



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

Mohamed El Khattabi, Maarten L. Van Roosmalen, Dennis Jager, Heidi Metselaar, Hjalmar Permentier, Kees Leenhouts, Jaap Broos

► To cite this version:

Mohamed El Khattabi, Maarten L. Van Roosmalen, Dennis Jager, Heidi Metselaar, Hjalmar Permentier, et al.. Lactococcus lactis as expression host for the biosynthetic incorporation of tryptophan analogues in recombinant proteins. *Biochemical Journal*, 2007, 409 (1), pp.193-198. <10.1042/BJ20070909>. <hal-00478844>

HAL Id: hal-00478844

<https://hal.science/hal-00478844v1>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

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

Mohamed EL KHATTABI^{*,†}, Maarten L. VAN ROOSMALEN^{*}, Dennis JAGER^{*},
Heidi METSELAAR^{*,‡}, Hjalmar PERMENTIER[#], Kees LEENHOUTS^{*,1} and Jaap
BROOS[¶]

^{*} Biomade Technology Foundation, Nijenborgh 4, 9747 AG Groningen. The Netherlands.

[#] Mass Spectrometry Core Facility, Centre for Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen. The Netherlands.

[¶] Department of Biophysical Chemistry and Groningen Biomolecular Science and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4, 9747 AG Groningen. The Netherlands.

¹ corresponding author: leenhouts@biomade.nl; Tel: +31-50-3638146; Fax: +31-50-3634429.

[†] Present address: Department of Cell Biology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

[‡] Present address: Department of Medical Microbiology, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen. The Netherlands.

Page heading title: Trp analogue incorporation with Trp auxotroph of *L. lactis*

SYNOPSIS

Incorporation of tryptophan (Trp) 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 possibility 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 in recombinant proteins. The Trp analogues 7-azatryptophan, 5-fluorotryptophan and 5-hydroxytryptophan were incorporated with efficiencies of >97%, >97% and 89%, respectively. Interestingly, 5-methyltryptophan could be incorporated with 92% efficiency. Successful biosynthetic incorporation of 5-methylTrp in recombinant proteins has not been reported before.

Key words: *Lactococcus lactis*, tryptophan auxotroph, 5-methyltryptophan, tryptophan analogues, IAA, glyphosate.

Abbreviations used: IAA, 3 β -indoleacrylic acid; CDM, chemically defined medium; glyphosate, N-phosphonomethylglycine; 5-FTrp, 5-fluorotryptophan; 5-OHTrp, 5-hydroxytryptophan; 7-AzaTrp, 7-azatryptophan; 5-methylTrp, 5-methyltryptophan; PA, protein anchor.

INTRODUCTION

Tryptophan (Trp) is an attractive intrinsic fluorescence probe in protein chemistry [1;2]. Its emission maximum (λ^{\max}) and the quantum yield (Q) are highly sensitive parameters for monitoring changes in 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-azatryptophan (7-AzaTrp), 5-hydroxytryptophan (5-OHTrp) and fluorotryptophans can be efficiently biosynthetically incorporated in 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]. Here we explored the possibility to use *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 tryptophan 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 for the Trp analogues 5-fluoroTrp (5-FTrp), 5-OHTrp, 7-AzaTrp and 5-methyltryptophan (5-methylTrp) in a recombinant model protein. This is the first report for the efficient incorporation of 5-methylTrp in a recombinant protein.

EXPERIMENTAL

Bacterial strains, plasmids, media and growth conditions

L. lactis strains and plasmids used in this 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 [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 the media with 80 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). L-Trp, DL-5-FTrp, L-5-OHTrp, DL-7-AzaTrp 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 harboring 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 or glyphosate (both from Sigma-Aldrich). The growth of the cultures was followed by optical density measurement at 600 nm using a Bio-Rad SmartSpec™ 3000 UV-VIS spectrophotometer (Bio-Rad Laboratories, Richmond, Calif.).

Expression conditions

Expression of PA6 and PA90 was induced by the addition of filtered sterilised culture supernatant of the nisin-producing strain NZ9700 [10] to the strains harboring plasmid pPA6 and pPA90. Nisin containing medium was added to the cultures at 1000-fold dilution (end concentration approximately 10 ng/ml). For efficient incorporation of Trp analogues, *L. lactis* strains harboring plasmids pPA6 or pPA90 were grown overnight in GM17 at 30°C. Subsequently, the cultures were diluted 50-fold in 400 ml fresh GM17 and grown for approximately 4 h at 30°C (OD₆₀₀: 0.8). The cells were harvested by centrifugation at 7,000 g for 10 min, washed with saline (0.9% NaCl) and

1 finally, resuspended into 100 ml CDM supplemented with 0.5% glucose. After a
2 starvation period of 45 min at 30°C, nisin and 1 mM (L-enantiomer) of either Trp or Trp
3 analogue were added. The cells were subsequently incubated for approximately 24 h at
4 30°C. After spinning down the cells, PA90 or PA6 were precipitated from the culture
5 using 5% trichloro acetic acid (TCA) during for 1 h at 0°C, followed by 20 min
6 centrifugation at 13,000 g and 0°C. Samples were analyzed using SDS-PAA gel
7 electrophoresis. Per lane TCA precipitate originating from 0.4 ml culture was loaded.

9 General DNA techniques and transformation

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

16 Generation of *trpBA* deletion strain

18 Oligonucleotide primers were designed, based on the *trp* operon sequence of *L. lactis*
19 strain MG1363 (G. Buist, personal communication), to amplify the two regions flanking
20 *trpB* and *trpA*, respectively. Recognition sequences for restriction enzymes (underlined)
21 were introduced by nucleotide substitutions (italic). Region F1, 5' of the *trpB* gene, was
22 amplified by PCR using the primers F1.fw: 5'-
23 GAGCGATTCTAGACTTTCCTAATGCTTTTGG-3' and F1.rev: 5'-
24 ATCCTTGGTTCTGCAGTTGATTGTAGGTCATGTATTTC-3'. Region F2, 3' of the
25 *trpA* gene, was amplified by PCR using the primers F2.fw: 5'-
26 CGGATATCCTGCAGGTCCACTTTACAAATGG-3' and F2.rev: 5'-
27 GGATTGATGCCATGGTCGCCTGTGACACCTTAG-3'. Fragment F1 (1235 bp) was
28 digested with *Xba*I and *Pst*I. Fragment F2 (1048 bp) was digested with *Pst*I and *Nco*I
29 and both fragments were ligated into the *Xba*I and *Nco*I sites of *L. lactis* gene
30 replacement vector, pORI280 (14) carrying an erythromycin resistance (*Em*^r) and LacZ
31 gene as markers. The ligation was transferred to *L. lactis* strain LL108 by
32 electroporation and resulted in plasmid pPA189. Vector pPA189 was purified and
33 transferred into *L. lactis* strain PA1001 to conduct a gene-replacement strategy as
34 described before (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^r* 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'-CCCTTCATGGATGGAAGAAC-3') and Del.rev (5'-GTTCTACGATTCCACTTGG-3'). A PCR reaction on the chromosomal DNA of the deletion mutant results in that case in a DNA fragment of 613 basepair (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.

Mass spectrometry

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 μ l of 2.5 ng/ μ l porcine trypsin (Promega, Leiden, The Netherlands) dissolved in 100 mM ammonium bicarbonate and incubated for 1-2 h at 37 °C. Of the overlaying liquid, 0.75 μ l was spotted on a MALDI target and mixed immediately with an equal volume of 10 mg/ml α -cyano-hydroxycinnamic acid (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 instrument (Applied Biosystems, Foster City, CA, USA) and a 4700 Proteomics Analyzer MALDI-TOF/TOF instrument (Applied Biosystems). Spectra were calibrated externally with standard peptides.

RESULTS

L. lactis PA1001 is not auxotrophic in Trp

L. lactis 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 hydrolyzing enzyme AcmA [16;17]). The entire peptidoglycan binding domain is called PA90 and contains 5 Trp residues. PA6 is part of PA90 and contains 1 Trp residue. In the plasmids containing the genes that encode for 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 similar growth rates as with Trp 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 was completely inhibiting 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 enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) [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 (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 tryptophan synthase. Tryptophan synthase is the enzyme that converts indole-3-glycerol-phosphate and L-serine into L-tryptophan, which is the last step in the biosynthesis of Trp. Its presence is wide spread among microorganisms and plants. The functional enzyme is a tetramer, formed by the two subunits α and β (2 α and 2 β subunits) [20], which are encoded by *trpA* and *trpB* genes, respectively. In *L. lactis* strain IL1403, tryptophan synthase is part of an operon containing seven structural genes in the order *trpEGDCFBA* [21;22]. An operon with similar organisation is also present in the genome of *L. lactis* strain MG1363 (G. Buist, personal communication), which is the parent strain of PA1001.

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 organisation 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 to 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 which can be biosynthetically incorporated is mainly determined by the substrate specificity of Trp-tRNA synthetase. This enzyme charges tRNA^{trp} 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, NH₂) or introduction of a nitrogen or sulphur atom in the indole nucleus [3;23]. Methylated Trp analogues are more difficult to incorporate biosynthetically using *E. coli*. Only the successful incorporation of 4-methyltryptophan has been reported using this host [23]. Surprisingly, when 5-methylTrp was used with PA1002 as expression host a good yield of PA6 was obtained, comparable to the yield of the other Trp analogues tested (Figure 4B). This suggests that 5-methylTrp can be biosynthetically incorporated using *L. lactis*.

Mass spectrometry

MALDI-TOF was used to establish the incorporation efficiency of the Trp analogues into PA6. PA6 bands were cut out from the SDS-PAA gel and the proteins were digested with trypsin yielding a fragment of 11 amino acids containing the single Trp residue in PA6. In Figure 5, MALDI-TOF spectra are shown for this tryptic fragment of PA6 containing 5-OHTrp, 5-methylTrp, 5-FTrp, and 7-AzaTrp respectively. In the case of 7-AzaTrp and 5-FTrp, no significant peak of the peptide containing Trp is visible, indicating that the incorporation efficiency is >97%. The incorporation efficiencies were 89% and 92% for 5-OHTrp and 5-methylTrp, respectively.

DISCUSSION

In this manuscript 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 in 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 for 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 λP_L 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. Recently, efficient incorporation of 5-FTrp was reported with a non-Trp auxotrophic strain of *E. coli* using IAA as 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 with 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 did only partly restore the growth rate. At this moment, the biological activity of these 2 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 auxotrophic strain PA1002 that result in high to very high incorporation efficiencies of the 4 Trp analogues tested into the secreted model recombinant protein PA6. The expression of protein anchor (PA) proteins was under

control of the nisin promotor, a promotor system known to be tightly regulated. The PA proteins used in this study are essentially homologous *L. lactis* proteins, only PA6 contains the heterologous c-myc sequence. However, a broad range other proteins of pro- and eukaryotic origin, including membrane proteins, have been expressed in *L. lactis* expression systems [5-8]. Therefore, it is likely that Trp analogs 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 2 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, NH₂), this analogue seems not to be accepted by the *E. coli* translation machinery. Early work on Trp analogue incorporation reported both chromosome-based expression of proteins in Trp starved *E. coli* cells upon addition of 5-methylTrp [26;27] and no expression under these conditions [28]. Recently, it was reported that 5-methylTrp can not be incorporated in a recombinant protein using an *E. coli* auxotroph [23].

5-methylTrp is an interesting analogue since its electron-donating methyl group lowers the ionization potential of the indole moiety by ~0.12 eV [29]. A lowering of the ionization potential will stimulate photo-induced electron transfer from the excited probe to nearby acceptors like peptide bonds [29]. For Trp embedded in a protein, this process is regarded the dominant non-radiative depopulation process, and the photophysical properties of Trp (quantum yield, fluorescence decay kinetics) are strongly dependent on this process. The ability of changing the ionization potential of a Trp position to a higher value, via 5-fluoro substitution [29], or 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 λ^{\max} , ¹L_b emission has been reported [31]. Likely, biosynthetic incorporation of 5-methylTrp will result more often in ¹L_b 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.

ACKNOWLEDGEMENTS

This work was supported by NanoNed grant 7919 from the Technology Foundation STW in the Netherlands.

REFERENCES

- 1 Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, Springer, New York
- 2 Eftink, M. R. (1991) Fluorescence techniques for studying protein-structure. Meth. Biochem. Anal. **35**, 127-205
- 3 Ross, J. B., Szabo, A. G. and Hogue, C. W. (1997) Enhancement of protein spectra with tryptophan analogs: fluorescence spectroscopy of protein-protein and protein-nucleic acid interactions. Methods Enzymol. **278**, 151-190
- 4 Broos, J., Gabellieri, E., Biemans-Oldehinkel, E. and Strambini, G. B. (2003) Efficient biosynthetic incorporation of tryptophan and indole analogs in an integral membrane protein. Protein Sci. **12**, 1991-2000
- 5 Arnau, J., Hjerl-Hansen, E. and Israelsen, H. (1997) Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. **48**, 331-338
- 6 Miyoshi, A., Poquet, I., Azevedo, V., Commissaire, J., Bermudez-Humaran, L., Domakova, E., Le Loir, Y., Oliveira, S. C., Gruss, A. and Langella, P. (2002) Controlled production of stable heterologous proteins in *Lactococcus lactis*. Appl. Environ. Microbiol. **68**, 3141-3146
- 7 Nouaille, S., Ribeiro, L. A., Miyoshi, A., Pontes, D., Le Loir, Y., Oliveira, S. C., Langella, P. and Azevedo, V. (2003) Heterologous protein production and delivery systems for *Lactococcus lactis*. Genet. Mol. Res. **2**, 102-111
- 8 Kunji, E. R. S., Slotboom, D. J. and Poolman, B. (2003) *Lactococcus lactis* as host for overproduction of functional membrane proteins. Biochim. Biophys. Acta-Biomem. **1610**, 97-108
- 9 de Vos, W. M. (1999) Gene expression systems for lactic acid bacteria. Curr. Opin. Microbiol. **2**, 289-295
- 10 Kuipers, O. P., deRuyter, P. G. G. A., Kleerebezem, M. and deVos, W. M. (1997) Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol. **15**, 135-140
- 11 Terzaghi, B. E. and Sandine, W. E. (1975) Improved medium for Lactic streptococci and their bacteriophages. Applied Microbiology **29**, 807-813

- 12 Poolman, B. and Konings, W. N. (1988) Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino-acid transport. J. Bacteriol. **170**, 700-707
- 13 Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, New York
- 14 Leenhouts, K., Venema, G. and Kok, J. (1998) A lactococcal pWV01-based integration toolbox for bacteria. Methods in Cell Science **20**, 35-50
- 15 Holo, H. and Nes, I. F. (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55**, 3119-3123
- 16 Buist, G., Kok, J., Leenhouts, K. J., Dabrowska, M., Venema, G. and Haandrikman, A. J. (1995) Molecular-cloning and nucleotide-sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell-separation. J. Bacteriol. **177**, 1554-1563
- 17 Steen, A., Buist, G., Leenhouts, K. J., El Khattabi, M., Grijpstra, F., Zomer, A. L., Venema, G., Kuipers, O. P. and Kok, J. (2003) Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. J. Biol. Chem. **278**, 23874-23881
- 18 Leone, M., Rodriguez-Mias, R. A. and Pellecchia, M. (2003) Selective incorporation of 19F-labeled Trp side chains for NMR-spectroscopy-based ligand-protein interaction studies. Chembiochem. **4**, 649-650
- 19 Steinrucken, H. C. and Amrhein, N. (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase. Biochem. Biophys. Res. Commun. **94**, 1207-1212
- 20 Miles, E. W. (2001) Tryptophan synthase: a multienzyme complex with an intramolecular tunnel. Chem. Rec. **1**, 140-151
- 21 Bardowski, J., Ehrlich, S. D. and Chopin, A. (1992) Tryptophan biosynthesis genes in *Lactococcus lactis* subsp *lactis*. J. Bacteriol. **174**, 6563-6570
- 22 Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarne, K., Weissenbach, J., Ehrlich, S. D. and Sorokin, A. (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp *lactis* IL1403. Genome Res. **11**, 731-753
- 23 Budisa, N., Pal, P. P., Alefelder, S., Birle, P., Krywcun, T., Rubini, M., Wenger, W., Bae, J. H. and Steiner, T. (2004) Probing the role of tryptophans in *Aequorea victoria* green fluorescent proteins with an expanded genetic code. Biol. Chem. **385**, 191-202
- 24 Ross, J. B. A., Rusinova, E., Luck, L. A., and Rousslang, K. W. (2000) Spectral enhancement of proteins by in vivo incorporation of tryptophan analogues. In Topics in Fluorescence Spectroscopy Vol. 6: Protein Fluorescence (Lakowicz, J. R., ed), pp. 17-42, New York, Kluwer Academic/ Plenum Publishers.

- 1 25 Kim, H. W., Perez, J. A., Ferguson, S. J. and Campbell, I. D. (1990) The specific
2 incorporation of labeled aromatic-amino-acids into proteins through growth of
3 bacteria in the presence of glyphosate - Application to fluorotryptophan labeling to
4 the H⁺-ATPase of *Escherichia coli* and NMR-studies. FEBS Lett. **272**, 34-36
- 5 26 Lark, K. G. (1969) Incorporation of 5-Methyltryptophan into Protein of
6 *Escherichia coli* 15T⁻ (555-7). J. Bacteriol. **97**, 980-982
- 7 27 Ezekiel, D. H., Carlson, N. and Jenkins, T. (1973) Incorporation of 5-
8 methyltryptophan into protein of *Escherichia coli*. Biochim. Biophys. Acta **312**,
9 751-764
- 10 28 Pardee, A. B., Shore, V. G. and Prestridge, L. S. (1956) Incorporation of
11 azatryptophan into proteins of bacteria and bacteriophage. Biochim. Biophys. Acta
12 **21**, 406-407
- 13 29 Liu, T. Q., Callis, P. R., Hesp, B. H., de Groot, M., Buma, W. J. and Broos, J.
14 (2005) Ionization potentials of fluorindoles and the origin of nonexponential
15 tryptophan fluorescence decay in proteins. J. Am. Chem. Soc. **127**, 4104-4113
- 16 30 Eftink, M. R., Selvidge, L. A., Callis, P. R. and Rehms, A. A. (1990) Photophysics
17 of indole-derivatives - experimental resolution of L_a and L_b transitions and
18 comparison with theory. J. Phys. Chem. **94**, 3469-3479
- 19 31 Broos, J., Tveen-Jensen, K., de, W. E., Hesp, B. H., Jackson, J. B., Canters, G. W.
20 and Callis, P. R. (2007) The emitting state of tryptophan in proteins with highly
21 blue-shifted fluorescence. Angew. Chem., Int. Ed. **46**, 5137-5139
- 22 32 Gasson, M. J. (1983) Plasmid complements of *Streptococcus lactis* NCDO-712
23 and other Lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**, 1-9
- 24 33 Bosma, T., Kanninga, R., Neef, J., Audouy, S. A. L., van Roosmalen, M. L., Steen,
25 A., Buist, G., Kok, J., Kuipers, O. P., Robillard, G. and Leenhouts, K. (2006)
26 Novel surface display system for proteins on non-genetically modified Gram-
27 positive bacteria. Appl. Environ. Microbiol. **72**, 880-889
- 28

Table 1. Strains and plasmids used in this study

| Strains and plasmids | Relevant features ^a | Source or reference |
|---|---|---------------------|
| <i>L. lactis</i> subsp. <i>cremoris</i> | | |
| MG1363 | <i>lac</i> ; <i>prtP</i> ; plasmid-free derivative of NCDO712 . | [32] |
| NZ9000 | MG1363 containing an insertion of the <i>nisRK</i> genes into the <i>pepN</i> gene. | [10] |
| NZ9700 | Nisin producing strain. | [10] |
| PA1001 | A derivative of strain NZ9000 lacking the entire <i>acmA</i> and <i>htrA</i> genes. | [33] |
| PA1002 | A derivative of strain PA1001 lacking the entire <i>trpB</i> and <i>trpA</i> genes. | This study |
| LL108 | Cm ^r , a derivative of MG1363 allowing the permissive replication of pORI-derivatives due to multiple insertions of its native <i>repA</i> into the chromosome. | [14] |
| Plasmids | | |
| pPA6 | Cm ^r , nisin inducible expression-secretion vector derived from pNZ8048, containing the 1 st peptidoglycan binding motif (LysM domain) of <i>acmA</i> fused C-terminally to <i>c-myc</i> ^b . | This study |
| pPA90 | Cm ^r , nisin inducible expression-secretion vector derived from pNZ8048, containing the three peptidoglycan binding motifs (LysM domains) of <i>acmA</i> ^c . | This study |
| pORI280 | Em ^r , LacZ, conditionally replicating cloning and reporter plasmid suitable for use in gene-replacement strategies. | [14] |

| | |
|--------|---|
| pPA189 | Em ^r , LacZ, gene-replacement vector. This study Derivative of pORI280 containing DNA sequences 5' and 3' of the <i>trpB</i> and the <i>trpA</i> genes, respectively. |
|--------|---|

1

2 ^a Cm^r: chloramphenicol resistance. Em^r: erythromycin resistance. LacZ: β -galactosidase.

3

Figure legends

Figure 1. Schematic linear representation of the relevant parts of plasmids pPA6 and pPA90. P_{nisA}: inducible nisin promoter; ss: signal sequence to enable secretion of the protein; c-myc: heterologous epitope EQKLISEEDL from a human onco gene; lollypop: terminator sequence. Spacer 1 and LysM1 in pPA6 encode the first binding domain in the lactococcal AcmA protein (amino acid [aa] GNTN.....QKLVL as in [16]). Plasmid pPA90 contains the entire sequence of *L. lactis* AcmA peptidoglycan binding domain (from spacer 1 to LysM3, aa sequence GNTN....YLRIK as in [16]). The position of the Trp codons in the LysM domains is indicated.

Figure 2. Growth of *L. lactis* strains PA1001 and PA1002 in CDM in absence or presence of Trp.

Cells were grown in CDM containing 1 mM Trp overnight at 30°C. Subsequently, the cells were washed with saline, then inoculated into fresh CDM without Trp during 45 min, followed by the addition Trp sources or no Trp at all (T=0).

Figure 3. Growth-inhibition of *L. lactis* strain PA1001 by Trp biosynthesis pathway inhibitors.

Cells were grown in CDM containing 1 mM Trp overnight at 30°C. Subsequently, the cells were washed with saline, 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 absorbance at 600 nm at the indicated times.

Figure 4. Coomassie stained SDS-PAA gels showing the proteins produced by *L. lactis* strain PA1002. The equivalent of 0.4 ml of the cell-free supernatants (TCA precipitated) of strain PA1002 producing either secreted PA6 or PA90 were loaded into each lane. Biomass of *L. lactis* strain PA1002 containing the plasmids pPA6 or pPA90 was formed in GM17 for approximately 4 h. Subsequently, the cells were cultivated for 24 h in CDM supplemented with 0.5% glucose and nisin, and containing (A) 1 mM Trp ("WT"); 1 mM 5-FTrp ("5F"); 1 mM 5-OHTrp ("5OH"); 1 mM 7-AzaTrp ("7aza") or

(B) 1mM 5-FTrp ("5F"); 1 mM 5-OHTrp ("5OH") or 1 mM 5-methylTrp ("5Meth") as the only tryptophan source. The position of the proteins PA6 and PA90 are indicated. The relative positions of the molecular weight (MW) standards are indicated on the right side of panel A. The MW standard is shown in panel B on the left side. The MW are indicated in kDa.

Figure 5. MALDI-TOF mass spectra of PA6 with different tryptophan analogues digested with trypsin.

The mass region of the SGDTLWGISQR peptide is shown: the monoisotopic mass of the protonated peptide with 5-OHTrp, 5-methylTrp, 5-FTrp, and 7-AzaTrp incorporated is 1235.6, 1233.6, 1237.6, and 1220.6 Da, respectively. The monoisotopic peak of the peptide with unmodified tryptophan is indicated with an asterisk at m/z 1219.6.

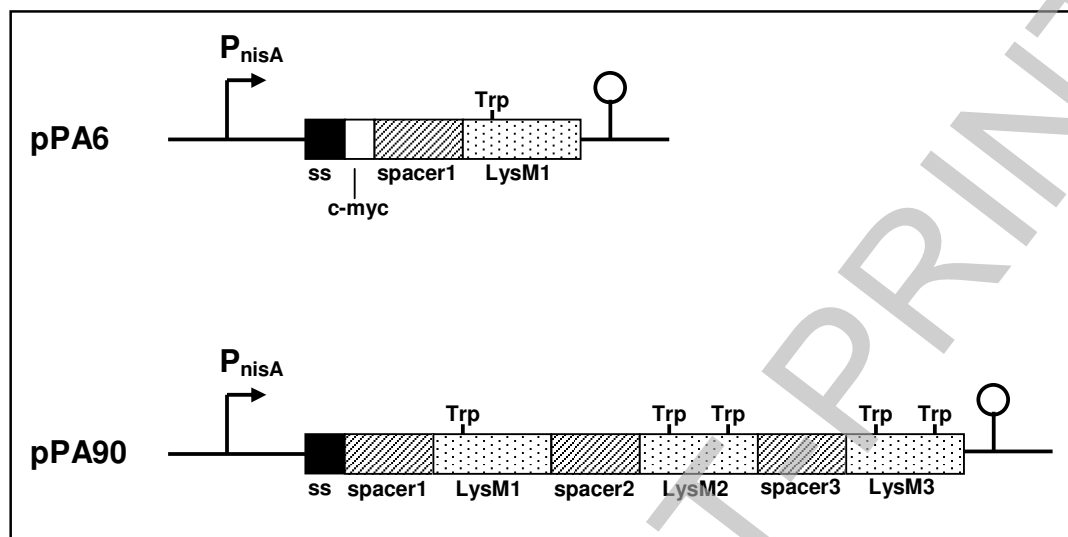
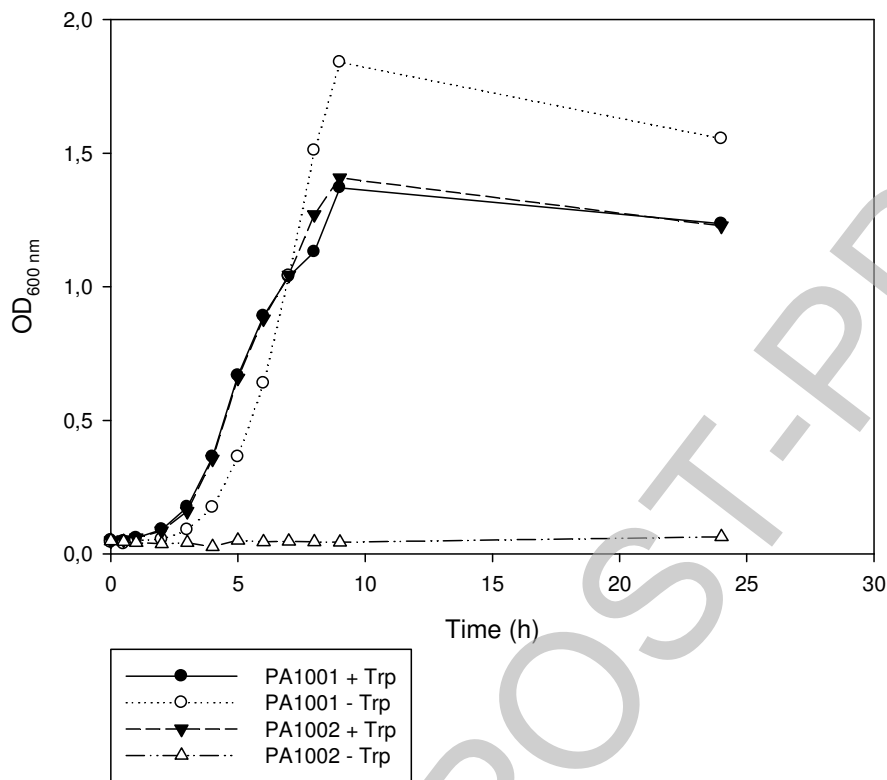


Figure 1.

1



2

3

4

5

6

7

8

9

Figure 2.

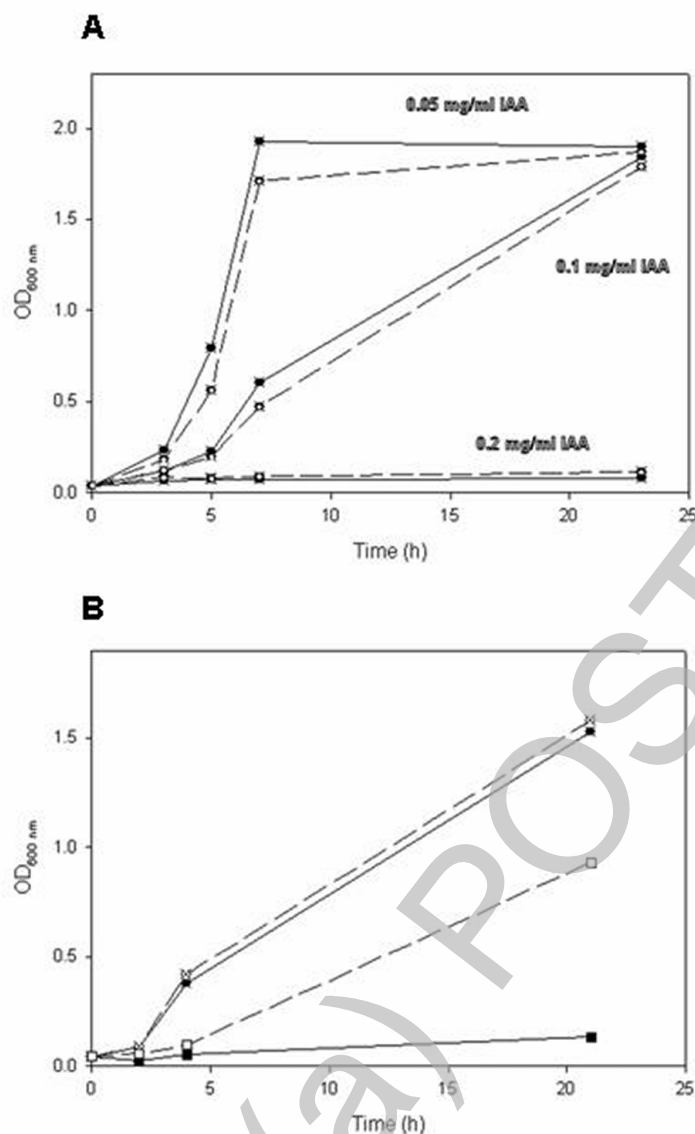


Figure 3.

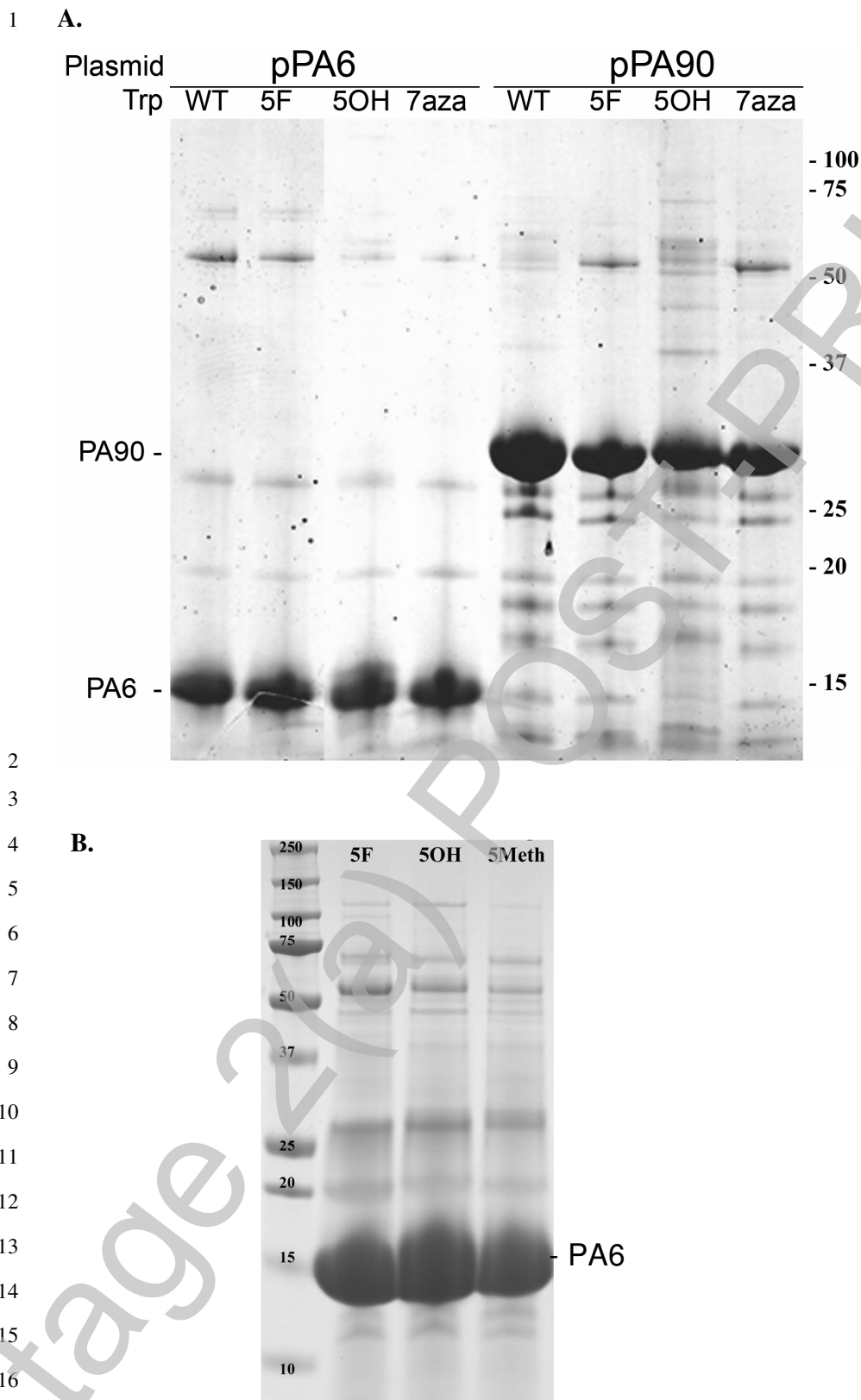
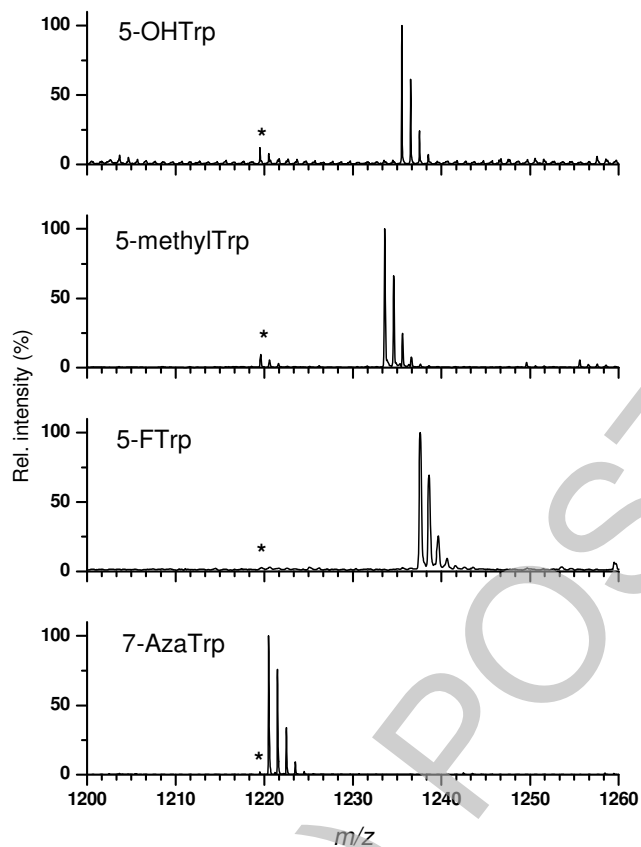


Figure 4.

1
2
3



4
5
6
7
8
9
10
11
12
13

Figure 5.