Chapter 2

Development of Chemically Defined Media to Express Trp-Analog-Labeled Proteins in a *Lactococcus lactis* Trp Auxotroph

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Abstract

Chemically defined media (CDMs) for growth of *Lactococcus lactis* strains contain about 50 components, making them laborious and expensive growth media. However, they are crucial for metabolism studies as well as for expression of heterologous proteins labeled with unnatural amino acids. In particular, the *L. lactis* Trp auxotroph PA1002, over-expressing the tryptophanyl tRNA synthethase enzyme of *L. lactis*, is very suitable for the biosynthetic incorporation of Trp analogs in proteins because of its most relaxed substrate specificity towards Trp analogs. Here we present two much simpler chemically defined media for *L. lactis*, which consist of only 24 or 31 components, respectively, and with which the *L. lactis* Trp auxotroph shows similar growth characteristics as with a 50-component CDM. Importantly, the expression levels of two recombinant proteins used for evaluation were up to 2-3 times higher in these new media than in the 50-component medium, without affecting the Trp analog incorporation efficiency. Taken together, the simplest CDM media known so far for *L. lactis* are presented, optimally suited for the production of Trp-analog-labeled recombinant proteins. Since *L. lactis* shows also auxotrophy for Arg, His, Ile, Leu Val, and Met, our simplified media may also be useful for the biosynthetic incorporation of analogs of these five amino acids.

Introduction

For the residue-specific labeling of a recombinant protein with a pseudoisosteric unnatural amino acid, an auxotrophic expression host is needed, which, during protein expression, is cultured in a synthetic medium supplemented with the unnatural amino acid and lacking the natural amino acid homolog. Most pseudoisosteric unnatural amino acids have so far been introduced using *E. coli* as expression host grown in the synthetic 15-component M9 medium (Ross *et al.*, 1997; Twine & Szabo, 2003; Budisa, 2004; Connor & Tirrell, 2007; Broos, 2014). However, recently we reported the use of *L. lactis* as an expression host for the incorporation of Trp analogs in recombinant proteins. The Gram-positive *L. lactis* is known as an attractive expression host for proteins, including membrane proteins (Kunji *et al.*, 2003). It can grow under aerobic or anaerobic conditions and it has a relatively low proteolytic activity. Tightly controlled gene expression systems are available (Kuipers *et al.*, 1997; Morello *et al.*, 2008). Moreover, *L. lactis* is auxotrophic towards Arg, His, Ile, Leu, Val, and Met (Berntsson *et al.*, 2009; Zhang *et al.*, 2009). For the incorporation of Trp analogs, a *L. lactis* Trp auxotroph strain, PA1002, has been developed (El Khattabi *et al.*, 2008). Compared to *E. coli*, the translation machinery of PA1002 was found to be more relaxed towards Trp analogs. The variety of Trp analogs translated by PA1002 could be significantly enhanced *via* the plasmid-based co-expression of the tryptophanyl tRNA synthethase enzyme (lacTrpRS) of *L. lactis* (Petrovic *et al.*, 2013a; Shao *et al.*, 2015a). This made it possible to biosynthetically incorporate Trp analogs with bulky substituents in recombinant proteins, which had not been biosynthetically incorporated before. Thus, methylated, brominated, chlorinated, and difluoro-substituted Trp analogs can now be introduced with high efficiency, while maintaining a high alloprotein yield. Using this expression system, we recently also reported the incorporation of β-(1-azulenyl)-L-alanine, which is an amino acid featuring special spectroscopic and electronic properties, including an intense blue color (Shao *et al.*, 2015a). Currently, PA1002, co-expressing lacTrpRS, is the most versatile expression system known for the incorporation of Trp analogs. For Trp-analog-labeled protein production a chemically defined medium (CDM) is needed to ascertain that no natural Trp is available during protein expression, while keeping the cells viable for 16 h.

The synthetic media used for *L. lactis* are much more complex than those for *E. coli* (Otto *et al.*, 1983; Poolman & Konings, 1988; Jensen & Hammer, 1993; Zhang *et al.*, 2009; Aller *et al.*, 2014). They contain about 50 components making them relatively labor intensive and costly compared to the synthetic M9 medium used for *E. coli*. In this work we explored the possibility to simplify a 50-component CDM for *L. lactis* while maintaining the growth characteristics, alloprotein expressions levels, and Trp analog incorporation efficiency. This goal could be reached by leaving out more than half of the components of the CDM used so far for PA1002. Interestingly, an alloprotein expression yield up to 2-3 times higher than in the 50-component CDM was obtained with the newly developed media, as exemplified by two model proteins.

Results

Evaluation of less important components in a 50-component CDM. Previous *L. lactis* Trp auxotroph PA1002 expression experiments were conducted with a CDM consisting of 50 components and using phosphate salts for pH buffering (Poolman & Konings, 1988) (Table 1). In the present work we refer to this medium as CDMbasis. To simplify this medium, knowledge is needed about the importance of each component for cell growth. Such information has recently been provided by Zhang et al., who developed two new synthetic media for *L. lactis* consisting of 57 components (Zhang *et al.*, 2009). All CDMbasis ingredients, except one, are also present in the media developed by Zhang et al. Proper buffering of *L. lactis* growth media is important as lactic acid is produced during growth, resulting in lowering the pH below 4.5, a pH regime where the cells do not grow. MOPS has been found as an excellent buffering component for *L. lactis* media (Jensen & Hammer, 1993; Aller *et al.*, 2014) and was included in the new minimal CDMs (mCDMs) developed in this work. The concentrations of the canonical amino acids are the same or very similar to either those in CDMbasis or the media developed by Zhang et al., except for glutamic acid, of which the concentration is 7-8 times higher in CDMbasis (Table 1). In the new mCDMs, all non-amino-acid concentrations are the same as the concentrations reported by Zhang et al. Twenty six of the non-amino-acid components were labelled by Zhang et al. as “somewhat important (S)” and “least important (L)” for growth. These compounds were left out in

formulating a new mCDM. The above changes resulted in a new medium, mCDM20, consisting of 31 components, including the 20 canonical amino acids (Table 1). To test this medium, cells were grown in GM17 at 30 ^{o}C till exponential phase, harvested, washed 3 times with PBS, and resuspended in either CDMbasis or mCDM20. The yields of biomass, expressed as OD_{600}, of *L. lactis* PA1002 after 16 h at 30 ^{o}C are presented in Figure 1. With CDMbasis an OD_{600} of 3.0 was measured while for mCDM20 the OD_{600} became 2.8. It can be concluded that leaving out most of the non-amino-acid components in CDM does not affect *L. lactis* PA1002 cell growth under the tested conditions.

Table 1. Composition of CDMbasis, mCDM20, mCDM7, mCDM11 and mCDM13

Constituents	CDMbasis (g/L)	mCDM20 (g/L)	mCDM7 (g/L)	mCDM11 (g/L)	mCDM13 (g/L)
KH_2PO_4	2.5	2.6	2.6	2.6	2.6
$K_2HPO_4.3H_2O$	3	5.6	5.6	5.6	5.6
Ammonium citrate	0.6				
Sodium acetate	1				
Potassium acetate		0.76	0.76	0.76	0.76
MOPS		12.7	12.7	12.7	12.7
L-tyrosine	0.29	0.33			0.33
L-glutamic acid	5	0.65		0.65	0.65
L-aspartic acid	0.455	0.455			
L-alanine	0.2375	0.2375			
L-glutamine	0.39	0.39		0.39	0.39
L-aspargine	0.35	0.35			0.35
L-arginine	0.125	0.72	0.72	0.72	0.72
L-lysine	0.4375	0.4375			
L-isoleucine	0.215	0.24	0.24	0.24	0.24
L-methionine	0.125	0.06	0.06	0.06	0.06
L-phenylalanine	0.275	0.275			
L-serine	0.3375	0.3375		0.338	0.338
L-threonine	0.225	0.225			
L-valine	0.325	0.7	0.7	0.7	0.7
L-glycine	0.175	0.175		0.175	0.175
L-histidine	0.15	0.17	0.17	0.17	0.17
L-leucine	0.475	1	1	1	1
L-proline	0.675	0.675			
L-cysteine	0.25	0.25			
Pyridoxal-chloride	0.002				

Vitamin B3	0.001	0.0009	0.0009	0.0009	0.0009
Vitamin B1	0.001				
Vitamin B2	0.001	0.0009	0.0009	0.0009	0.0009
Vitamin B5	0.001	0.0012	0.0012	0.0012	0.0012
Na-p-aminobenzoate	0.01				
Vitamin H	0.01				
Folic acid	0.001				
Vitamin B12	0.001				
Orotic acid	0.005				
Thymidine	0.005				
Inosine	0.005				
DL-6,8-thioctic acid	0.0025				
Pyridoxamine dichloride	0.005				
Pyridoxal HCl		0.0048	0.0048	0.0048	0.0048
$MgCl_2.6H_2O$	0.2				
$CaCl_2.2H_2O$	0.05				
$FeCl_2.4H_2O$	0.005				
$ZnSO_4.7H_2O$	0.005	0.005	0.005	0.005	0.005
$CoCl_2.6H_2O$	0.003				
$CuSO_4.5H_2O$	0.0002				
$MnSO_4.H_2O$	0.05				
$MgSO_4$		0.53	0.53	0.53	0.53
Adenine	0.01				
Uracil	0.01				
Xanthine	0.01				
Gugnine	0.01				
Glucose	20	15	15	15	15
Tryptophan	0.204	0.204	0.204	0.204	0.204

Importance of individual amino acids in mCDM for the growth of PA1002. For a further simplification of mCDM20, the importance of each of the 20 amino acids for growth was investigated. As the medium is developed for a Trp auxotrophic strain, in all subsequent experiments Trp was included. Six amino acids are essential for *L. lactis* growth namely Met, Ile, Leu, Val, Arg, and His (Zhang *et al.*, 2009). Using the leave one out (LOO) approach, thus leaving the other 19 amino acids in the medium, the effect on growth of these 6 amino acids was investigated. The OD_{600} of the 20 cultures after o/n growth are presented in Fig. 1. These data show that media without Met, Ile, Leu, Val, Arg,

or His result in significantly lower OD_{600} values (<1.5) than obtained for mCDM20 (2.8). Thus for good growth of Trp auxotroph strain PA1002 these 6 amino acids, like Trp, need to be present in the medium. This medium is called here mCDM7 (Table 1).

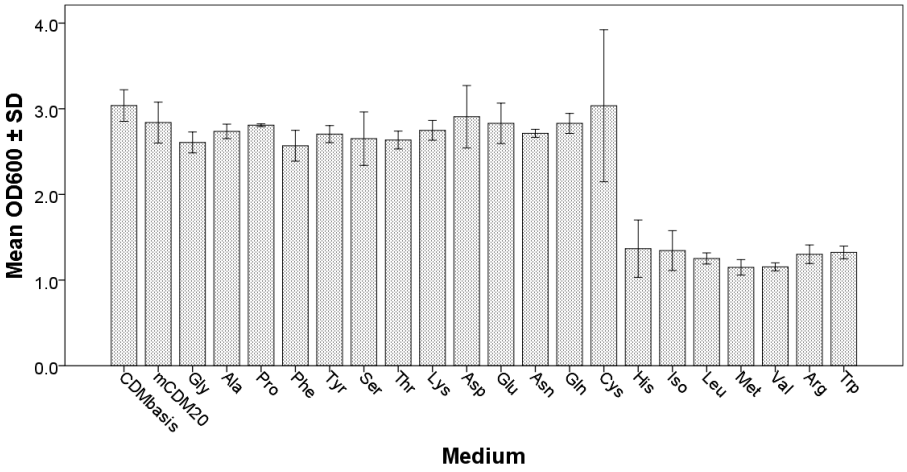

Figure 1. The optical density (OD_{600}) after o/n incubation of PA1002 cultures subjected to the leave one out approach for the 20 canonical amino acids.

Leaving one of the other 13 amino acids out from mCDM20 had no or only a minor effect on the OD_{600} (Figure 1), suggesting that these amino acids are not important for growth. However, with mCDM7 only an OD_{600} of 1.0 was obtained after o/n growth (Table 2, entry A1), significantly less than with mCDM20 (Table 2, entry A2). The discrepancy between these experiments can be explained by interaction phenomena taking place between the amino acids. To investigate this, the 13 amino acids were divided in "somewhat important (S)" and "less important (L)" amino acids, following the classification of Zhang et al. (2009).

Adding all 8 "S" amino acids to mCDM7 resulted in an unfavorable growth medium for PA1002 as the OD_{600} after o/n incubation was only 0.6 (Table 2, entry A3). Including all 5 "L" amino acids in mCDM7 enhanced the growth, as the OD_{600} became 1.6 (Table 2, entry A4), a cell density significantly higher than found for mCDM7 (OD_{600} = 1.0), but lower than when mCDM20 was used. This

medium, mCDM7 plus all 5 "L" amino acids was used to investigate the impact of each "S" amino acid on growth (Table 2, entries A5-A12). Including Ser or Gly in this medium resulted in a much higher OD_{600} of 2.0 and 2.2, respectively, while including one of the other 6 "S" amino acids minimally affected the growth (Table 2). Of the 5 "L" amino acids, only Glu and Gln turned out to be quite important for growth as the OD_{600} increased from 1.0 for mCDM7 to 1.8-1.9 when one of these amino acids were included in mCDM7 (Table 2, entries A13-17). Based on the outcomes of the above first screens of "S" and "L" amino acids, a new mCDM medium (mCDM11) was composed, consisting of mCDM7 + Ser, Gly, Glu, and Gln. This medium well supports growth of PA1002 as an OD_{600} of 2.4 is obtained in an overnight culture (Table 3, entry B1), compared to an OD_{600} of 3.1 for mCDM20.

Table 2. The OD_{600} of *L. lactis* PA1002 after 16 h at 30°C in mCDM7 complemented with amino acids as indicated.

Treatment	"S Amino acids"								"L Aminio acids"					OD_{600}
	Pro	Asn	Ser	Thr	Ala	Gly	Lys	Tyr	Glu	Phe	Asp	Gln	Cys	
A1	0	0	0	0	0	0	0	0	0	0	0	0	0	1.0
A2	1	1	1	1	1	1	1	1	1	1	1	1	1	2.7
A3	1	1	1	1	1	1	1	1	0	0	0	0	0	0.6
A4	0	0	0	0	0	0	0	0	1	1	1	1	1	1.6
A5	1	0	0	0	0	0	0	0	1	1	1	1	1	1.4
A6	0	1	0	0	0	0	0	0	1	1	1	1	1	1.3
A7	0	0	1	0	0	0	0	0	1	1	1	1	1	2.0
A8	0	0	0	1	0	0	0	0	1	1	1	1	1	1.7
A9	0	0	0	0	1	0	0	0	1	1	1	1	1	1.4
A10	0	0	0	0	0	1	0	0	1	1	1	1	1	2.2
A11	0	0	0	0	0	0	1	0	1	1	1	1	1	1.3
A12	0	0	0	0	0	0	0	1	1	1	1	1	1	1.7
A13	0	0	0	0	0	0	0	0	1	0	0	0	0	1.9
A14	0	0	0	0	0	0	0	0	0	1	0	0	0	1.1
A15	0	0	0	0	0	0	0	0	0	0	1	0	0	1.1
A16	0	0	0	0	0	0	0	0	0	0	0	1	0	1.8
A17	0	0	0	0	0	0	0	0	0	0	0	0	1	1.3

"0" represent it is not added to mCDM7

"1" represent it is added to mCDM7

The one at a time approach was used to explore whether one or two amino acids present in mCDM20 but not in mCDM11 are responsible for this difference in OD_{600} and the results of these experiments are presented in Table 3. Only Asn and Tyr increased the OD_{600} from 2.4 to 2.7 and 2.6, respectively (entries B3 and B7 in Table 3). When Tyr and Asn were both added to mCDM11 (entry B8 in Table 3) an OD_{600} value similar to the OD_{600} for mCDM20 was obtained, thus showing that the amino acids Asp, Ala, Lys, Phe, Thr, Pro, and Cys, present in mCDM20 are not supporting additional growth. This medium is called mCDM13, a new CDM composed of 24 chemicals including 13 amino acids (Table 1), in which *L. lactis* Trp auxotroph PA1002 grows as well as in the 50-component CDMbasis.

Table 3. The OD_{600} of *L. lactis* PA1002 after 16 h at 30°C in mCDM11 complemented with amino acids as indicated

Treatment	Pro	Asn	Thr	Ala	Lys	Tyr	OD_{600} after 16 h incubation
B1	0	0	0	0	0	0	2.4
B2	1	0	0	0	0	0	2.3
B3	0	1	0	0	0	0	2.7
B4	0	0	1	0	0	0	2.3
B5	0	0	0	1	0	0	2.4
B6	0	0	0	0	1	0	2.1
B7	0	0	0	0	0	1	2.6
B8	0	1	0	0	0	1	3.2

"0" represent it is not added to mCDM11

"1" represent it is added to mCDM11

The OD_{600} in mCDM20 is 3.1

The production level of recombinant protein in L. lactis PA1002 grown in CDMbasis, mCDM20 or mCDM13. The simplification of the CDMbasis aims to express Trp-analog-labelled protein efficiently and cost-effectively. Two different proteins, LmrR and W20 lysM tandem were used to investigate the impact of either CDMbasis, mCDM20, and mCDM13 on the production levels of these proteins. LmrR is a transcriptional regulator found in *L. lactis* and its gene was cloned in a pNSC8048 vector behind a P_{nis} promotor. W20 lysM tandem protein consists of two engineered lysin motifs from the *L. lactis*

enzyme N-acetylglucosaminidase (AcmA). The gene of this single-Trp containing protein was cloned in a nisin inducible expression-secretion vector derived from pNZ8048. W20 lysM tandem protein is thus secreted into the medium. The production levels of these two proteins each labeled with either 5-fluoroTrp (5FW), or 5-methylTrp (5MeW) in *L. lactis* PA1002 grown in CDMbasis, mCDM20 and mCDM13, respectively, were determined after o/n induction with nisin. In Figures 2 and 3 the OD_{600} values of the cultures are presented. Under all tested conditions, the growth is the lowest in mCDM13 and the presence of 5MeW in the medium is more toxic for cell growth than 5FW. Expression levels of the proteins, per volume unit cell culture, were determined for all 6 alloproteins by lysing the cells with glass beads and

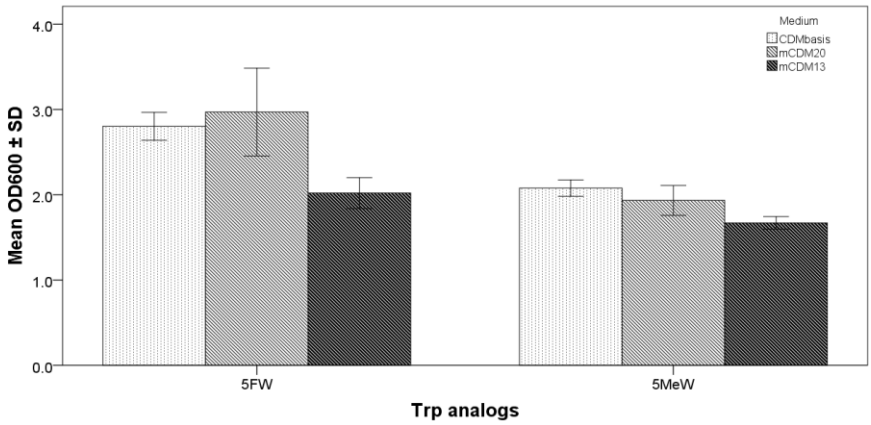

Figure 2. The optical density (OD_{600}) after o/n incubation of PA1002 cultures expressing LmrR protein in different synthetic media supplemented with a Trp analog.

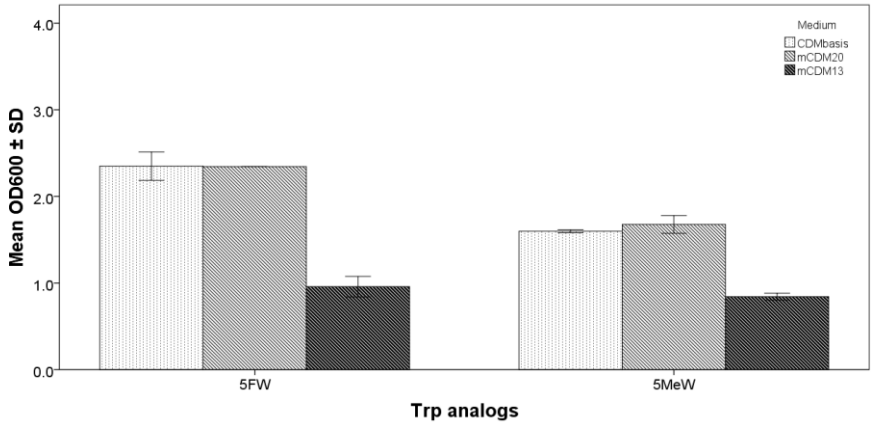

Figure 3. The optical density (OD600) after o/n incubation of PA1002 cultures expressing W20 lysM protein in different synthetic media supplemented with a Trp analog.

loading the whole cell extracts on an SDS-PAGE gel. Protein band intensities were determined and the results are presented in Table 4. For W20 LysM tandem protein labelled with 5FW, expression in mCDM20 and mCDM13 are up to 2.3 times higher compared to CDMbasis as expression medium. Expression of 5MeW-labelled W20 lysM tandem protein is also higher in mCDM20 than in CDMbasis but lower in mCDM13 (-35%). For LmrR, a different picture was observed as the expression level is 2.3-2.7 times higher for both the 5FW and 5MeW-labelled protein in mCDM13. Note that high alloprotein expression levels were obtained in mCDM13, but that in this medium the lowest amount of biomass (OD_{600}) was produced (Figure 2), indicating that cells grown in mCDM13 produce protein more efficiently than in the other 2 media investigated. Taken together, the highest expression levels of the two test proteins were obtained in one of the two newly developed media.

Table 4. Expression levels and Trp analog incorporation efficiencies of W20 lysM proteins and LmrR proteins

Protein		5FW				5MeW		
		Position	CDMbasis	mCDM20	mCDM13	CDMbasis	mCDM20	mCDM13
W20	Expression level (AU)		3000±500	7000±1300	3900±1200	2700±800	4100±70	1700±20
	% analog	20	98%	96%	96%	95%	94%	96%
LmrR	Expression level (AU)		5300±400	7700±900	7100±400	7700±500	18000±200	21000±1100
	% analog	67	≥99%	98%	97%	≥99%	96%	≥99%
		76	≥99%	≥99%	≥99%	≥99%	≥99%	96%
		120	≥99%	≥99%	≥99%	≥99%	≥99%	≥99%

The incorporation efficiency of Trp analogs in CDMbasis, mCDM20 and mCDM13. Besides a high expression level of alloprotein, also the labeling efficiency is of importance. The incorporation efficiency of Trp analogs into LmrR and W20 lysM tandem protein was determined by MALDI-TOF (see Table 4). In CDMbasis W20 lysM protein could be labeled with 5FW or 5MeW with an incorporation efficiency of 98% and 95%, respectively, and similar incorporation efficiencies were obtained when mCDM20 or mCDM13 were used. LmrR contains 3 Trp residues at positions 76, 96 and 120 and labeling efficiencies at these 3 positions were determined when cells were cultured in CDMbasis, mCDM20 or mCDM13, supplemented with either 5FW or 5MeW. In all LmrR samples, incorporation efficiencies at the 3 positions were 96% or higher. In summary, a very high Trp analog incorporation efficiency can be obtained when PA1002 is cultured in the two newly developed media.

Discussion

In this report new synthetic media have been developed, specifically for the production of recombinant alloproteins by the *L. lactis* Trp auxotroph PA1002, a derivative of *L. lactis* strain MG1363 (El Khattabi *et al.*, 2008). Several CDMs for *L. lactis* have been developed over the years for metabolic studies (Otto *et al.*, 1983; Poolman & Konings, 1988; Jensen & Hammer, 1993; Zhang *et al.*,

2009; Aller *et al.*, 2014) and defined media also allow the expression of heterologous proteins labeled with unnatural amino acids (El Khattabi *et al.*, 2008; Berntsson *et al.*, 2009; Petrovic *et al.*, 2012; Petrovic *et al.*, 2013b; Petrovic *et al.*, 2013a; Shao *et al.*, 2015a). Most of these media support growth for prolonged incubation times but for the production of alloproteins by Trp auxotroph PA1002, a less demanding role of the CDM is required. Here the medium is only used to keep the cells viable during alloprotein expression for 16 h when cell growth is minimal or even absent because of the toxicity of the Trp analogs. Development of CDMs for *L. lactis* gave insight into the importance of most components (Zhang *et al.*, 2009). Steered by this information, two much simpler CDMs, mCDM20 and mCDM13, were developed. Both new media support high-density growth of PA1002 in the presence of Trp and without inducer. When used as expression medium in the PA1002-based protocol for the production of Trp-analog-labeled alloproteins, protein yields per culture volume up to 2-3 times higher than when using the starting-off CDMbasis were obtained, suggesting adverse effects of one or more of the components not present in mCDM13 and mCDM20. In comparing the mCDM13 and mCDM20 media, the latter supports higher cell densities in all alloprotein expression experiments presented in Figures 2 and 3. However, the amount of W20 alloprotein produced per cell is similar (5MeW-labeled W20 lysM tandem) to much higher (5FW-labeled W20 lysM tandem) in mCDM13. For the non-secreted LmrR, the expression yields are significantly higher in cells cultured in mCDM13 compared to mCDM20. This benefits the purification of the expressed protein, making the simpler mCDM13 the most attractive medium for alloprotein production. As preparation of the synthetic medium is the most labor intensive part of the PA1002-based alloprotein expression protocol, the new media make this system significantly less labor intensive and more cost efficient. In conclusion, the simplest synthetic media known for *L. lactis* are presented in this report which are optimally suited for the efficient production of Trp-analog-labeled alloproteins.

Experimental Procedures

Bacterial strain and plasmids. The *L. lactis* Trp auxotroph PA1002 (El Khattabi *et al.*, 2008), which harbors the pMG36e-trprs plasmid for expressing lacTrpRS (Petrovic *et al.*, 2013a), was used in this study. To test the system with two different alloproteins, plasmid pNZ8048-PA295 (containing the gene for the W20 LysM tandem protein(Petrovic *et al.*, 2012) or plasmid pNSC8048-lmrR (containing the gene for the multidrug transcriptional repressor protein LmrR (Agustiandari *et al.*, 2008)) was electrotransformed into *L. lactis* PA1002 containing the pMG36e-trprs plasmid.

Cultivation and protein expression. The 50-component CDMbasis was prepared essentially as described before (Berntsson *et al.*, 2009). For the preparation of mCDM media, a 1.18 × basic buffer, pH 6.5-6.6, was used consisting of KH_2PO_4, K_2HPO_4, MOPS, $MgSO_4$, and potassium acetate. 845 ml basic buffer (filter sterilized) was mixed with 75 ml 20% glucose (autoclaved), 20 ml 50× vitamin mix (filter sterilized), and 50 ml 20× amino acid mix without Trp (filter sterilized). To this mixture was added 10 ml 0.05% $ZnSO_4$ solution (filter sterilized). Cells were cultured without shaking at 30 ^{o}C. For cultivation, 1 ml overnight culture of *L. lactis* Trp auxotroph PA1002 in GM17 medium with 5 µg/ml of chloramphenicol and 75 µg/ml erythromycin was used to inoculate 50 ml fresh GM17 with 5 µg/ml of chloramphenicol and 75 µg/ml erythromycin and incubated until an OD_{600} of 0.8 was reached. The cells were centrifuged at 5500×g for 8 min and the pellet was resuspended in PBS. This procedure was repeated two more times. Subsequently, the cells were resuspended in 50 ml synthetic medium (Table 1), excluding Trp, and this culture was left for 30 min at 30 ^{o}C. The expression of the recombinant proteins, W20 lysM tandem or LmrR, was induced by adding 8 ng/ml nisin and 1 mM Trp or Trp analog and the culture was left at 30 ^{o}C for 16 h (Petrovic *et al.*, 2013a).

SDS PAGE gel electrophoresis and protein expression level determination. For W20 lysM tandem protein samples, the supernatant of the centrifuged cell culture was used, because W20 lysM tandem is secreted by *L. lactis* into the medium. For LmrR, the cell pellet was resuspended into 20 mM Tris-HCl buffer, pH 8.0, with 50 mM NaCl, followed by the addition of 10 mM $MgSO_4$, 100 µg/ml DNase I, complete protease inhibitor (Roche), and glass beads (50-100

mesh). After opening the cells using a MINI-beadbeater (BioSpec Products), and centrifugation, the supernatant was mixed with loading buffer and loaded on a SDS-PAGE gel. Gels were stained with 0.05% Coomassie Brilliant Blue R-250. The protein band intensity was evaluated using the program ImageMaster (Pharmacia).

Protein purification and mass spectrometry. W20 lysM tandem was purified with a HiTrap Phenyl HP column (GE Healthcare) using a fast protein liquid chromatography (FPLC) system (Äkta FPLC) as described elsewhere (Petrovic *et al.*, 2012). LmrR was purified using two columns, a streptactin sepharose and a HiTrap heparin HP column as described before (Agustiandari *et al.*, 2008).

For mass spec analysis, the buffer of the two proteins was changed to 100 mM ammonium bicarbonate buffer using an Amicon concentrator (MWCO 10 kDa). 1 µl 100 µg/ml trypsin was added to 20 µl protein solution (around 0.1 mg/ml protein) and incubated for 2-3 h at 37 °C. A 1 µl sample was spotted on a MALDI plate and mixed immediately with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamate (LaserBio Labs) in 50 % acetonitrile/ 0.1 % (v/v) trifluoroacetic acid. Spots were measured using a Voyager DE-PRO MALDI-TOF (time of flight) instrument (Applied Biosystems). The incorporation efficiency of Trp analog into proteins was calculated after measuring the peak areas of the peptide containing either Trp or Trp analog.

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