the biosynthesis of Trp. We have investigated this approach for L. lactis and we also report the construction of a Trp auxotroph of L. lactis by deleting the Trp synthase genes. Best results were obtained using the Trp auxotrophic mutant strain and conditions are described that result in the efficient replacement (89–97%) of Trp by the Trp analogues 5-FTrp, 5-OHTrp, 7-AzaTrp and 5-methylTrp (5-methyltryptophan) in a recombinant model protein. This is the first report on the efficient incorporation of 5-methylTrp into a recombinant protein.

EXPERIMENTAL

Bacterial strains, plasmids, media and growth conditions

L. lactis strains and plasmids used in the present study are listed in Table 1. Plasmids pPA6 and pPA90 used to express the studied proteins PA6 and PA90 respectively are based on the nisin-inducible expression vector pNZ8048 [10]. The relevant parts of the plasmids are described in Figure 1. L. lactis strains were grown at 30°C in M17 medium, supplemented with 0.5% glucose (GM17) [11] or in CDM (chemically defined medium) [12], supplemented with 0.5% glucose and 1 mM of all amino acids, except Trp. Antibiotics (chloramphenicol or erythromycin) were added at a concentration of 5 µg/ml for the maintenance of the plasmids. Media were solidified by 1.5% agar and β-galactosidase activity of LacZ was detected by supplementing...
incorporation of Trp analogues, *L. lactis* strains harbouring plasmids pPA6 or pPA90 were grown overnight in GM17 at 30°C. Subsequently, the cultures were diluted 50-fold in 400 ml of fresh GM17 and grown for approx. 4 h at 30°C (DMc 0.8). The cells were harvested by centrifugation at 7000 g for 10 min, washed with saline (0.9% NaCl) and, finally, resuspended into 100 ml of CDM supplemented with 0.5% (v/v) trichloroacetic acid for 1 h at 0°C, followed by 20 min centrifugation at 13000 g and 0°C. Samples were analysed using SDS/PAGE. Per lane trichloroacetic acid precipitate originating from 0.4 ml of culture was loaded.

**General DNA techniques and transformation**

Restrictions, ligations and PCRs were performed according to the enzyme supplier’s instructions and essentially as described by Sambrook et al. [13]. Genomic DNA was isolated as described earlier [14]. Plasmids were transferred to *L. lactis* strains by electroporation using a Gene Pulser (Bio-Rad Laboratories) [15].

**Generation of trpBA deletion strain**

Oligonucleotide primers were designed, based on the trp operon sequence of *L. lactis* strain MG1363 (G. Buist, personal communication), to amplify the two regions flanking the *trpBA* operon (italic). Region F1, 5′ of the *trpB* gene, was amplified by PCR using the primers F1.fw: 5′-GACGGCTTCAGACTTTCTCTG-GTTTTTGG-3′ and F1.rev: 5′-ATCCCTGGTCTGAG-3′. Region F2, 3′ of the *trpA* gene, was amplified by PCR using the primers F2.fw: 5′-CGGATAAACCTGAGAGGAC-3′ and F2.rev: 5′-GATGATGGTTAGCTTATTTTCTTTATT-3′. Fragment F1 (1235 bp) was digested with XbaI and PstI. Fragment F2 (1048 bp) was digested with PstI and NcoI and both fragments were ligated into the XbaI and NcoI sites of *L. lactis* gene replacement vector, pORI280 [14], carrying an Em′ (erythromycin resistance) and LacZ gene as markers. The ligation was transferred to *L. lactis* strain LL108 by electroporation and transformation.

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**Table 1 Strains and plasmids used in the present study**

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant features</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>L. lactis subsp. cremoris</strong></td>
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<tr>
<td>MG1363</td>
<td>lac, prPB, plasmid-free derivative of NCD0712</td>
<td>[32]</td>
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<tr>
<td>NZ9000</td>
<td>MG1363 containing an insertion of the nisRK genes into the pepM gene</td>
<td>[10]</td>
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<tr>
<td>N23700</td>
<td>Nisin-producing strain</td>
<td>[10]</td>
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<tr>
<td>PA1001</td>
<td>A derivative of strain NZ9000 lacking the entire acmA and htrA genes</td>
<td>[33]</td>
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<td>PA1002</td>
<td>A derivative of strain PA1001 lacking the entire trpB and trpA genes</td>
<td>Present study</td>
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<tr>
<td>LL108</td>
<td>Cmr, a derivative of MG1363 allowing the permissive replication of pORI-derivatives due to multiple insertions of its native repA into the chromosome</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pPA6</td>
<td>Cmr, nisin-inducible expression–secretion vector derived from pNZ8048, containing the 1st peptidoglycan-binding motif (LysM domain) of acmA fused C-terminally to c-myc</td>
<td>Present study</td>
</tr>
<tr>
<td>pPA90</td>
<td>Cmr, nisin-inducible expression–secretion vector derived from pNZ8048, containing the three peptidoglycan-binding motifs (LysM domains) of acmA</td>
<td>Present study</td>
</tr>
<tr>
<td>pORI280</td>
<td>Em′, LacZ, conditionally replicating cloning and reporter plasmid suitable for use in gene-replacement strategies</td>
<td>[14]</td>
</tr>
<tr>
<td>pPA189</td>
<td>Em′, LacZ, gene-replacement vector. Derivative of pORI280 containing DNA sequences 5′ and 3′ of the trpB and the trpA genes respectively</td>
<td>Present study</td>
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**Figure 1 Schematic linear representation of the relevant parts of plasmids pPA6 and pPA90**

Abbreviations: Pnis, inducible nisin promoter; ss, signal sequence to enable secretion of the protein; c-Myc, heterologous epitope EQKLISEEDL from a human oncogene; lollypop, terminator sequence. Spacer 1 and LysM1 (lysin motif domain) in pPA6 encode the first binding domain in the lactococcal AcmA-protein (amino acid (aa) GNTN...GKKVL as in [16]). Plasmid pPA90 contains the entire sequence of *L. lactis* AcmA peptidoglycan-binding domain (from spacer 1 to LysM3, as sequence GNTN...LYLRIK as in [16]). The position of the Trp codons in the LysM domains is indicated.

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**Expression conditions**

Expression of PA6 and PA90 was induced by the addition of filtered sterilized culture supernatant of the nisin-producing strain NZ97000 [10] to the strains harbouring plasmid pPA6 and pPA90. Nisin-containing medium was added to the cultures at 1000-fold dilution (end concentration approx. 10 ng/ml). For efficient growth of the cultures was followed by attenuance (growth suppression). The position of the Trp codons in the LysM domains is indicated.

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**Figure 1 Schematic linear representation of the relevant parts of plasmids pPA6 and pPA90**

The media with 80 μg/ml X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside), L-Trp, DL-5-FTrp, L-5-OHTrp, DL-7-D-galactopyranoside), L-Trp, DL-5-FTrp, L-5-OHTrp, DL-7-Dihydroxytrp and DL-5-methylTrp were from Sigma–Aldrich. Fresh stock solutions were prepared in dilute NaOH.

Conditions for the inhibition of tryptophan biosynthesis were as follows. *L. lactis* strain PA1001 harbouring the plasmid pPA90 was grown at 30°C for 16 h in GM17 medium. The cells were harvested by centrifugation, washed with saline and diluted into CDM with or without 1 mM Trp, containing different concentrations of IAA (3β-indoleacrylic acid) or glyphosate (N-phosphonomethylglycine) (both from Sigma–Aldrich). The growth of the cultures was followed by attenuation (D) measurement at 600 nm using a Bio-Rad SmartSpec™ 3000 UV–Vis spectrophotometer (Bio-Rad Laboratories, Richmond, CA, U.S.A.).
resulted in plasmid pPA189. Vector pPA189 was purified and transferred into *L. lactis* strain PA1001 to conduct a gene-replacement strategy as described previously [14], and that results in this case in the removal of the *trpBA* genes from the chromosome (from nt 1002272 to 1004262 according to EMBL/GenBank® databases with accession number AM406671) without leaving the Em' and LacZ genes and any other plasmid sequences in the chromosome. The deletion in the chromosome was confirmed by PCR using primers flanking the deleted gene region, Del.fw (5′-CCCTTCTAGGATGGAGAAC-3′) and Del.rev (5′-GTCTTACGATTCACCTTGG-3′). A PCR reaction on the chromosomal DNA of the deletion mutant results in that case in a DNA fragment of 613 bp, whereas the chromosomal DNA of the wild-type as a template generates in such a PCR assay a DNA fragment of 2603 bp. A strain with the *trpBA* deletion was designated PA1002.

MS

Coomassie-stained bands of PA6 protein were excised from the gel and washed several times with water and acetonitrile. After the last wash step, the bands were dried completely and resuspended in 20 μl of 2.5 mg/μl porcine trypsin (Promega, Leiden, The Netherlands) dissolved in 100 mM ammonium bicarbonate and incubated for 1–2 h at 37 °C. Of the overlying liquid, 0.75 μl was spotted on a MALDI (matrix-assisted laser-desorption ionization) target and mixed immediately with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamate (LaserBio Labs, Sophia Antipolis Cedex, France) in 50% acetonitrile with 0.1% (v/v) trifluoroacetic acid. Spots were air-dried and positive-mode reflectron mass spectra were recorded on a Voyager DE-PRO MALDI–TOF (time-of-flight) instrument (Applied Biosystems, Foster City, CA, U.S.A.) and a 4700 Proteomics Analyzer (Applied Biosystems) MALDI–TOF/TOF instrument. Spectra were calibrated externally with standard peptides.

RESULTS

*L. lactis* PA1001 is not auxotrophic in Trp

*L. lactis* strain PA1001 is a strain that has been optimized for the inducible expression of proteins that contain the peptidoglycan-binding domain of the lactococcal cell-wall-hydrolysing enzyme AcmA [16,17]. The entire peptidoglycan-binding domain is called PA90 and contains five Trp residues. PA6 is part of PA90 and contains one Trp residue. In the plasmids containing the genes that encode PA90 or PA6, the genes are located behind a tightly regulated nisin promoter (Figure 1). Upon expression by the addition of nisin to the growth medium, PA90 and PA6 are secreted into the culture supernatant by means of a signal peptide present in the N-terminus of the recombinant proteins. Secretion into the growth medium simplifies the isolation of the recombinant proteins.

*L. lactis* strain PA1001 was grown in CDM containing no Trp or 1 mM L-Trp to investigate its growth characteristics. Growth rates were similar in the presence or absence of Trp, showing that PA1001 is not auxotrophic in Trp and that the biosynthetic route for Trp is efficient enough to sustain growth rates similar to those obtained when Trp is added to the medium (Figure 2).

Inhibition of the Trp biosynthesis pathway

In *E. coli*, inhibition of the Trp biosynthesis pathway by inhibitors like IAA or glyphosate has been used in prototroph *E. coli* strains to stimulate the incorporation of Trp analogues [18]. This approach was explored for *L. lactis* PA1001 in CDM. A concentration of 0.2 mg/ml of IAA completely inhibited the growth of *L. lactis* PA1001 (Figure 3A). However, addition of Trp did not elevate the IAA-inhibitory effect, suggesting that the growth-inhibitory effect caused by IAA in *L. lactis* differed from that in *E. coli*.

Trp biosynthesis was also inhibited by glyphosate (Figure 3B). A glyphosate concentration of 10 mM had no effect on the growth, but 30 mM inhibited the growth of *L. lactis* strain PA1001 in CDM. In this case, the growth was partially restored after the addition of Trp. Glyphosate inhibits the function of the EPSP (enzyme 5-enolpyruvylshikimate-3-phosphate synthase) [19], which acts upstream in the formation of chorismate, a precursor of all aromatic amino acids. Growth inhibition may result from the limitation of the other aromatic amino acids phenylalanine and tyrosine. This was tested by the addition of 1 mM extra of these two amino acids to CDM (final concentrations 2.7 and 2.1 mM respectively), but no stimulation in the growth rates were observed (results not shown). An increase in growth rate in the presence of glyphosate was only achieved by the addition of Trp. This result indicates that the Trp biosynthesis pathway is inhibited by glyphosate and that the concentrations of the aromatic amino acids phenylalanine and tyrosine in CDM were not limiting for the growth. Apparently, the biosynthesis routes for phenylalanine and tyrosine under the tested conditions are more efficient than for Trp.

Deletion of the *trpBA* genes from the chromosome of *L. lactis* strain PA1001

Since growth was only partially recovered in the case of inhibition with glyphosate, it is likely that incorporation of Trp analogues will not be very efficient. For this reason, a Trp auxotroph *L. lactis* strain was generated by deleting the genes coding for the enzyme Trp synthase. Trp synthase is the enzyme that converts indole-3-glycerol-phosphate and L-serine into L-Trp, which is the last step in the biosynthesis of Trp. Its presence is widespread among micro-organisms and plants. The functional enzyme is a tetramer, formed by the two subunits α and β (two α- and two β-subunits) [20], which are encoded by *trpA* and *trpB* genes respectively. In *L. lactis* strain LL1403, Trp synthase is part of an operon containing seven structural genes in the order *trpEGDCFBA* [21,22]. An
Cells were grown in CDM containing 1 mM Trp overnight at 30°C. Subsequently, the cells were washed with saline and then inoculated into fresh CDM containing either IAA (A) or glyphosate (B). The cultures contained the inhibitor without any Trp (solid line) or in combination with 1 mM Trp (dashed line). The concentrations of IAA tested are indicated in (A). Two concentrations of glyphosate were used, a permissive concentration of 10 mM (circles) and an inhibitory concentration of 30 mM (squares). The growth was followed by measuring the D₆₀₀ at the indicated times.

The trpB and trpA genes are located at the end of the trp operon. Therefore both genes can be deleted at the same time without disturbing the organization of the rest of the operon. The DNA sequences upstream of trpB and downstream of trpA, amplified by using PCR, were inserted into the conditionally replicating plasmid pORI280 [14]. Deletion of the trpBA genes from the chromosome of strain PA1001 by the use of the pORI280-derivative pPA189 was confirmed by PCR and Trp auxotrophy was confirmed by the absence of growth on CDM lacking Trp (Figure 2). Growth of the generated L. lactis strain PA1002 in CDM + Trp was similar to that of the parent strain PA1001 (Figure 2).

**Production of recombinant proteins in the presence of Trp analogues using Trp auxotroph PA1002**

L. lactis strain PA1002 carrying plasmids pPA90 or pPA6 was used to express PA90 or PA6 respectively. L. lactis strain PA1002 did not grow when inoculated in CDM that was supplemented with the Trp analogues 5-FTrp, 5-OHTrp or 7-AzaTrp. However, proteins could be produced after the formation of biomass in the absence of nisin in rich growth medium (GM17) followed by washing of the cells and resuspending in CDM supplemented with Trp or a Trp analogue and nisin (Figure 4A). Compared with expression in the presence of Trp, production levels of PA6 and PA90 were only minimally affected by the presence of the Trp analogues.

The range of Trp analogues that can be biosynthetically incorporated is mainly determined by the substrate specificity of Trp-tRNA synthetase. This enzyme charges tRNATrp (Trp tRNA) with Trp and the protein from E. coli only accepts minor changes in the indole moiety of the Trp structure like small substituents (F, OH and NH₂) or introduction of a nitrogen or sulphur atom in the indole nucleus [3,23]. Methylated Trp analogues are more difficult
The incorporation of 5-methylTrp into a recombinant protein is reported for the first time. The incorporation efficiency for this Trp analogue was 92% using the *L. lactis* PA1002 Trp auxotroph.

Replacing Trp with a Trp analogue in a protein can facilitate its characterization using spectroscopy techniques like absorbance, fluorescence or NMR. For many applications, a high-to-complete incorporation efficiency is desired. Most Trp analogue-containing proteins have been produced using *E. coli* (reviewed in [3, 24]). Good results have been obtained by using one of the available Trp auxotrophs and the incorporation efficiency was found to be dependent on the promoter used to express the recombinant proteins. A tightly controlled promoter system is needed, otherwise the recombinant protein is expressed during the biomass generation phase, in medium containing Trp. Best results have been obtained with the T5, tac and λPc promoters [24]. Starvation of the washed cells after growth in Trp-containing medium to deplete the level of Trp was also found to be critical for high incorporation efficiency. Previously, efficient incorporation of 5-FTrp was reported with a non-Trp auxotrophic strain of *E. coli* by using IAA as an inhibitor of the biosynthesis of Trp [18]. A similar approach using glyphosate has been reported [25]. This approach can be useful for expression organisms for which no Trp auxotroph is available like *L. lactis*. We have explored the potential of this approach but found that the growth-inhibitory effect of IAA on *L. lactis* in CDM without Trp is not related to inhibition of the Trp biosynthesis, since addition of Trp did not alleviate the inhibition of the cell growth rate (Figure 3). Similarly, an inhibitory effect on the growth of *L. lactis* was found at 30 mM glyphosate, but addition of 1 mM Trp only partly restored the growth rate. At this moment, the biological activity of these two compounds towards this Gram-positive organism is unclear. These results prompted us to make a Trp auxotroph of *L. lactis* and this was achieved via deletion of the *trpBA* genes. Growth conditions were found for the *L. lactis* Trp auxotroph strain PA1002 that resulted in high-to-very-high incorporation efficiencies of the four Trp analogues tested into the secreted model recombinant protein PA6. The expression of PA (protein anchor) proteins was less efficiently incorporated than 7-AzaTrp and 5-FTrp using *L. lactis* systems [5–8]. Therefore it is likely that Trp analogues can be efficiently incorporated into many other proteins expressed in *L. lactis* PA1002.

In several studies with *E. coli*, 5-OHTrp turned out to be less efficiently incorporated than 7-AzaTrp and 5-FTrp using the same expression system [24]. *L. lactis* PA1002 also shows a lower preference for 5-OHTrp than the other two Trp analogues. However, this Trp auxotroph is the first organism allowing the biosynthetic incorporation of 5-methylTrp. Although closely resembling the structure of allowed substituents at the 5-position (F, OH and NH₂), this analogue seems not to be accepted by *L. lactis* because the PA proteins used in the present study are essentially homologous *L. lactis* proteins; only PA6 contains the heterologous c-myc sequence. However, a broad range of other proteins of prokaryotic and eukaryotic origin, including membrane proteins, have been expressed in *L. lactis* expression systems [5–8]. Therefore it is likely that Trp analogues can be efficiently incorporated into many other proteins expressed in *L. lactis* PA1002.

**DISCUSSION**

In the present study, the construction of a Trp auxotroph of *L. lactis* is presented and we demonstrate the quantitative biosynthetic incorporation (> 97%) of the Trp analogues 7-AzaTrp and 5-FTrp into a secreted recombinant model protein. An 89% efficiency for the incorporation of 5-OHTrp into the recombinant PA6 protein using this Trp auxotroph was found. The biosynthetic incorporation of 5-methylTrp into a recombinant protein is reported for the first time. The incorporation efficiency for this Trp analogue was 92% using the *L. lactis* PA1002 Trp auxotroph.
embedded in a protein, this process is regarded the dominant non-radiative depopulation process, and the photophysical properties of Trp (quantum yield and fluorescence decay kinetics) are strongly dependent on this process. The ability to change the ionization potential of a Trp position to a higher value, via 5-fluoro substitution [29], or to a lower value, via 5-methyl substitution, can become an interesting experimental approach to study this quenching process in more detail.

An anisotropy study using 5-methylindole revealed that it shows mixed emission from the $^1L_a$ and $^1L_b$ states, while indole was found to emit only from $^1L_a$ [30]. Trp embedded in a protein emits from $^1L_a$; only for one protein showing an extremely blue-shifted $\lambda_{\text{max}}$, $^1L_a$ emission has been reported [31]. It is likely that, biosynthetic incorporation of 5-methylTrp will result more often in $^1L_a$-emitting proteins. The impact of the protein matrix on the spectroscopic parameters of 5-methylTrp can now be experimentally explored using L. lactis PA1002 as expression host.

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REFERENCES


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