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“Protein secretion in reduced genome strains from *Streptomyces lividans* TK24”

Tesis

QUE PARA OBTENER EL GRADO DE

MAESTRA EN BIOTECNOLOGÍA

PRESENTA

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"One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away." - *Stephen Hawking*

SUMMARY

Genome reduction is a strategy that aims to improve the efficency of heterologous expression. Non-essential gene clusters from the secondary metabolism in *S. lividans* TK24, a common expression system, were deleted. The effect of this deletion on the expression and secretion for both native and heterologous proteins was not studied previously. In this work the native secretomes from ten reduced genome strains were characterized first. As a second step, monitoring heterologous protein secretion with mRFP allowed to know the effect of the genomic reduction on the expression and secretion of a heterologous protein by *S. lividans* TK24. Secreted monomeric Red Fluorescent Protein (mRFP) amounts considered low (strain R1.9: 9.235 ± 0.447 mg/gDCW) when compared with the wild type reported yields (61.551 ± 19.101 mg/gDCW) are reported. These values are seven to twenty-five times lower than the expected minimum. This suggests that, although the system has been previously proved as efficent for heterologous expression by Novakova *et al.* (2016), a different approach to study and understand the effect of the deletions in the cell's metabolism and general functioning might be needed.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BCA	Bicinchoninic Acid Assay
BSA	Bovine serum albumin
ddH_2O	Sterilized distillated water
dH_2O	Distillated water
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
h	Hour(s)
IM	Inner membrane
(k)bp	(Kilo) base pairs
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute(s)
mRFP	Monomeric Red Fluorescent Protein
NaCl	Sodium chloride
OD_{600}	Optical density at 600 nm
OM	Outer membrane
ON	Overnight
PMSF	Phenylmethylsulfonyl fluoride
RGS	Reduced Genome Strains
RT	Room temperature
s	Second(s)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TAE buffer	Tris-acetate-EDTA buffer
TCA	Trichloroacetic acid
TEMED	Tetramethylethyleendiamine
TTBS	Tris-buffered saline
WT	Wild type

TABLE OF FIGURES

1 Introduction

The transport of proteins across membranes is essential for prokaryotic cellular function, underscored by the fact that around one third of all proteins produced by a cell are secreted out of the cytoplasm (Tsirigotaki *et al.*, 2017). *Streptomyces lividans* is a Gram-positive model well known for its ability to secrete a wide variety of metabolites directly to the extracellular space making it a suitable host cell factory for the heterologous expression of polypeptides of bacterial and eukaryotic origin (Hamed *et al.*, 2017). Some of the advantages that make *S. lividans* secretion an attractive biotechnology platform are the absence of an outer membrane, the availability of advanced genetic and synthetic biology tools (Kieser *et al.*, 2000); low protease activity and the avoidance of inclusion body formation (Anne *et al.*, 2017; Koepff *et al.*, 2017). *S. lividans* TK24 is the most commonly used strain for heterologous protein production. TK24 has been previously successfully applied for heterologous expression of a number of industrially important proteins, including active trimeric murine tumor necrosis factor alpha (mTNF-α) (Lammertyn *et al.*, 1997; Pozidis *et al.*, 2001), a *Jonesia sp.* xyloglucanase of 100 kDa (Sianidis *et al.*, 2006) and thermostable cellulase (celA) (Hamed *et al.*, 2017). While these examples are currently available for industrial usage, here we attempt to explore strategies that can enhance the efficiency of heterologous protein production through the TK24 system.

A common approach in the Synthetic Biology field is the deletion of non-essential gene clusters thereby increasing growth efficiency and streamlining the use of carbon and nitrogen sources for heterologous protein production. This method also creates simplified cells with predictable behavior that act as a platform to build in various genetic systems for specific purposes (Morimoto *et al.*, 2008) with the hypothesis that these modifications would improve the performance of host cells in general (Bu *et al.*, 2019). Genome reduction has been used in numerous species such as *E. coli* (Posfai *et al.*, 2006), *Bacillus subtillis* (Morimoto *et al.*, 2008) and *Streptomyces coelicolor* (Gomez-Escribano and Bibb, 2011) with varying degrees of success. A Blast Analysis against the genome of the closely related bacterium *S. coelicolor* A3 revealed that at least 507 genes from *S. lividans* TK24 have no clear ortholog in *S. coelicolor* A3 which makes them potential candidates for removal (Ruckert *et al.*, 2015). As an attempt to acquire efficient expression of secondary metabolites from *Streptomyces lividans*

TK24, reduced genome strains (RGS) were constructed by Novakova et al, in 2017. This collection of strains was expected to be suitable for heterologous protein expression.

2 Study of literature

2.1 Bacterial protein secretion

An essential prokaryotic cell function is the transport of proteins. Protein secretion is the process in which proteins are conveyed from the cytoplasm into the cellular membrane, the environment or another cell. Bacteria have developed numerous ways of transporting proteins between locations through those systems, which are essential for the growth of bacteria and are used in an array of processes. Some secretion systems are found in almost all bacteria and secrete a wide variety of substrates, while others have been identified in only a small number of bacterial species or are dedicated to secreting one or a few proteins (Green and Mecsas, 2016).

More than one-third of the bacterial proteome that is synthesized by cytoplasmic ribosomes is exported to extracytoplasmic locations, where the polypeptides acquire their native functional states (Tsirigotaki *et al*., 2016). These proteins are either membrane-embedded or secreted to the trans side of the plasma membrane; for example, to the periplasm (comprising the ‘secretome’).

The cell envelope is a complex, dynamic, multilayered structure that serves to protect bacteria from their unpredictable and often hostile surroundings. The cell envelopes of most bacteria fall into one of two major groups: Gram-negative bacteria have an inner cytoplasmic membrane surrounded by a thin layer of peptidoglycan (PG) and an outer membrane containing lipopolysaccharide. Gram-positive bacteria lack a protective outer membrane, but the PG layers are many times thicker than those in Gram-negative organisms (Bagnoli & Rapuoli, 2017). At least 17 diverse secretion systems have been identified for Gram-negative bacteria and around 7 protein translocation systems were reported for Gram-positive bacteria (Forster and Marquis, 2012; Tsirigotaki *et al.*, 2017).

2.1.1 Signal peptides

Proteins destined for secretion are synthesized as preproteins with an N-terminally extended sequence named the signal peptide (SP). Signal peptides typically have three common regions termed N, H and C. In bacteria a signal peptide is a ~20- to 30-residue N-terminal sequence that targets proteins and consists of an N-terminal basic region (N), a hydrophobic patch (H), and a three-residue motif for signal peptidase cleavage (C) (Payne *et al.*, 2012). Although signal peptides share conserved physicochemical properties they differ in sequence. The signal peptide's role is to guide the nascent preprotein to the secretion channel and plays a foundational role in protein sorting and localization.

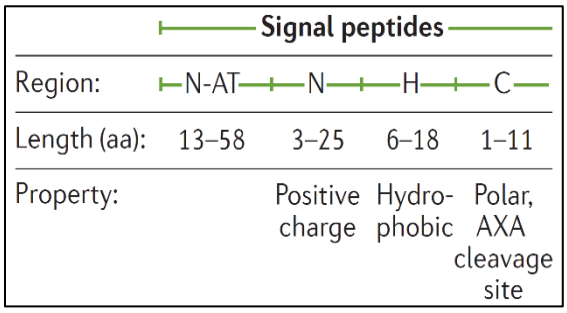

Figure 2.1.1: Pre-proteins contain signal peptides fused to mature domains at the amino terminus, which act as sorting and targeting tags in the cytoplasm. The N-terminal region (N) is mostly positively charged. The helical hydrophobic region (H) engages either SRP, SecA or trigger factor. The mainly polar carboxy-terminal region (C) contains the AXA SPase cleavage motif. (Tsirigotaki *et al.*, 2017).

2.1.2 Protein secretion in G-positive bacteria

Gram-positive organisms have dynamic cell envelopes that protects the cell and mediates the interactions with the environment. Major components of the cell envelope include peptidoglycan (PG), teichoic acids (TAs), capsular polysaccharides (CPS), surface proteins and phospholipids (Bagnoli & Rapuoli, 2017). Despite its complex structure, the cell envelop of Gram-positive bacteria is permeable to proteins. Secreted proteins can be released directly into the culture medium, where they obtain their native conformation, as it does not contain an outer membrane (Anné *et al.*, 2016).

The secretion pathways for Gram-positive bacteria (Fig. 2.1.1) include: The General Secretion System (Sec), Twin-Arginine Transport Pathway (Tat), Non-classically Secreted Protein Pathway, Type IV secretion, Type VII Secretion System, Flagella Export Apparatus and Holins. However, from a biotechnological point of view, only the Sec and Tat systems are useful.

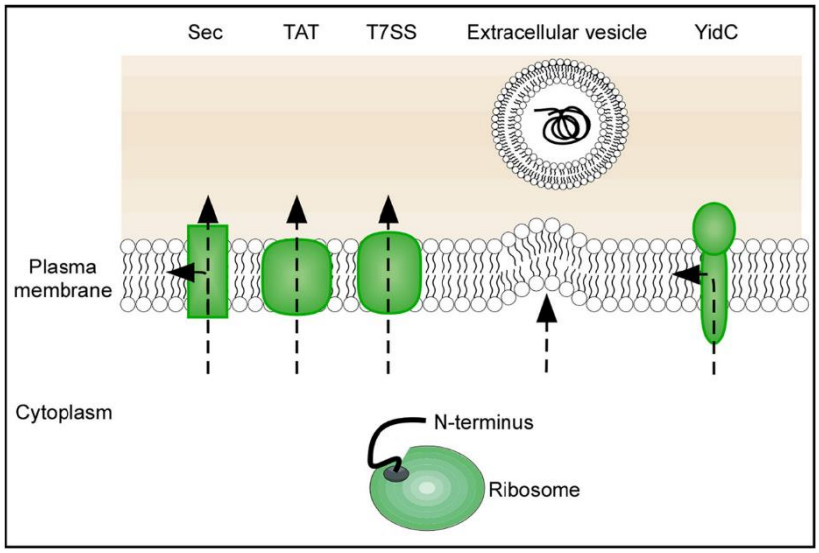

Figure 2.1.2: Schematic overview of 5 main protein export pathwyas in *Streptomyces*. Sec-pathway that mediates unfolded proteins secretion; Tat-pathway for the export of folded proteins; T7SS secrete small proteins (less than 100 residues), which play a role in virulence; extracellular vesicles export 'non-classical secreted proteins'; YidC, an insertase for membrane proteins that works alone or in complex with SecYEG. (Hamed *et al.*,

2.1.2.1 *Streptomyces lividans* TK24 and its secretion pathways

Streptomycetes are Gram-positive, aerobic soil bacteria with a fungal-like growth, mainly recognized as antibiotic producers, since more than 60% of the nearly 6000 antibiotics of microbial origin are produced by Streptomycetes (Anné & Mellaert, 1993). Besides the wide antibiotic variety produced by these actinomycetes, *Streptomyces spp.* are proficient secretors of hundreds of polypeptides and are therefore frequently suggested as host for production of heterologous proteins of industrial or medical relevance. These characteristics render Streptomycetes among the most important industrial microorganisms (Koepff *et al.*, 2017).

2.1.2.1.1 The SEC system

The SEC secretion system is the main protein translocation route in bacteria. In *Streptomyces lividans* TK24 it transports more than 78% of the secretome (Tsolis *et al.*, 2018). Sec-dependent secretory proteins are N-terminally tagged with a signal peptide comprising 15 to 40 amino acids defined by a canonical AxA motif (Payne *et al.*, 2012; Tsirigotaki *et al.* 2017) which functions to keep the protein in a non-folded state and to trigger the translocase in an allosteric manner (Hamed *at al.*, 2018; Koepff *et al.*, 2017). Once the polypeptide is in a "translocation competent state" (unfolded and soluble) protein secretion occurs co-translationally or post-translationally. In either case the central component of the Sec system is the transmembrane SecYEG channel (Fig 2.1.1.1), which translocates proteins into or across, the plasma membrane (Tsirigotaki *et al.*, 2016).

2.1.2.1.2 Twin-arginine translocation

In *S. lividans* TK24, ~21% of the secretome represents potential Tat substrates (Tsolis *et al.*, 2018). Contrary to Sec, the Tat pathway translocates proteins that are already intracellularly folded, possibly containing cofactors. Tat signal peptides N-region contains a conserved S/T-R-R-x-FLK motif and their H-region is less hydrophobic than the one from its Sec homologue (Hamed *et al.*, 2018). The Tat translocon consists three integral membrane proteins: TatA proteins family, that form the translocation channel; and TatB and TatC, that assemble to form a multivalent receptor complex to which Tat signal peptides bind (Fig. 2.1.1.1).

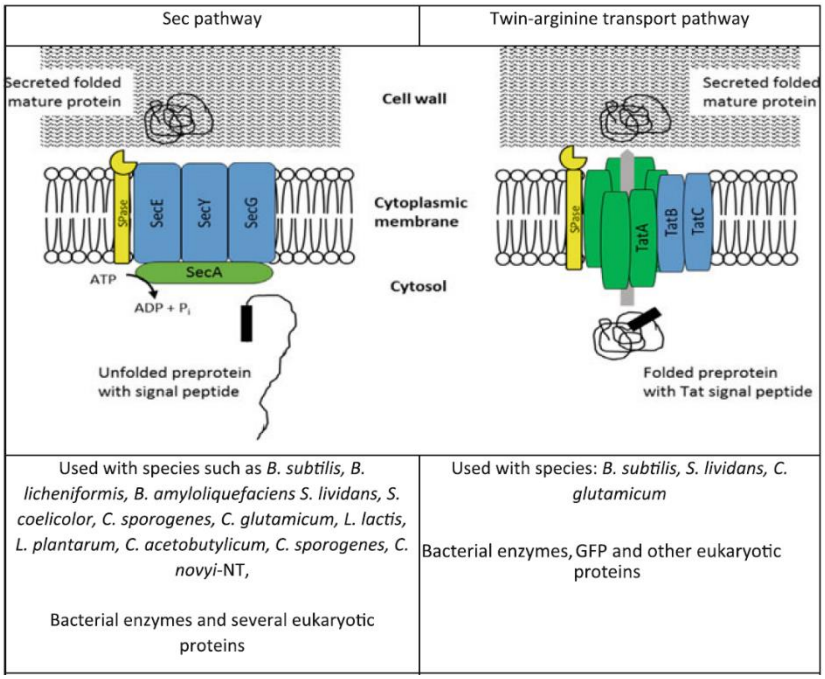

Figure 2.1.1.1: Sec and Tat graphic description and main possible biotechnological applications (edited from Anné *et al.*, 2016)

2.2 Protein secretion biotechnology

The biopharmaceuticals and industrial enzymes market have come a long way since 1982 when the first biopharmaceutical product, recombinant human insulin, was launched. Bacterial systems remain the most attractive hosts to produce recombinant proteins due to low cost and high productivity. Although a wide variety of bacterial host systems are currently used in industrial processes, such as *Bacillus subtilis, Anabaena sp.* and *Staphylococcus carnosus* (Terpe, 2006), *Escherichia coli* remains to be the most common host of choice because of its well-understood genetics, the wide variety

of available genetic tools and largescale production systems already settled. However, for some industrial processes, using *E. coli* as host has two major disadvantages: first, because of *E. coli* being a Gram-negative bacterium, it has an outer membrane (OM) composed of lipopolysaccharides (LPS) that acts as an effective permeability barrier, hindering secreted proteins from being released into the extracellular medium; second, many proteins overexpressed in *E. coli* become misfolded and accumulate in the cytoplasm as inclusion bodies. These inclusion bodies need to be solubilized and proteins refolded, an often-cumbersome process with poor recovery, to become active, increasing productions cost of recombinant proteins. (Bagnoli & Rappuoli, 2017).

In principle, all bacteria can be used for heterologous protein production, but missing information about their regulation and mechanism, as well as the fact that there are no commercial vectors and promoter systems are the reasons why these systems are not so frequently used (Terpe, 2006). One such example is the Gram-positive soil bacteria *Streptomyces*; the lack of the OM represents a major advantage of this genus over E. coli for production of secreted recombinant proteins as they are directly deposited extracellularly facilitating downstream processing (Hamed *et al.*, 2018).

2.2.1 Actinomycetes and *Streptomyces* for heterologous protein production (Hamed *et al.*, 2018)

Many *Streptomyces spp.* secrete large quantities of extracellular proteins including hydrolytic enzymes and proteinaceous enzyme inhibitors. Combined with non-pathogenicity and a well-established fermentation technology the genus always been attractive for research groups to investigate the feasibility of using *Streptomyces* for heterologous protein production (Anné & Mellaert, 1993).

Genome sequencing (Ruckert *et al.*, 2015) and RNAseq data (Busche *et al.*, 2018) confirmed that *S. lividans* TK24 is a very suitable host. Limited restriction-modification capabilities and low endogenous protease activity are some of its attractive features. Numerous vector systems (derivatives of plasmid pIJ101 (Kieser *et al.,* 2000)) are available tools for genetic manipulation of streptomycetes (Hamed *et al.,* 2018). Recently, the CRISPR/Cas approach is also available as an instrument for gene editing in actinomycetes (Tong *et al.,* 2015) including *S. lividans* (Cobb, Wang and Zhao, 2015; Jia *et al.,* 2017). Several heterologous prokaryotic and eukaryotic proteins have been successfully produced in *S. lividans*. Some, hardly producible in *B. subtilis* or *E. coli*,

could successfully be secreted from *S. lividans* such as Endoglucanase from *Thermobifida fusca* (Jung *et al.,* 1993), CelA from *Rhodothermus marinus* (Hamed *et al.*, 2017), xyloglucanase from *Jonesia sp*. (Sianidis *et al.,* 2006), functional periplasmic alkaline phosphatase from *Thermus thermophilus* (Diaz *et al.,* 2008) and monomeric red fluorescent protein and enhanced green fluorescent protein (Hamet *et al.,* 2018).

However, unsuccessful heterologous protein secretion is a problem encountered by many hosts and proteins (Hamed *et al.*, 2018). Different approaches have been proposed to increase secretion yields such as increase expression through promoters' manipulation, and modulation of components directly related to the protein secretion such as adaptation and overexpression from signal peptides.

In the past few years '-omics' technologies (genomics, proteomics and transcriptomics) have come into the spotlight, as a proposal to form a basis for a more rational approach to develop strains with better and higher production of a wanted product. Together with bioinformatical analysis, -omics technologies are expected to provide a more accurate understanding, at system level, of the maintenance and expression of non-native DNA and recombinant protein synthesis.

2.3 Reduced genome bacteria

The current wide availability of genome information and high-throughput technologies has enabled the systematic improvement of industrial microbes through genome engineering. Restructuring of microbial genomes has been shown to have several advantages over conventional approaches for strain improvement (Fujio, 2007).

Genome reductions are rationally designed deletions to create simplified cells with predictable behavior that act as a platform to build in various genetic systems for specific purposes (Morimoto *et al.*, 2008). This approach is believed to improve metabolic efficiency and decrease the redundancy among genes and regulatory circuits while preserving good growth profiles and protein production. (Pósfai *et al.,* 2006). By deleting blocks of nonessential genes, the production of unwanted by-products can be reduced, genome stability can be increased, and streamline metabolism without physiological compromise can be accomplished (Lee *et al.,* 2009).

Researchers have successfully constructed reduced-genome strains from different bacteria. Both Gram-negative and Gram-positive cases have been reported: In 2006,

Pósfai and collaborators created 15% genome-reduced strains from *E. coli* that showed high electroporation efficiency and accurate propagation of recombinant genes and plasmids (reported as unstable in other strains). Lee and partners created another 14.3% reduced-genome *E. coli* strain in 2008 and reported ~83% increase in L-threonine production compared with the wild-type strain yields. As for Gram-positive bacteria, in 2008, Morimoto reported a *Bacillus subtilis* strain depleted of 20% of its genomic sequence. When compared with wild-type cells, the productivity of extracellular cellulase and protease from transformed plasmids was remarkably enhanced. In 2011 Gómez-Escribano engineered nine *Streptomyces coelicolor* strains with four endogenous secondary metabolite gene clusters deleted (actinorhodin, prodiginine, creatine phosphokinase and calcium dependent antibiotic) to increase the level of secondary metabolite production. They found some strains to produce levels four to ten times higher than the parental strain.

3 Experimental previous work

3.1 Monitoring protein secretion in *Streptomyces* using fluorescent proteins (Hamed *et al.*, 2018)

The group applied fluorescent protein technology to study protein secretion in *S. lividans* TK24. When fused to Sec (SPSec) and Tat (SPTat) signal peptides, green and monomeric red fluorescent proteins secretion can be directed through the respective export pathway. A high copy number plasmid over-expression was compared to a chromosomally integrated spSec-mRFP gene and it was found that the first option is the most suitable for the heterologpous expression for both fluorescent proteins. Secretion under high- and low-level synthesis in various media was observed. Fluorimetric detection of SPSec-mRFP recorded folded states, while immuno-staining detected even non-folded topological intracellular intermediates. It was found that high-level synthesis of heterologous secretory proteins has a drastic effect on the endogenous secretome.

3.2 Increased heterologous production of the antitumoral polyketide mithramycin A by engineered *S. lividans* TK24 strains (Novakova *et al.*, 2018): The reduced genome strains origin.

A collection of derivative strains of *Streptomyces* TK24 was constructed by sequential deletion of known, potentially interfering, secondary metabolite gene clusters using a protocol based on the positive selection of double crossover events with blue pigment indigoidine-producing gene.

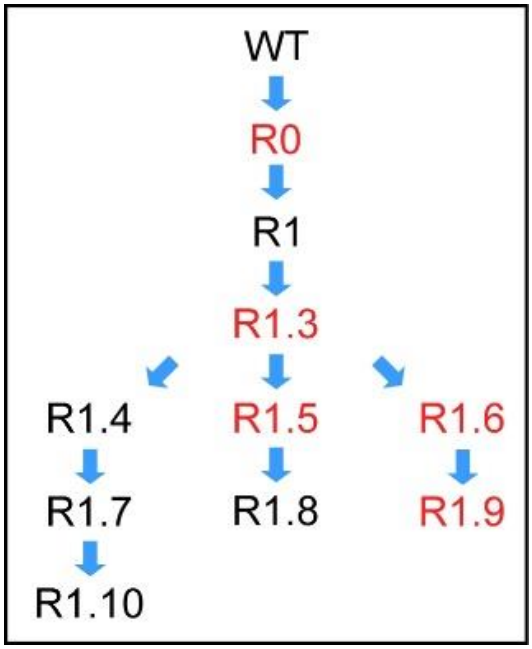

Figure 3.2-1: Derivative strains tree

The five different gene clusters correspond to the following secondary metabolites (Fig. 3.2-2): blue-pigmented polyketide antibiotic actinorhodin (cluster C), red-pigmented antibiotic undecylprodiogiosin (cluster B), calcium dependent antibiotic (cluster D), coelimycin 1 (cluster A) and the brown pigment

melanin (cluster E). In addition, extracellular glycan encoded genes *matA* and *matB*, and region encoding transcription factor (HrdD) were also deleted in some cases.

As shown in figure 3.2-1, all reduced-genome strains (RGS) have a parental strain (R0) missing the actinorhodine cluster from which the rest was designed.

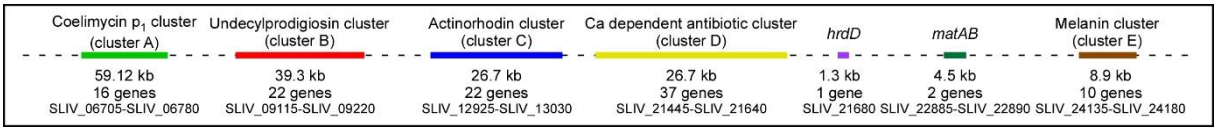

Figure 3.2-2: *S. lividnas* TK24 selected secondary metabolites gene clusters description (Clusters arranged according to the order at the chromosome)

In 2018 Novakova and collaborators engineered the previously described strains to improve antitumoral polyketide mithramycin A (MTM) production. The group reported an increase of MTM production from 850 mg/L (wild-type strain) to nearly 3000 mg/L in medium SM17 produced by *S. lividans* RedStrep 1.3 (ΔB ΔC ΔD).

In summary, a collection of *S. lividans* RGS was generated for heterologous expression of actinomycetes biosynthetic gene clusters. The collection was proven to be suitable for increased heterologous gene expression according to the first experiments performed. However, the characterization of basic microbiological features, such as bacterial growth, and the evaluation of the bacterial secretory machinery emerged as an interesting approach to become acquainted on the effect of the deletions in comparison with the wild type strain well known performance.

4 Justification

The project “STREPSYNTH” aimed to set up a Streptomyces-based new industrial production platform for high value-added biomolecules. As part of the first stage of this project and because *Streptomyces lividans* is a formidable protein secretion factory for heterologous proteins, several gene clusters for secondary metabolites have been deleted from the *S. lividans* TK24 chromosome, resulting in 14 strans with 0.32-1.98% genome reductions (RGS). The effect that such deletions have on cellular growth, the secretory machinery and the native secretome of RGS’s from *S. lividans* compared to that of the wild type strain has not been evaluated.

5 Hypothesis and objectives of this thesis

5.1 Hypothesis

If secondary metabolites are not involved in essential catabolic and anabolic activities, the deletion of their biosynthetic gene clusters, will not diminish and might improve heterologous protein secretion.

5.2 Objectives

Determine the effect of the deletion of secondary metabolites biosynthetic gene clusters on the cellular growth, total secretome and on the secretion of a heterologous protein (mRFP) via SEC pathway in reduced genome strains of *Streptomyces lividans* TK24.

5.2.1 Specific objectives

- Determine the cellular growth performance from reduced genome strains (RGS) of *S. lividans* TK24 compared to the wild type strain well-known pattern.
- Evaluate the amounts of native secreted proteins from reduced genome strains (RGS) of *S. lividans* TK24.
- Identify differences in the protein profiles from the wild type strain compared to the RGS’s.
- Monitor heterologous protein secretion of mRFP in *S. lividans* TK24 wild type strain and RGS at late exponential and stationary phases.

6 Materials and methods

6.1 Buffers

The buffers often used in the laboratory are listed in table 1.

Table 1: Common buffers

Buffer	Components	pH if adjusted
Tris 1.5M pH 8.8	1.5 M Tris powder	8.8
Tris 1M pH 8	1 M Tris powder	8.0
Tris 0.5M pH 6.8	0.5 M Tris powder	6.8
6x SDS sample buffer	350 mM Tris pH 6.8 30% glycerol 10% SDS 0.06% bromophenol blue	
SDS electrophoresis buffer	25 mM Tris 192 mM glycine 0.1% SDS	
Transfer buffer	20% MeOH 0.1% SDS 192 mM glycine 25 mM Tris	
Tris-acetate-EDTA (TAE) buffer	40 mM Tris 20 mM CH_3COOH 1 mM EDTA	
TTBS buffer	20 mM Tris 150 mM NaCl 0.3% Tween-20	7.5
MES buffer	700mM MES	6.9
PTC buffer	103 g sucrose 0.25 g K_2SO_4 2.03 g $MgCl_2$•6H2O 2.94 g $CaCl_2$•$2H_2O$	7.2

	80 mL TES-buffer 2 mL spore element solution.* *Spore element solution: (per L) 40 mg $ZnCl_2$ 200 mg $FeCl_3 \cdot 6H_2O$ 10 mg $CuCl_2 \cdot 2H_2O$ 10 mg $MnCl_2 \cdot 4H_2O$ 10 mg $Na_2B_4O_7 \cdot 10H_2O$ 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

6.2 Antibiotics

The antibiotics used during the thesis are listed in table 2.

Table 2: Antibiotics. Stock and final concentration in the media of antibiotics used during this thesis.

Antibiotic	Stock concentration	Final concentration
Thiostrepton	50mg/mL	Submerged culture: 10ug/mL Plates: 50ug/mL

6.3 Microbiological techniques

6.3.1 Bacterial strains

Strains used in this study were *S. lividans* TK24 as a wild type and 10 reduced genome strains; RGS strains and the biosynthetic cluster deleted in each of them are described in figure 6.3.1.1 (Clusters arranged as presented on figure 3.2-2). All strains carry a high copy number plasmid pIJ486 containing the coding region of the Vsi-signal peptide fused with 5' region of the mRFP gene, under the control of vsi promoter from *Streptomyces venzuelae.*

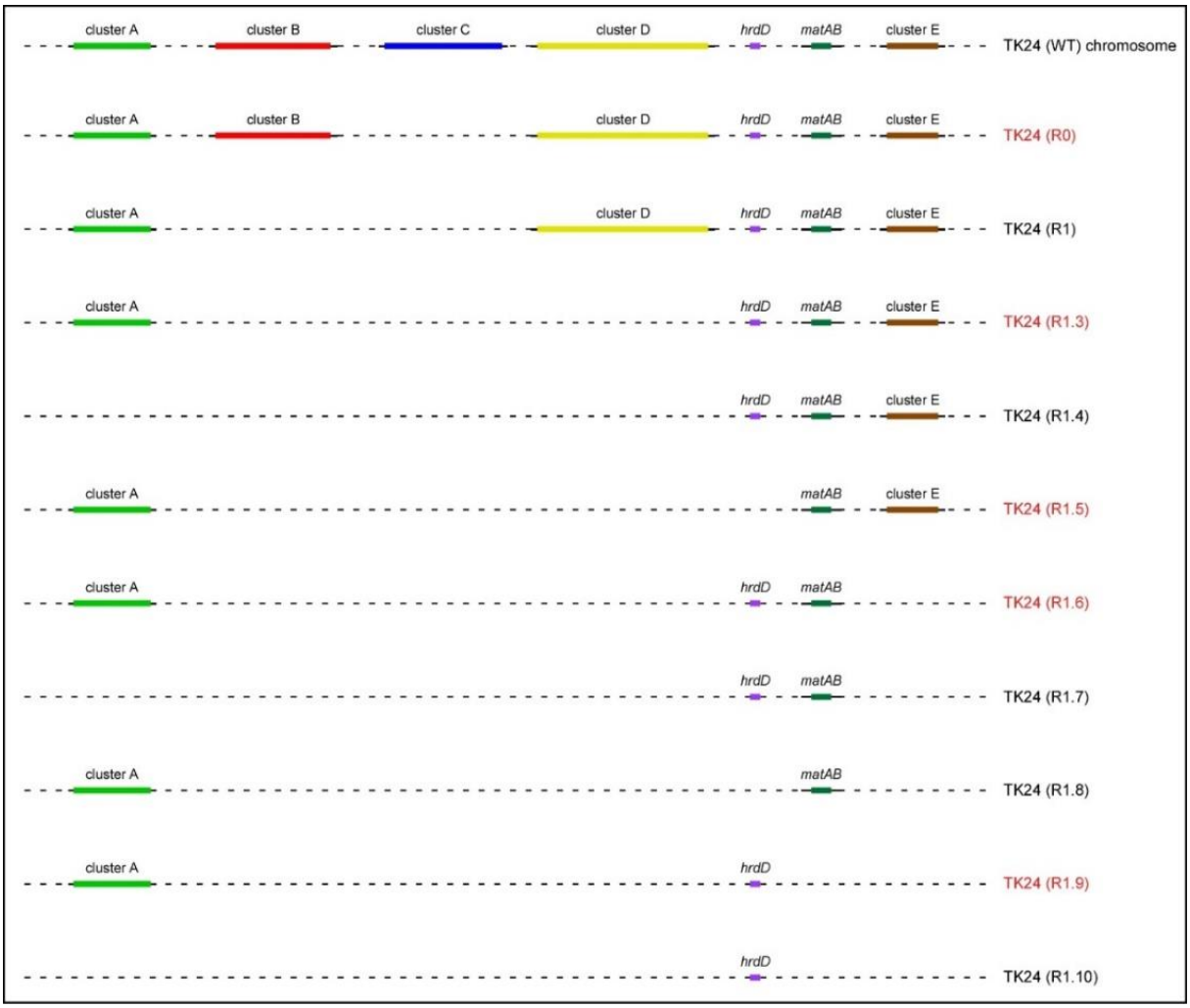

Figure 6.3.1-1: Graphic description from secondary metabolites gene clusters deleted from TK24 (Clusters arranged according to the order at the chromosome presented on figure 3.2-2).

6.3.2 Media

Table 3

Medium	Composition per liter of medium
Minimal Medium (MM)	10 g Glucose, 3 g $(NH_4)_2SO_4$, 2.6 g K_2HPO_4, 1.8 g NaH_2PO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 25 mL minor elements solution (per liter: 40 mg $ZnSO_4 \cdot 7H_2O$, 40 mg $FeSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, 40 mg $MnCl_2 \cdot 4H_2O$)

Nutrient Broth (NB)	8 g NB, pH 6.9 (containing 5 g/L peptic digest of animal tissue, 3 g/L beef extract)
Phage medium (Hamed *et al.*, 2017)	10 g Glucose, 5 g Tryptone, 5 g yeast extract, 5 g Lab Lemco powder, 0.74 g $CaCl_2$•$2H_2O$, 0.5 g $MgSO_4$•$7H_2O$, pH: 7.2
R2	103 g sucrose, 025 g K2SO4, 10.12 g $MgCl_2$•$6H_2O$, 10 g glucose, 0.1 g casaminoacids (Difco), 1 g yeast extract (Difco), 5 g Lab Lemco Powder (Oxoid), + 100 mL TES (pH 7.2, 0.25M) + 2 mL Spore element Sol. (see above) + 10 mL KH_2PO_4 (0.5%) solution. Before use: add 1/100 vol. 36.8% $CaCl_2$•$2H_2O$ solution add 1/1000 vol. 2mM $CuSO_4$ solution.
S	4 g pepton (Difco), 4 g Yeast extract (Difco), 0.5 g $MgSO_4.7H_2O$, 2 g KH_2PO_4, 4 g K_2HPO_4 (or 5.2g K_2HPO_4•$3H_2O$. Before use: add 50 mL sterile glucose solution (10 g glucose in 150 mL H_2O) to 266 mL medium and 0.8% glycine (final concentration)
Tryptic Soy Broth (TSB)	30 g TSB (containing 17 g casein peptone (pancreatic)), 5 g NaCl, 3 g soya peptone (papain digest), 2.5 g K_2HPO_4, 2.5 g Glucose)

6.3.3 Growth conditions

S. lividans TK24 and its derivatives were precultured in 50 mL Phage medium supplemented with thiostrepton (10 µg/mL) if necessary, and grown at 28 °C with

continuous shaking at 240 rpm for 48 h. After growth, the optical density of preculture was measured at 600 nm (OD_{600}) and the mycelia were harvested by centrifugation (3800 x g; 15 min; SIGMA 3-16K centrifuge) and washed twice with sterilized water. After homogenizing the mycelium in 50 mL of sterilized water, the strains were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of MM or NB.

In order to have the same number of mycelia per each inoculum this equation was used (Hamed *et al.*, 2018):

$$\text{Volume of inoculum} = (\text{Final volume of culture} \times 0.25) / OD_{600}$$

The flasks were shaken at 240 rpm and 28 °C for 48-120 hours, pH was controlled using 100 mM MES buffer (pH 6.9) (Hamed *et al.*, 2018).

6.3.4 Bacterial dry cell mass and growth curve determination

To quantify the dry cell weight (DCW), a 10 mL culture sample was taken at the indiated time points. Samples were entrifuged at 3800 x g for 15 min (SIGMA 3-16KL refrigerated centrifuge). The bacterial pellets were harvested, resuspended in sterilized water and vacuum filtered using a 0.2 µm pore size filter (predried and preweighted; PORAFIL® MV; Macherey-Nagel). The filter was dried (overnight 12-24 h in an oven at 60ºC) and DCW determined by weight difference.

6.4 Molecular biology

6.4.1 Protoplasts formation

A preculture from S. lividans in 5 mL rich medium (Phage or NB medium) was grown. Culture was homogenized and inoculated 50 mL S medium (containing glucose and 0.8% glycine) was inoculated with 1-2 mL from preculture and incubated at 30°C/280 rpm for 24 h. Culture was harvested by centrifugation at 5000 rpm for 5 min. Cell pellet was washed with 10-15 ml 0.9% NaCl followed by centrifugation. Second washing step with 10-15 mL PTC buffer. Cell pellet was resuspended in sterile 5.5 mL PTC containing 10 mg/mL lysozyme and incubated at 30°C/120 rpm for 15-30 min. Protoplast formation was confirmed by microscopy and incubation prolonged in lysozyme solution prolonged if necessary. 10 ml PTC buffer was added to the protoplast suspension, homogenized by pipetting, and centrifugated at 800 rpm.

Protoplasts were washed with 10-15 mL PTC buffer and centrifuged. Finally, protoplasts were resuspended in PTC buffer to get an OD ~1.0. and divided in aliquots stored at -20°C or –70°C.

6.4.2 Transformation

The production of monomeric red fluorescence protein (mRFP) in *S. lividans* TK24 and RGS strains were achieved by cloning mRFP (Hamed *et al.*, 2018) encoding gene in a multi-copy plasmid pIJ486 after a strong constitutive promoter and the Sec-dependent signal peptide of *Streptomyces venezuelae* subtilisin inhibitor (vsi) to form pIJ486-sp^{Sec}-mRFP.

Protoplasts were thawed in warm water bath. Plasmid DNA was added to 200 µL from protoplast suspension per transformation and mixed by pipetting. 500 µL 35% PEG6000 solution (Filter sterilized solution of 35% PEG6000 in PTC buffer, PEG6000 > NBS Biologicals) was added and homogenized by pipetting. After 5 min at RT transformed protoplasts were plated on non-selective regeneration medium R2 with antibiotic. Plates were incubated at 30°C for 24h for regeneration of protoplast.

6.5 Protein quantification

6.5.1 TCA precipitation

In parallel to the procedure described in section 6.3.4, where the cell pellet was taken for DCW cuantification, proteins in the supernatant were precipitated using 20 % trichloroacetic acid (TCA) for 30 min at 4°C, followed by two acetone washes for 15 min at -20°C. After each step a centrifugation was performed for 30 min at 20,000 g at 4°C. The pellet was resuspended in a small amount of Tris buffer with basic pH (8.8).

6.5.2 Bradford protein assay

Concentrate Biorad protein assay dye reagent was diluted 1:5 in dH_2O and aliquoted per 1 mL. The volume of protein solution with unknown concentration added to aliquots of Bradford reagent could vary between 1 and 20 µL. OD was measured at 595 nm with microplate reader (TECAN, software i.control 1.7). Standard curve was acquired by plotting absorbance of BSA standards (0, 2, 5, 10, 15 and 20 µg) against their

concentration using Microsoft Office Excel software. The concentration of other test samples was derived from this curve.

6.5.3 BCA protein assay

Pierce™ BCA Protein Assay Kit was used to quantify protein samples. 50 volumes of reagent “A” were mixed with 1 volume of reagent “B” and aliquoted per 1 mL. The volume of protein solution with unknown concentration added to aliquots could vary between 1 and 20 µl. OD was measured at 595 nm with microplate reader (TECAN, software i.control 1.7). Standard curve was acquired by plotting absorbance of BSA standards (0, 2, 5, 10, 15 and 20 µg) against their concentration using Microsoft Office Excel software. The concentration of test samples derived from this curve.

6.5.4 Fluorescence assays and quantification of fluorescent proteins

Fluorescence measurements were carried out in an Infinite® M200 microplate reader (Tecan) (mRFP: excitation at 550 nm/emission at 580 nm). To compare strain performance, mRFP fluorescence intensities, obtained at the transition to stationary phases were measured. Error propagation was applied to calculate DCW-specific mRFP production.

The quantification of mRFP was carried out by cloning mRFP as His-tagged versions in pIMBB316. Plasmid was transformed in E. coli strain BL21 followed by purification of His-mRFP by metal affinity chromatography (not shown). Calibration curves of both fluorescence measurements and protein mass were generated. The amounts of mRFP detected via fluorescence assays were compared with the amounts quantified via western blotting.

6.6 Protein techniques

6.6.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Extracellular protein fractions of cultures of *S. lividans* TK24 (WT and RGS) were obtained after centrifugation (10 min, 4200 x g, 4°C) to remove biomassSupernatant protein precipitation was carried out with trichloroacetic acid (TCA) (final concentration of 20% w/v; 4°C) where applicable. Proteins were separated by 12 % SDS–PAGE and

the precision Plus ProteinTM Standard (All Blue) from Bio-Rad was used. Proteins were visualized by immuno blotting with rabbit polyclonal antibodies against secreted proteins or by silver staining.

6.6.2 Silver staining

SDS-PAGE gels were fixed for 1h (fixing solution: 10% Acetic Acid (HOAc+ 40% Methanol); after rinsing the fixed gels with d.H_2O, the silver staining was performed as follows: the gel was then incubated in $CuCl_2$ solution for 15min (5mL of 20% $CuCl_2$ + 95mL d.H_2O), rinsed with d.H_2O, washed twice for 15 min (10% Ethanol + 5% $HOAc_9$), incubated in $KMnO_4$ solution for 15 min (100mL solution: 0.4mL of 1%$KMnO_4$ + 99.6mL d.H_2O), washed twice for 15 min or longer, incubated in silver solution for 15-20 min (0.1% $AgNO_3$ (100mL solution: 0.5mL 20%$AgNO_3$ +99.5mL d.H_2O), and rinsed under running d.H_2O for ~45sec. Finally, the staining was developed by adding 300mL a solution containing 6g K_2CO_3+ 60uL 37% Formaldehyde in d.H_2O for ~1min, and the reaction was stopped when bands reached required color intensity by adding a solution of 5% acetic acid in water.

6.6.3 Western blot

After SDS-PAGE, proteins were transferred from the resulting gel to a nitrocellulose blotting membrane using a semidry transfer system with transferring buffer for 40 min at 20 V.

The blot was blocked in 5% milk solution in TTBS, on a shaker for at least 30 min. It was then washed with TTBS 3 times for 5 min each.

The blot was incubated with primary antibody solution for 1 h or ON at 4°C on a shaker. The membrane was then washed with TTBS 3 times for 5 min each, followed by Incubation with secondary antibody solution for an hour on a shaker (both antibodies were diluted 1/50,000 in 1% milk in TTBS buffer). It was then washed with TTBS 3 times for 5 min each.

The blot was covered with ECL mix and incubated for 1 min. The chemiluminescence was imaged using CCD camera (LAS 3000, FujiFilm) for 20 minutes.

*mRFP antibodies were obtained from Immunosource or generated against lab-purified proteins at Davids Biotechnologie, Germany.

6.7 Miscellanea

APS was supplied by Serva (Germany). Most daily materials were supplied by Greiner Bio-One (Germany), like falcon tubes, 24-well plates and other. Chemicals were from Sigma. Bacto Soytone from DIFCO Laboratories.

6.8 Laboratory Instruments

Table 4: Laboratory Instruments

Name	Company	Use
Stuart 3D gyro-rocker SSL3	Cole-Parmer, UK	Shaker
Memmert IN55	Memmert GmbH, Schwabach	Incubation (30°C)
HP Scanjet G4010 Photo Scanner	HP, USA	Scanning coomassie stained gels
ImageQuant LAS 4000 Fujifilm™ CCD camera	GE Healthcare Life Sciences, USA Fujifilm, Japan	Western blot visualization
Laboratory centrifuge model Sigma 3-16 KL, with cooling	SciQuip, UK	Falcon centrifuge
MDF-U55V-PE Ultra Low Temperature Freezer	Panasonic, Japan	Storage
Mini-PROTEAN II electrophoresis cell	Bio-Rad, Germany	Tank for SDS-PAGE
		Laminar flow hood
PowerPac™ 300	Bio-Rad, Germany	Power supply
PowerPac™ Basic	Bio-Rad, Germany	Power supply
Sigma 1-16k refrigerated laboratory centrifuge	SciQuip, UK	Microcentrifuge tube centrifuge
Sigma 1-14 microcentrifuge	SciQuip, UK	Microcentrifuge tube centrifuge (RT)

Dry bath HB-48 one block	Witeg, Germany	Heat block
Infinite®-M200	Tecan Group Ltd., Switzerland	Micloplate reader
Mettler AJ100	Mettler-Toledo GmbH, Germany	Balance
Vacuubrand MZ 2C NT	VACUUBRAND GMBH, Germany	Pump (Membranes, TCA)

7 Results and discussion

7.1 RGS growth and native secretion

The first step towards RGS characterization was growth evaluation, measured as grams of dry cell weight (DCW); all strains were cultured in nutrient broth (NB) medium that has been previously described as the best alternative for native and heterologous proteins production (Hamed *et al.*, 2017; Hamed *et al.*, 2018; Tsolis *et al.*, 2018).

Mycelial organisms can grow as dispersed hyphal fragments or as pellets when they are cultured in liquid media (Stanbury *et al.*, 2017), and it has significant influence on the final products. Growth in pellets will be exponential until the density of the pellet results in a diffusion-limited environment at the center of the pellet that causes poor nutrients supply at the central biomass and failure to diffuse out potentially toxic products.

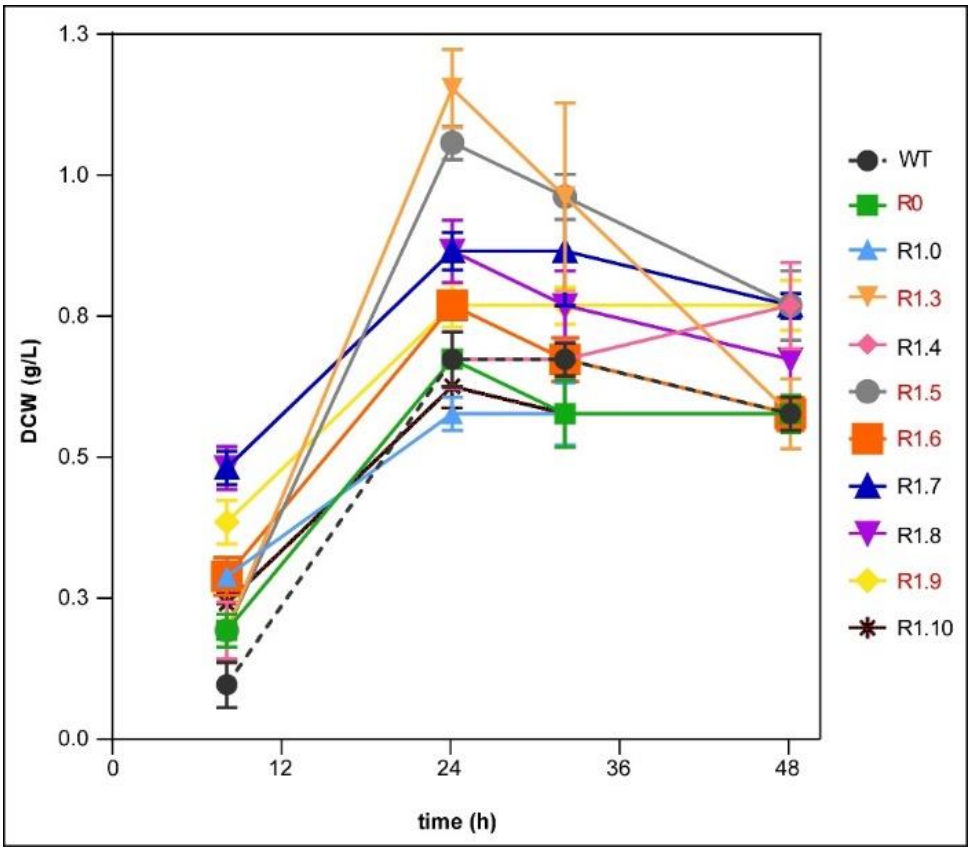

Figure 7.1-1: Comparison of cell growth from TK24 wild type (WT) and RGS in nutrient broth (NB) expressed as values of dry cell weight (DCW) (g/L). n = 3, values represent the mean ± SD (strains used for heterologous secretion tests shown in red). 8 hours taken as the first time point after standardized inoculation based on the growth at OD_{600} (Specified in methodology section 5.3.3)

Although DCW was determined only at 4 discrete times for each strain, it is visible that the wild type strain [dotted black line] shows three main stages of bacterial growth, as shown in figure 7.1-1,: exponential phase (0-24h), stationary phase (24-32h) and dead phase (32-48h); also, in all cases the highest biomass value was reached at 24 hours. This fact allowed us, for this work purposes, to define this time point as the late exponential phase.

For the experiments performed in this thesis, pellet formation was avoided by culturing all the strains under continuous agitation, and for some of the tested strains (R1.9 and R1.10) the genome reduction included deletion of the gene for aggregation (*matAB*). For that reason, the end of the stationary phase was considered as an important time point to be monitored.

The parental RGS [R0, green line] showed a different growth pattern than the wild type, as it presents a dead phase (a decline in biomass) between 24 and 32 h and then enters to the stationary phase, maintaining its biomass at 0.6±0.1 g/L for the rest of the evaluated time. The growth pattern of all others RGS were classified into wild type strain pattern or parental strain pattern. Strains R1.0, 1.4, R1.7 and R1.9 follow the wild type strain growth behavior with no appreciable dead phase before entering the stationary phase, although for R1.0 and R1.9 the cellular total biomass remains constant for the rest of the evaluated time whereas R1.4 increases after 32 hours of culture. Strains R1.3, R1.5, R1.6, R1.8 and R1.10, on the other hand, showed a parental strain pattern, although just R.1.10 overcome the dead phase, and resume its growth for the rest of the evaluated time. Growth stoppage may be due to either substrate limitation, the accumulation of auto-toxic products in the medium or a combination of the two factors. From the previously described growth patterns, it was noticed that at 48 hours of growth the strains were entering or finishing the stationary phase. Considering this last fact and the need of avoiding cell lysis from later time, 48 hours were defined as the time point for the stationary phase.

In all organisms, unicellular or mycelial, growth results in the consumption of nutrients and the excretion of microbial products. Both events influence the growth of the organism since, after a certain time, the growth rate decreases until growth ceases (dead phase). The mild differences observed regarding RGS cellular growth might be explained by the fact that stationary phase cells are not simply exponential phase cells

that have stopped growing, they are physiologically different cells (Stanbury *et al.*, 2017). Probably because different expression profiles of housekeeping genes, sigma factors deployment and quorum sensing responses, that together enable the cells to resume growth after the organism is adapted to its environment. The organism's adaptation to change its metabolism by changing gene expression is reinforced by the phenomenon of secondary metabolite biosynthesis.

After growth characterization, the second step was to study the effect of deleted gene clusters on the absolute amounts of total secreted proteins at late exponential (24h) and stationary phases (48h). This was done to determine if the protein secretion is affected (higher, lower or equivalent) in RGS in comparison to *S. lividans* TK24. Figure 7.1-2 shows total secreted proteins in volumetric units [A] (mg of protein/L of medium) to indicate the protein production of each strain in the media, and in specific units [B] (mg of protein/g of DCW) to evaluate each strain performance regardless of biomass production, since there is no direct correlation between biomass production and protein secretion.

According to the statistical analysis (data not shown), R1.3 is the strain with a significant difference (α=0.05) in the production of native total secretome at the late exponential phase (24h), with around one third of the protein obtained with the wild type strain (10.09±2.0477 and 33.60±1.99 mg/gDCW respectively). For the stationary phase (48 h), four strains presented significant difference in its native total secretome when compared with the wild type strain: R1.5 (25.78±2.29 mg/gDCW), R1.8 (23.85±11.86 mg/gDCW), R1.9 (24.41±6.54 mg/gDCW) and R1.10 (7.63±1.44 mg/gDCW), WT (52.94±9.01 mg/gDCW).

These data suggest that cluster deletion does not affect the secretion rate of proteins within 24 hours of fermentation in the RGSs. However, after 48 hours, genome edition may have a more significant detrimental effect. Two questions arise at this point: why is the wild type strain secreting a higher amount of total protein? Is it possible that regulation of protein export gene was deleted?

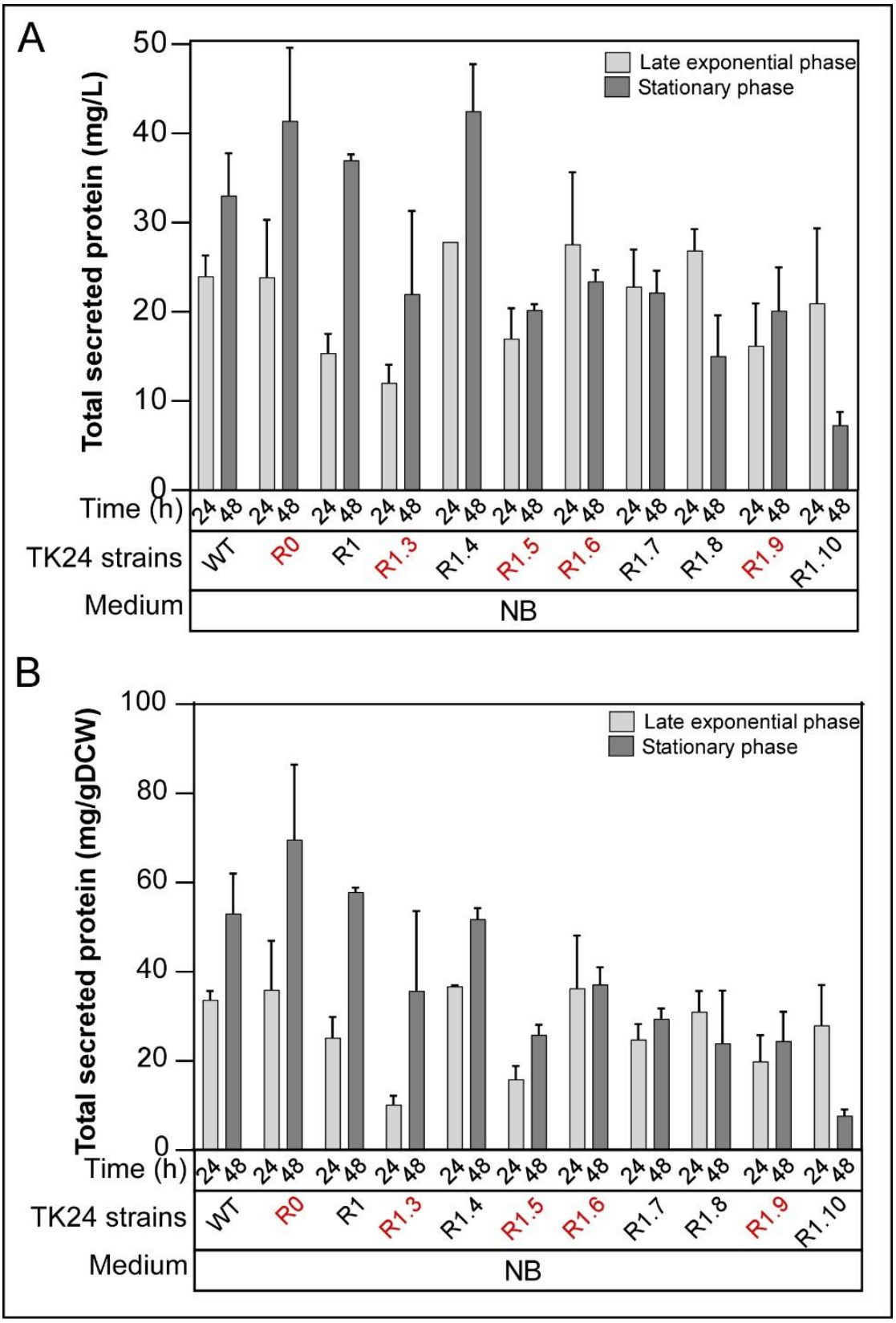

Figure 7.1-2: Amounts of secreted native proteins by RGS in volumetric (A) [mg/L] and specific (B) [mg/gDCW] units in nutrient broth (NB) at late exponential (24h) and stationary phase (48h). n = 3, values represent the mean ± SD (strains used for heterologous secretion tests shown in red).

For the first question, it is necessary to clarify that the production of lower amounts of total proteins does not necessarily indicate the RGS will have lower production of heterologous proteins. It is possible that with the reduction of its genome, the bacteria will be able to employ its stationary phase metabolism to express and secrete the heterologous protein, resulting in lower amount of total secreted protein but higher proportion of the heterologous protein; therefore, production could be enhanced as suggests one of the main supportive arguments for reduced genome microorganisms. However, as explained later, the results obtained indicate that in fact, the deletion might have affected the heterologous protein production. For the second question, a concrete answer cannot be given. It is known that the selected gene clusters include some regulating proteins, but its specific function has not yet been defined.

Next, the total secretome polypeptides of TK24 and its mutants, were harvested and loaded on SDS-PAGE (Fig. 7.1-3). The protein patterns showed clear differences between the strains compared with the wild type, as some proteins increased in intensity, some remained the same and others were lost (all marked with arrows on the right side of the figure). A very clear example of variation in the general profile are in strains R1.3 and R1.5 in comparison with all the other strains: the last band, below 14.4 kDa (most probably corresponding to the lividans subtilisin inhibitor (lsi)) is decreased in intensity. The bands (not identified) at 35.0 kDa, above 66.2 kDa and 116.0 kDa disappeared. Finally, a new group of bands (not identified) are seen below 35.0 kDa. A sequence analysis was not performed, and the previous description has been made only by gel observation. Non regarding the intensity, a common featu re between all profiles was the previously mentioned lsi band. Subtilisin inhibitor-like proteins group are encoded in a constitutive gene highly transcribed (Brawner *et al.*, 1991, Taguchi *et al.*, 1993) and can be taken as a reference for the correct function of the secretory machinery (Lammertyn *et al.*, 1997).

Theoretically, differences in the native total secretome were not expected, specially at the late exponential phase. It is possible that once late exponential phase is reached and during stationary phase, secondary metabolism begins to regulate the maintenance and well cellular functioning. Secondary metabolism may have evolved as an alternative strategy to switch off metabolic pathways of the primary metabolism by producing secondary metabolites that can act as metabolic regulators (Malik 1980 toooo old reference); in addition, secondary metabolism provide a mechanism whereby

accumulated intermediates and end products of primary metabolism are removed from cells and also its ability to regulate and integrate the cellular metabolism. Since the native secretome starts showing changes from 24 hours of growth, it is possible that the secondary metabolism is already acting and could explain differences in protein profiles observed by SDS-PAGE

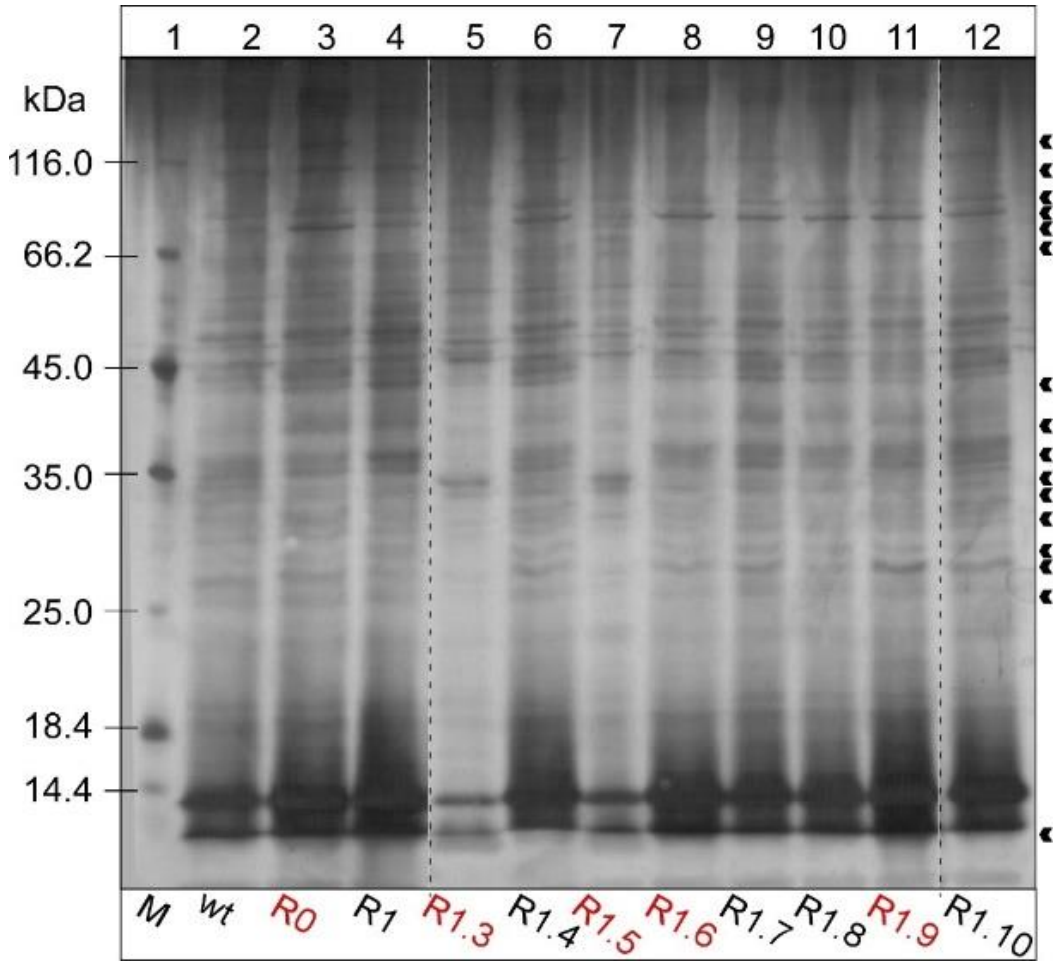

Figure 7.1-3: Native secretomes from RGS (strains used for heterologous secretion tests shown in red). Arrows indicate the presence, absence or intensity changes in bands from RGS with respect to the WT profile. Polypeptides loaded from culture supernatants (0.9–16 mL/lane) that are equivalent to 0.2 mg of dry cell weight from TK24 analyzed by SDS–PAGE and silver-staining. Lane 1, molecular weight markers.

7.2 Testing heterologous secretion

To test the effect of secondary metabolite gene clusters deletion on the expression and secretion of heterologous proteins, the monomeric red fluorescence protein (mRFP) was used as a test heterologous protein (Hamed *et al.*, 2018). The ORF of mRFP gene was fused to the 3' end of the *vsi*-encoded signal sequence (*sp*^{SecV}*-mRFP*) and cloned downstream the *vsi (*Subtilisin inhibitor from *Streptomyces venezuelae)* promoter into the high copy number plasmid pIJ486; the resulting plasmid was called pIJ486-*sp*^{SecV}*-mRFP* (Hamed *et al.*, 2018). The mRFP carrying plasmid (Fig. 7.2-1) was transformed to TK24 and its derivatives via protoplast transformation. In table 5 the strains where the plasmid transformation was successful are shown. 3 to 4 transformed colonies were selected to test the heterologous expression of the mRFP.

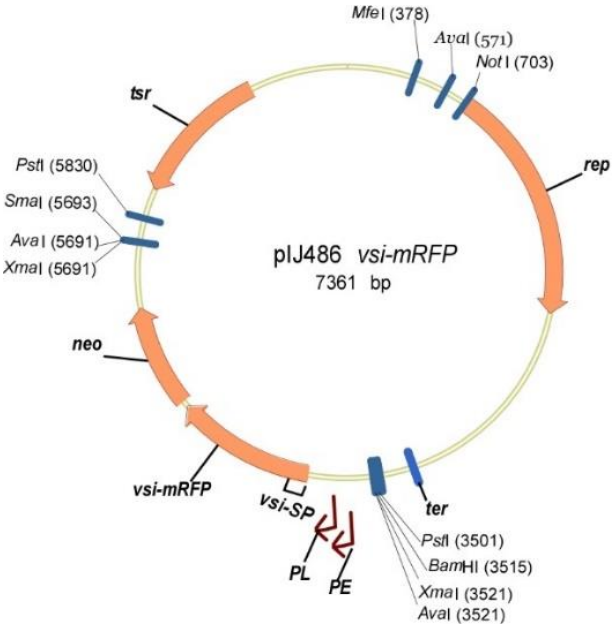

Figure 7.2-1: pIJ486-*sp*^{SecV}*-mRFP* plasmid map.

Strain	mRFP transformation
R0.0	✓ 3 colonies
R1.0	-
R1.3	✓ 4 colonies
R1.4	-
R1.5	✓ 4 colonies
R1.6	✓ 3 colonies
R1.7	-
R1.8	-
R1.9	✓ 3 colonies
R1.10	-

Table 5: Successfully transformed RGS.

To quantify the secreted mRFP, a His-tagged version was cloned in pIMBB643, transformed in *E. coli* BL21, and purified by immobilized metal affinity chromatography (IMAC). Calibration curves of fluorescence intensity as a function of absolute amounts of protein were generated (data not shown).

7.2.1 mRFP in *S. lividans* TK24 wild type strain

The first series of experiments that involved mRFP was performed ~~only on~~ using the wild type strain in order to have an experimental reference for expression, secretion, intracellular accumulation and detection levels of the heterologous protein. As reported by Hamed *et al.* in 2018, SP^{SecV}-mRFP is secreted through the Sec pathway and becomes extracellularly folded. Its secretion could clearly be detected in the culture supernatant since growing cells turned the media red after a couple of days (pictures in APENDIX II).

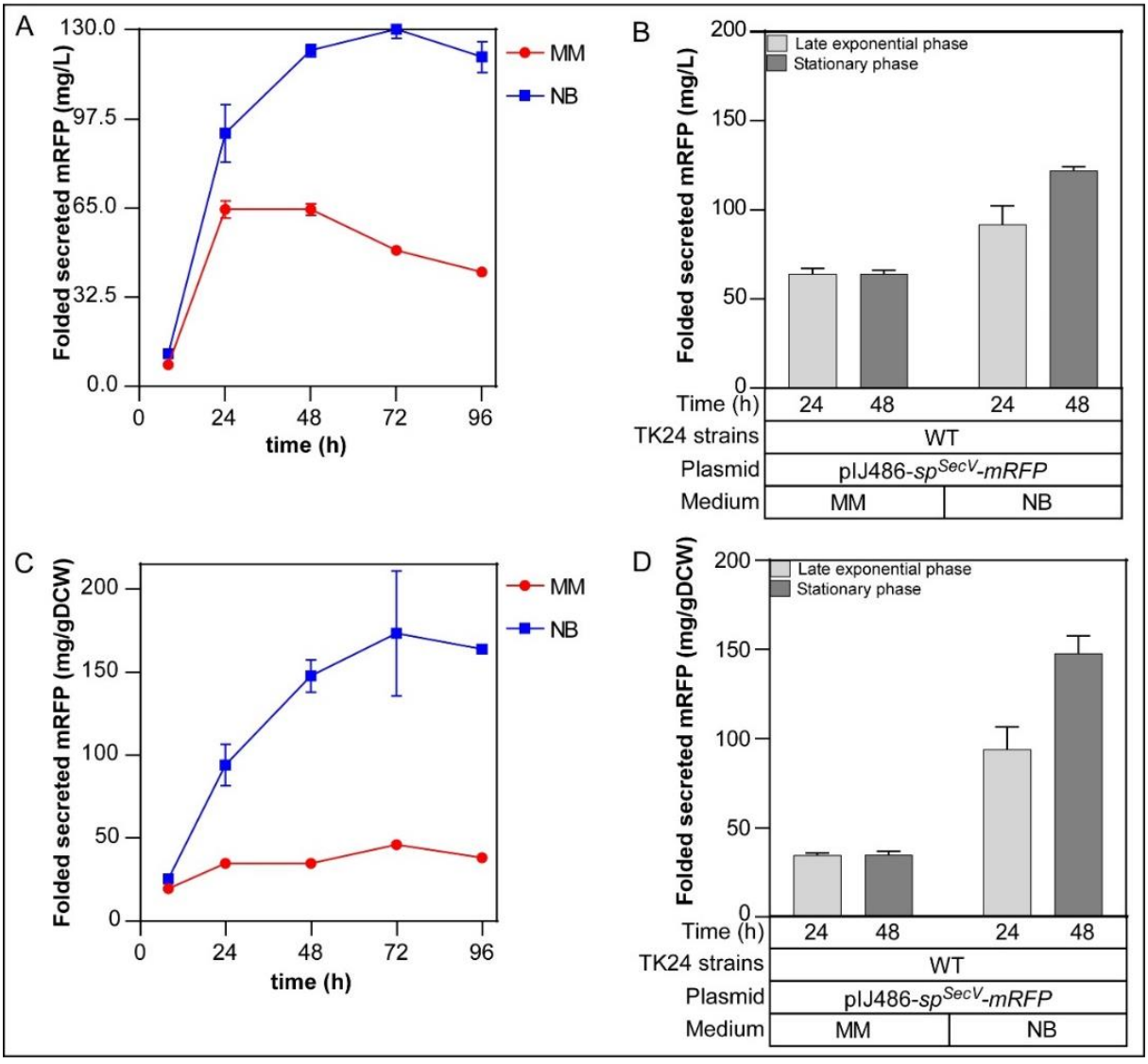

Figure 7.2.1-1: Secreted folded mRFP expressed in volumetric [mg/L] (A, B) and specific [mg/gDCW] (C, D) units secreted by *S. lividans* TK24 wild type (WT) strain carrying pIJ486-sp^{SecV}-*mRFP* in minimal medium (MM) and nutrient broth (NB) for the indicated time related to its growth curves in the same media. n = 3, values represent the mean ± SD.

The mRFP amounts were detected by fluorescence measurements (Fig. 7.2.1-1) and the experiment revealed that, for both tested media (MM as the cleanest, NB as the best media for heterologous expression), the amounts of secreted mRFP increased through time. For MM at 72h of growth the maximum specific production reported is 45.99±0.61 mg/gDCW (equivalent to 49.17 ±1.64mg/L). These amounts are almost four times lower than the maximum specific production reported for NB at 72h of growth: 173.30±37.75 mg/gDCW (equivalent to 129.74±3.10 mg/L). These values are among the highest levels of Sec secretion for heterologous proteins known for *S. lividans* (Vrancken and Anné, 2009; Kashiwagi *et al.*, 2017; Hamed *et al.*, 2018).

Figures 7.2.1-1 B and D show the mRFP amounts for the previously selected time points (late exponential and stationary phases). In the following sections, mRFP expression in RGS will be the tested at those time points (section 7.1). TK24 native secretome and the effect of mRFP expression and secretion in MM and NB was analyzed by SDS-PAGE. The secretome profile showed changes in both media. mRFP secretion (indicated with filled arrow) gave rise to several different proteins (indicated with stars) simultaneously depleted others (indicated with empty arrows) (Fig. 7.2.1-2).

The mRFP band intensity concurs with the amounts detected by fluorescence which indicate the secreted mRFP increases through time. The band identified as potentially belong to the subtilisin inhibitor (below 14 kDa) appears to be present, although low in intensity for both strains in MM. As previously mentioned, this band can be taken as a reference to evaluate the proper function of the bacterial secretory machinery.

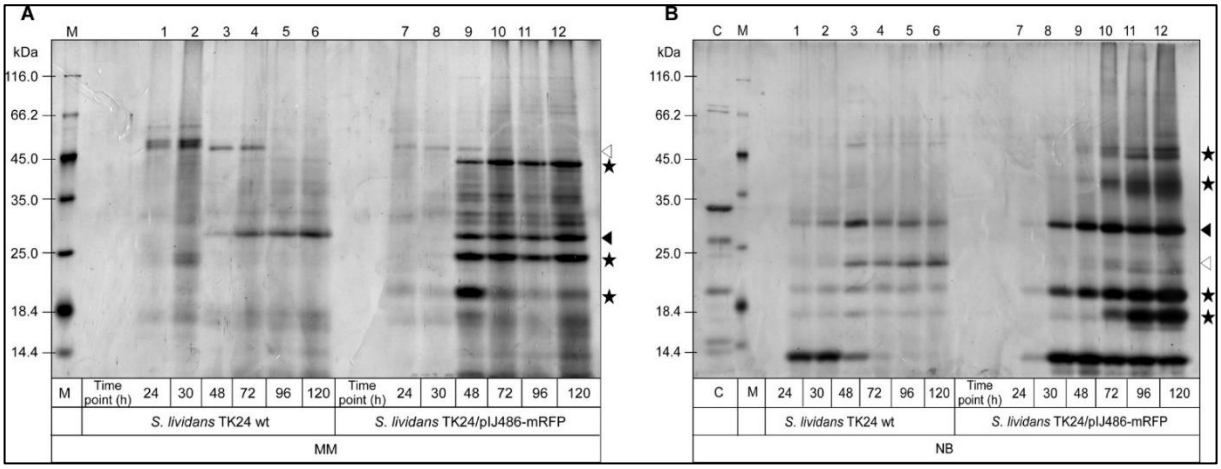

Figure 7.2.1-2: mRFP secreted by *S. lividans* TK24/pIJ486-*sp*^{SecV}-*mRFP* grown for the indicated times in minimal medium (MM) (A) and nutrient broth (NB) (B). Polypeptides (1-7 µg/lane) from culture supernatants (0.9-16 µL/lane) that are equivalent to 0.2 mg of dry cell weight, analyzed by SDS-PAGE and silver-staining. Filled arrow: secreted mRFP; empty arrow: secretome polypeptides with reduced abundance; asterisks: secretome polypeptides with increased abundance. Molecular weight marker as in figure 7.1-3.

The third experiment from this group of assays involved cytoplasmic contents of TK24/pIJ486-*sp*^{SecV}-*mRFP*. Significant amounts of cytoplasmic protein were observed which were uncorrelated to final secretion yields. The intracellular amount of folded mRFP (measured as mRFP fluorescence) was determined in the two media (Fig. 7.2.1-3) and it was found that in MM it increases with time while for NB a major accumulation amount is reached at 24 hours and after this time period the intracellular mRFP amount decreases, such behavior is complementary with the one observed in figure 7.2.1-1. Folded mRFP levels are three times higher in MM (3.09±0.072 mg/gDCW equivalent to 3.05±0.60mg/L) than in NB (1.08±0.27 mg/gDCW equivalent to 1.15±0.31 mg/L)

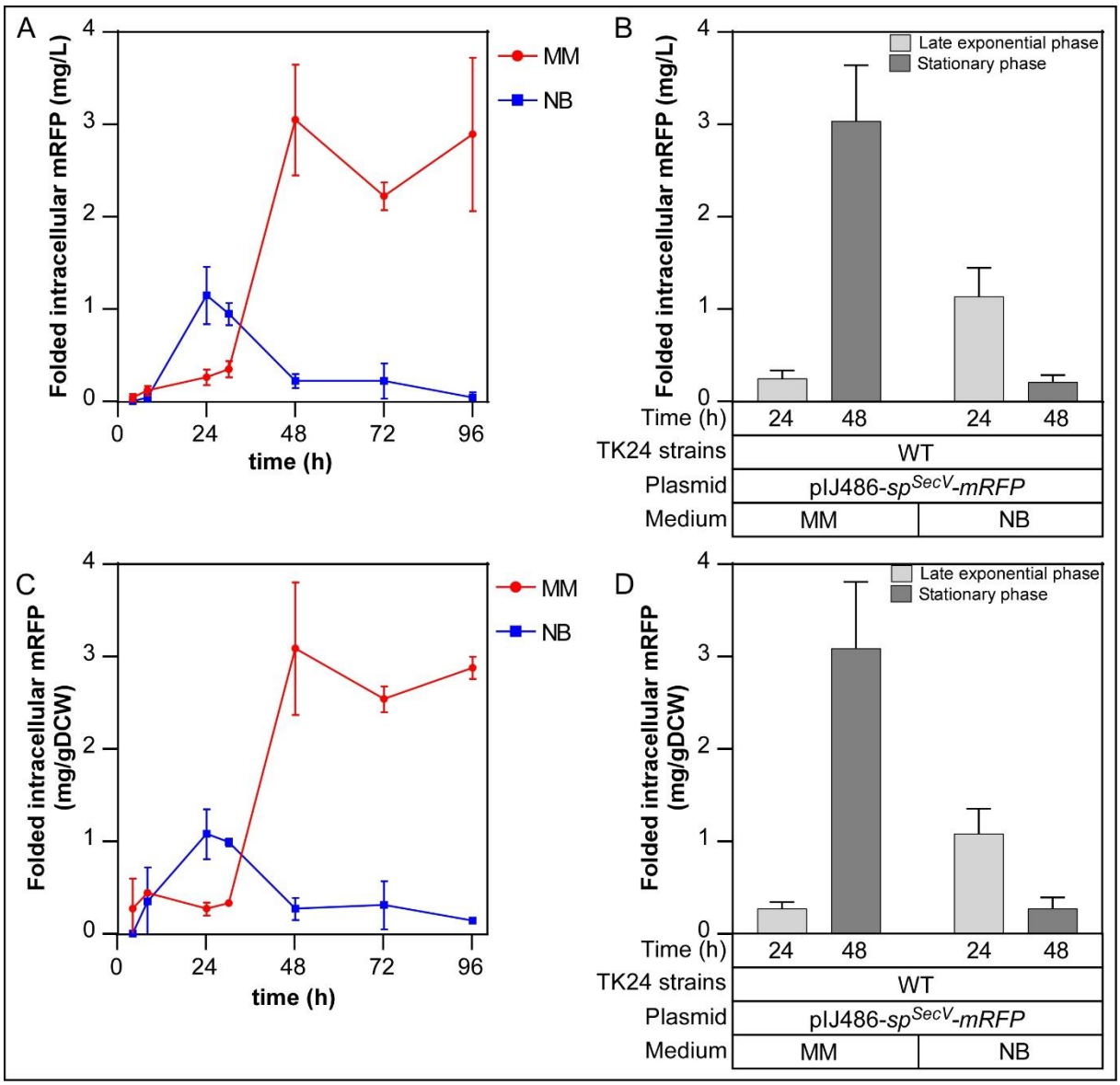

Figure 7.2.1-3: Intracellular (accumulated) mRFP expressed in volumetric [mg/L] (A, B) and specific [mg/gDCW] (C, D) units produced by *S. lividans* TK24 wild type (WT) strain carrying pIJ486-*sp*^{SecV}-*mRFP* in minimal medium (MM) and nutrient broth (NB) for the indicated time related to its growth curves in the same media. n = 3, values represent the mean ± SD.

which suggests MM maybe promoting the intracellular accumulation of the heterologous protein or/by impeding secretion.

Finally, total amounts of intracytoplasmic mRFP were determined by western blot (immuno-detection for mRFP). Antibodies allowed us to monitor folded and non-folded (i.e., non-fluorescent) cytoplasmic states of mRFP and three forms of mRFP were identified: a) A premature mRFP, with the signal peptide still attached [Figure 7.2.1-4, lanes 6 to 14, black circle], b) The second form is the mature folded mRFP [Figure 7.2.1-4, lanes 6 to 14, black arrow], that showed major accumulation in all media, c) third form is the early degraded mRFP state most probably by proteolytic activity.

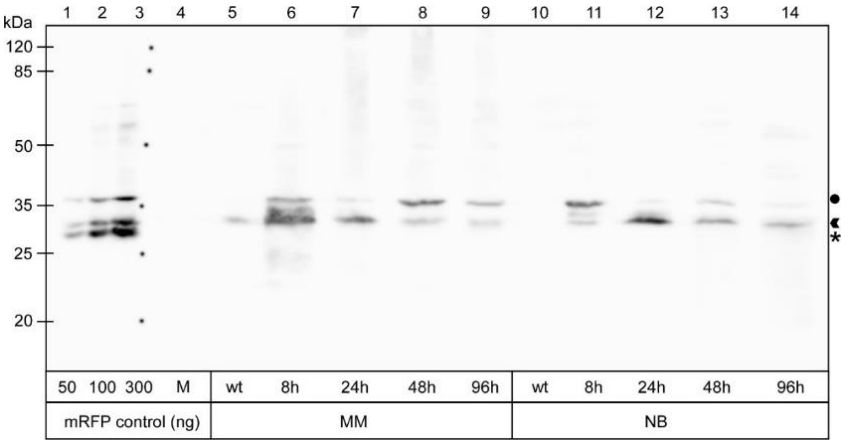

Figure 7.2.1-4: Western blot analysis for intracellular mRFP in TK24 grown in minimal medium (MM) and nutrient broth (NB). Total cell lysates loaded equivalent to 1mg of dry cell mass of TK24 carrying pIJ486-*sp*^{SecV}-*mRFP* grown for the indicated times in the indicated media, washed twice and loaded on 12% SDS-PAGE and visualized using mRFP-antibodies. Circle: mRFP stabilized preform state with its signal peptide attached; filled arrow: mature folded mRFP; asterisk: first stage of mRFP

By comparing both secreted and intracellular mRFP it can be inferred that *sp*^{SecV}-*mRFP* is synthesized at high levels in both MM and NB media. In NB most of the synthesized mRFP is secreted while a small amount remains folded intracellularly and is proteolytically degraded. On the other hand, in MM only ~30 % of the produced mRFP is secreted and the rest remains inside the cell, this suggests that the export capacity of the cell might be saturated or other mechanisms prevent its secretion. These results are similar to those previously described by Hamed *et al.* in 2018.

7.2.2 mRFP in *S.lividans* TK24 RGS

The last series of experiments comprises the assays for mRFP expression and secretion by RGS. Successful transformation of the plasmid pIJ486-*sp*^{SecV}-*mRFP* was done in R0, R1.3, R1.5, R1.6 and R1.9 strains. The following results cover the data generated by the best selected clone per strain.

The detected amounts of secreted folded mRFP, as shown in figure 7.2.2-1, were considerably lower in all RGS evaluated cases than that observed with the wild type strain as shown in graphs A and C. Graphs B and D illustrate the same results without data from TK24 wild type strain. It was observed that strain R1.9 together with R1.5 were the strains with the “highest” amount, at 24 hours of growth, of secreted mRFP [9.235 ± 0.447 mg/gDCW equivalent to1.478 ± 0.072 mg/L for R1.9 and 2.441 ± 0.050 mg/gDCW equivalent to 1.196 ± 0.001 mg/L for R1.5 respectively]. These values are seven to twenty-five times lower than the reported for the wild type strain [61.551 ± 19.101 mg/gDCW equivalent to 33.783 ± 18.582 mg/L]. As mentioned earlier, the indicated values correspond to the late exponential phase, which in contrast with the wild type model, was not expected to be the phase for the highest production of the

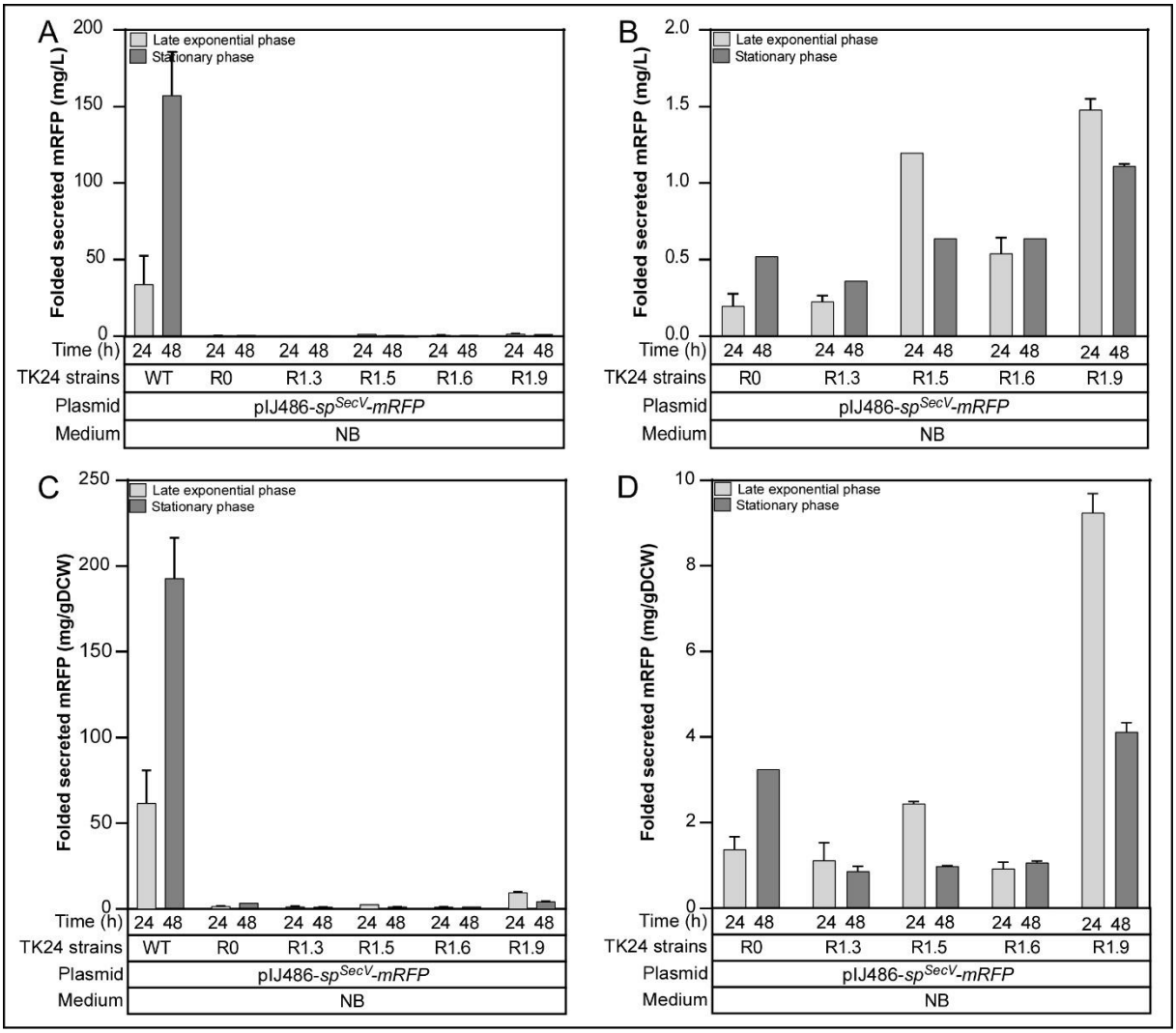

Figure 7.2.2-1: Folded secreted mRFP expressed in volumetric [mg/L] (A, B) and specific [mg/gDCW] (C, D) units secreted by RGS carrying pIJ486-*sp*^{SecV}-*mRFP* in nutrient broth (NB) for the indicated time related to its growth curves in the same media. n = 3, values represent the mean ± SD.

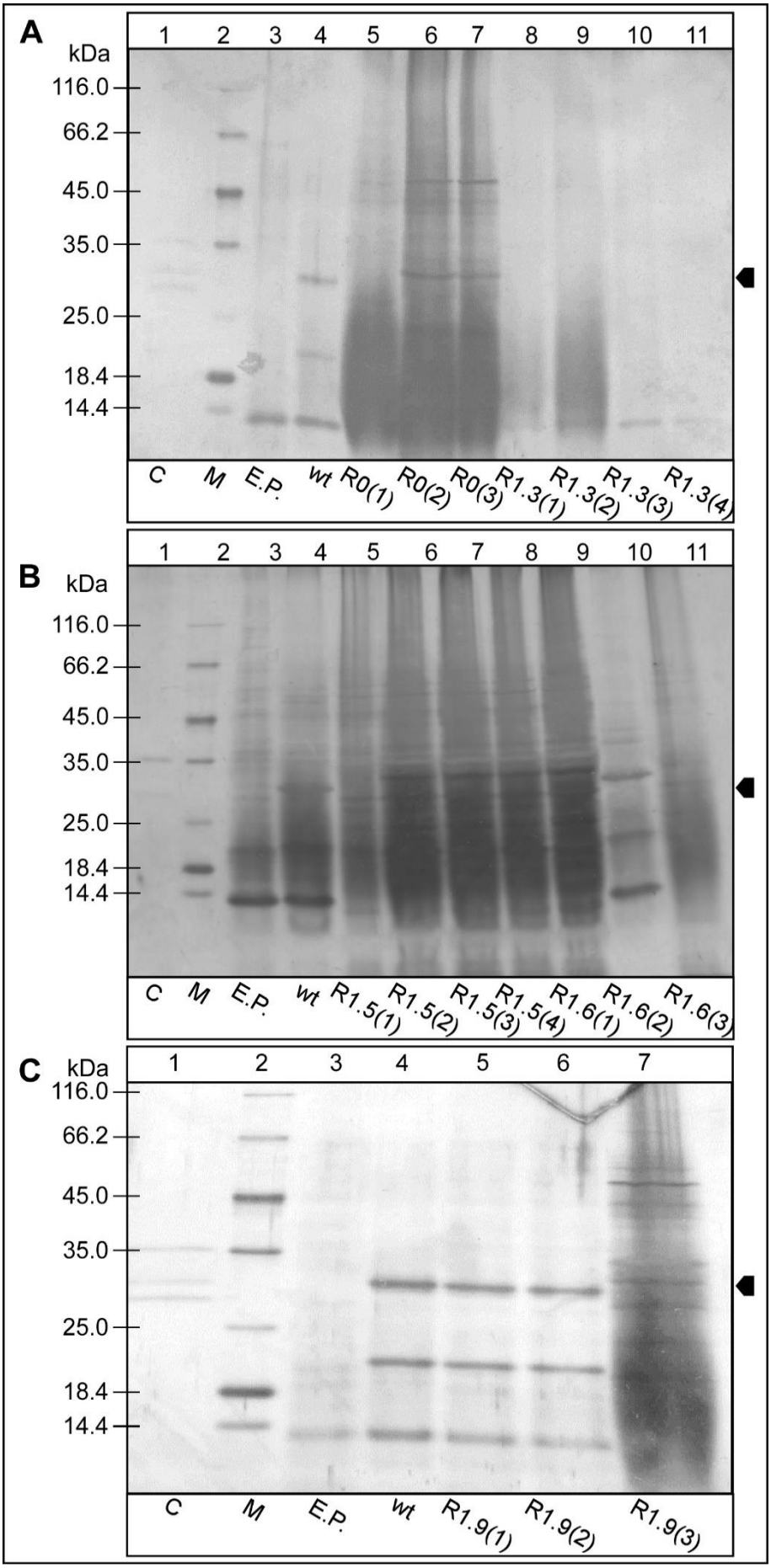

Figure 7.2..2-2: mRFP secreted by *S. lividans* TK24 RGS carrying plasmid pIJ486-*sp*^{SecV}-*mRFP*. Grown for 24h in nutrient broth media. Polypeptides equivalent to 0.1 mg of dry cell weight, analyzed by SDS-PAGE and silver-staining.

A) Tested colonies for strains R0 and R1.3.

B) Tested colonies for strains R1.5 and R1.6.

C) Tested colonies for strain R1.9.

Filled arrow indicates secreted mRFP. Purified mRFP control (50ng) in lane 1. Molecular weight marker in lane 2 as in figure 7.1-3.

heterologous protein. This behavior was observed in all cases but the parental strain (R0). The electrophoretic analysis (Figure 7.2.2-2) showed that not all the transformed colonies were able to produce the heterologous protein. For strain R1.3 (gel A, lanes 8-11) none of the four tested colonies (same clone) was able to secrete any amount of mRFP detectable by fluorescence. Changes on the native secretome are not visible as many bands were lost even for the wild type strain lane (lane 4 for all gels).

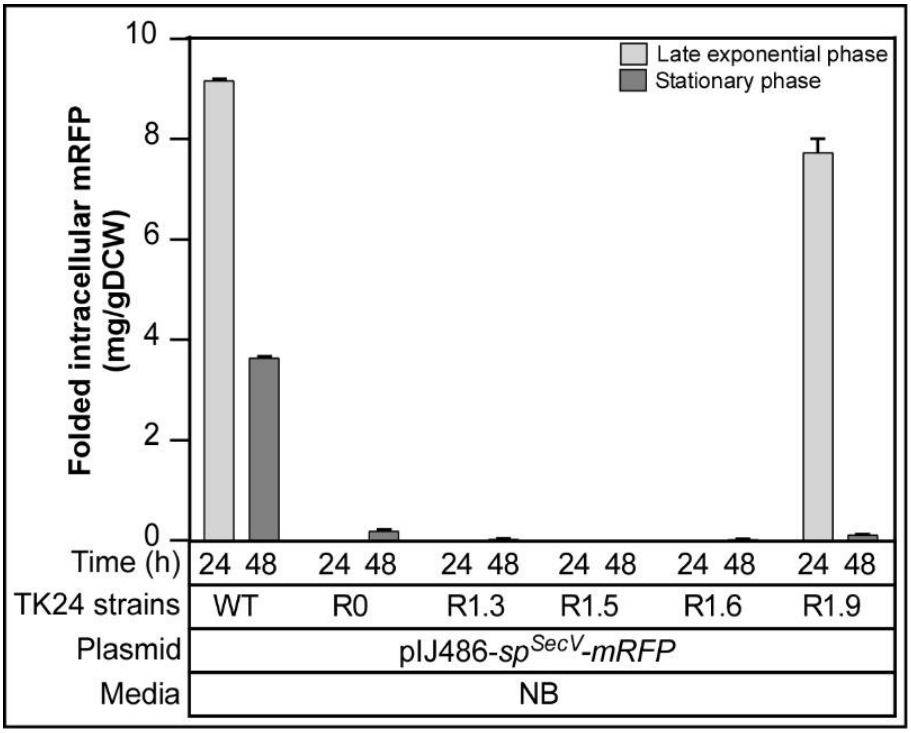

Figure 7.2.2-3: Amount of intracellular (accumulated) folded mRFP expressed in specific [mg/gDCW] (C, D) units produced by *S. lividans* TK24 RGS carrying pIJ486-*sp*^{SecV}-*mRFP* in nutrient broth (NB) for the indicated time related to its growth curves in the same media. n = 3, values represent the mean ± SD.

As analyzed and described in section 7.2.1, intracellular amounts of folded mRFP were also measured by fluorescence. For these experiments, the reported amounts were slightly higher than the previously reported values for the control (wild type strain). Strain R1.9 (Figure 7.2.2-3) showed a value equivalent to 7.73 ± 0.27 mg/g DCW. An extremely low amount from intracellular mRFP was detected for strains R0 and R1.3 at 48 hours of growth. As mentioned earlier, the relative fluorescence measurement can detect only the folded mature state of the mRFP, which is why inmuno-detection was also performed (Figure 7.2.2-4). This assay showed that for most of the strains, intracellular mRFP forms could not be detected. For strain R0 (gel A), two out of three tested colonies (same clone) showed intracellular protein accumulation. Although for strains R1.5 and R1.6 (gel B) it appears to be some bands, they do not belong to any of the mRFP possible intracellular forms. Finally, for strain R1.9, the detected form corresponds to the mature folded mRFP state.

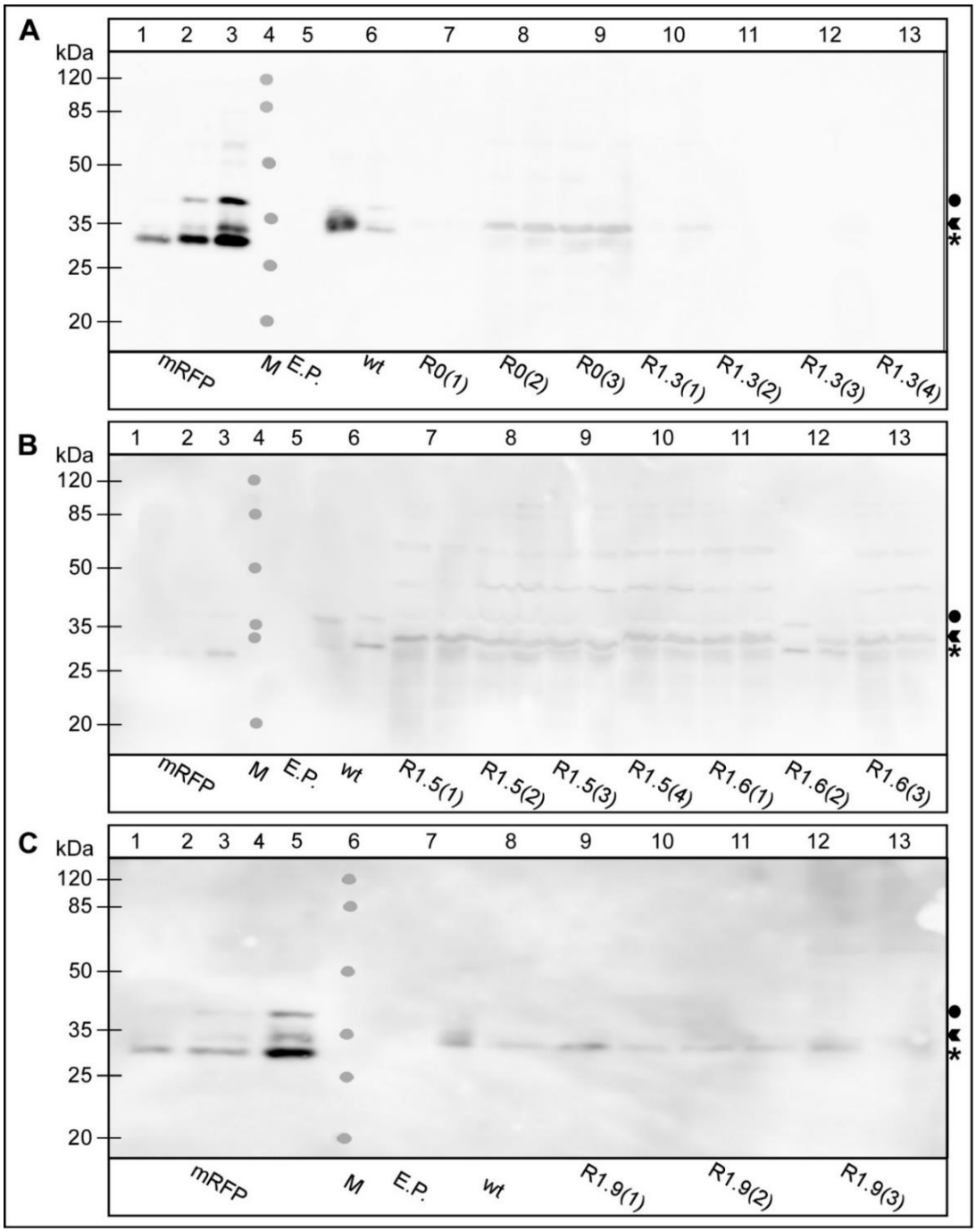

Figure 7.2.2-4: Western blot analysis for intracellular mRFP in TK24 RGS grown in nutrient broth (NB). Total cell lysates loaded equivalent to 0.1mg of dry cell mass washed twice and loaded on 12% SDS-PAGE and visualized using mRFP-antibodies.

A) Tested colonies for strains R0 and R1.3 (20 min exposure).
B) Tested colonies for strains R1.5 and R1.6 (14 min exposure).
C) Tested colonies for strain R1.9 (20 min exposure).

Circle: mRFP stabilized preform state with its signal peptide attached; filled arrow: mature folded mRFP; asterisk: first stage of mRFP intracellular degradation. Purified mRFP control in lanes 1(50ng), 2 (100ng) and 3 (300ng). Molecular weight marker in lane 6 as in figure 7.2.1-4.

A succesful study on engineered *Streptomyces avermitilis* strains was presented by Komatsu *et al.* in 2010. In this study nonessential genes were removed. Strains were depleted from around 17% of its total genome (more than 1.4 Mb out of 9.02Mb was deleted). The deletion mutants did not produce any of the major endogenous secondary metabolites found in the parental strain, showing that the native production was maybe altered as a consequence from the deletions. However, the suitability of the mutants as hosts for efficient production of foreign metabolites was shown by heterologous expression of three different exogenous biosynthetic gene clusters: streptomycin (from *Streptomyces griseus*), cephamycin C (from *Streptomyces clavuligerus*), and pladienolide (from *Streptomyces platensis*). In Komatsu's experiments, both streptomycin and cephamycin C were reported at levels higher than those of the native-producing species.

On the other hand, Novakova *et al.* showed, in 2016, to improve antitumoral polyketide mithramycin A (MTM) production with the same strains presented on this thesis.

The low amounts of secreted mRFP detected by relative fluorescence in combination with the low amounts of intracellular detected mRFP, both by relative fluorescence and immuno-detection, suggest that the cells are not being able to read the plasmid information as expected. Plasmid pIJ486-*sp*^{SecV}*-mRFP* has been reported to produce high expression levels by the wild type strain under the same conditions in NB. This was confirmed in the second stage of experiments presented on this thesis.

Although it has been previously demonstrated, in *S. lividans* TK24, that translational efficiency can be the primary barrier limiting its function for gene expression. And it is known that the capacity and diversity of constitutive promoters for protein expression in *Streptomyces* are still limited. In 2018, Zhao *et al*. demonstrated that heterologous promoters from eubacteria could be converted into powerful promoters when inserted in *S. lividans*. It is possible that including a stronger promoter in the pIJ486 plasmid, the efficiency on expression and secretion of the studied protein (mRFP) might be improved.

A recent transcriptomics analysis performed on the wild type strain (data not published yet) showed that the deleted clusters do not belong to the highly transcribed set of genes in *S. lividans* TK24. With this new information it is possible to propose a redesign on the deletions in order to improve the RGS performance and facilitate the study of

the cell's growth, metabolism and secretory machinery. Such redesign should be more precise in terms of the genes and clusters selection.

8 Conclusions

- Changes where observed on the cellular growth. At least for six RGS (R1.3, R1.5, R1.6, R1.7, R1.8 and R1.9) the highest biomass, reached during the monitored time points, overcame the wild type value (at 24 hours).

- In comparisson to the WT strain (52.94±9.01 mg/gDCW), the secretion of total proteins disminished for specific strains during stationary phase (48 h): R1.5 (25.78±2.29 mg/gDCW), R1.8 (23.85±11.86 mg/gDCW), R1.9 (24.41±6.54 mg/gDCW) and R1.10 (7.63±1.44 mg/gDCW).

- Any of the proven combinations for secondary metabolites cluster deletion gives a strain with an increased capacity of heterologous protein production in comparison with the wild type strain from *S. lividans* TK24.

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ACTA DE EXAMEN DE GRADO

No. 00266
Matrícula: 2173801906

"PROTEIN SECRETION IN REDUCED GENOME STRAINS OF STREPTOMYCES LIVIDANS TK24"

En la Ciudad de México, se presentaron a las 17:00 horas del día 26 del mes de noviembre del año 2019 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. JAVIER BARRIOS GONZALEZ
DR. OCTAVIO LOERA CORRAL
DR. FRANCISCO JAVIER FIGUEROA MARTINEZ
DR. ULISES CARRASCO NAVARRO

Bajo la Presidencia del primero y con carácter de Secretario el último, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRA EN BIOTECNOLOGIA

DE: CLARA LUZ MARCELIN LEYVA

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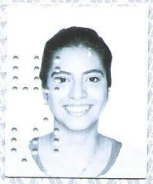
Aprobar

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.



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