Streptomycetes: Attractive Hosts for Recombinant Protein Production

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Enzymes are increasingly applied as biocatalysts for fulfilling industrial needs in a variety of applications and there is a bursting of interest for novel therapeutic proteins. Consequently, developing appropriate expression platforms for efficiently producing such recombinant proteins represents a crucial challenge. It is nowadays widely accepted that an ideal ‘universal microbial host’ for heterologous protein expression does not exist. Indeed, the first-choice microbes, as Escherichia coli or yeasts, possess known intrinsic limitations that inevitably restrict their applications. In this scenario, bacteria belonging to the Streptomyces genus need to be considered with more attention as promising, alternative, and versatile platforms for recombinant protein production. This is due to their peculiar features, first-of-all their natural attitude to secrete proteins in the extracellular milieu. Additionally, streptomycetes are considered robust and scalable industrial strains and a wide range of tools for their genetic manipulation is nowadays available. This mini-review includes an overview of recombinant protein production in streptomycetes, covering nearly 100 cases of heterologous proteins expressed in these Gram-positives from the 1980s to December 2019. We investigated homologous sources, heterologous hosts, and molecular tools (promoters/vectors/signal peptides) used for the expression of these recombinant proteins. We reported on their final cellular localization and yield. Thus, this analysis might represent a useful source of information, showing pros and cons of using streptomycetes as platform for recombinant protein production and paving the way for their more extensive use in future as alternative heterologous hosts.

Keywords: streptomycetes, recombinant proteins, heterologous expression, industrial enzymes, therapeutic proteins

INTRODUCTION

Nowadays, we witness the increasing application of enzymes in industrial sectors, including food, detergent, and textile manufactures (Trono, 2019) and the bursting of interest in proteins for therapeutic and diagnostic purposes (Tripathi and Shrivastava, 2019). Developing efficient bioprocessing strategies for protein production is consequently of utmost importance. Most of valuable industrial enzymes and therapeutic proteins are recombinant versions, produced by heterologous platforms (Adrio and Demain, 2014). However, an ideal ‘universal host’ for protein heterologous expression does not exist. Those microbes (as Escherichia coli or yeasts) that are still considered the first-choices to this purpose possess intrinsic limitations inevitably restricting their use. Production of heterologous proteins in E. coli is limited by self-cytotoxicity, incorrect folding,
aggregation into inclusion bodies, and/or lack of secretion (Adrio and Demain, 2014). In yeasts, recombinant protein production is often associated with hyper-glycosylation and product retention within the periplasmic space (Vieira Gomes et al., 2018).

In this scenario, bacteria belonging to the Streptomyces genus might represent a promising alternative platform for recombinant protein production. Streptomyces are Gram-positive, aerobic bacteria, characterized by a mycelial life style and commonly found in soils, where they secrete multiple hydrolytic enzymes to degrade complex organic substrates. This natural secretion capacity represents their most attractive feature for recombinant protein production. Secretion may prevent local accumulation of the overexpressed recombinant proteins, reducing toxicity to host cells and promoting correct folding (Anné et al., 2012). It facilitates downstream recovery decreasing production costs (Hamed et al., 2018). In addition, streptomyces are characterized by low endogenous proteolytic activity; they grow relatively fast and in inexpensive media; they do not produce pyrogenic lipopolysaccharides and endotoxins; they are not pathogenic; and they might express G + C-rich genes without codon usage optimization (Anné et al., 2012; Sevillano et al., 2013). Thanks to the extensive fermentation knowhow deriving from their use as antibiotic producers (Ndlovu et al., 2015), streptomyces are robust and scalable industrial strains, and a wide range of tools for their genetic manipulation have recently become available (Kieser et al., 2000). Notwithstanding these potential advantages, nowadays their use is not so common as it could be expected. To investigate this aspect, in this mini-review we cover – to the best of our knowledge – all studies published from 1980s to December 2019, in which streptomyces were used as heterologous hosts for recombinant protein production. Table 1 reports these 94 cases of proteins expressed in streptomyces. Figure 1 highlights the main results emerging from the analysis of Table 1 in terms of protein class, homologous source, heterologous host, and molecular tools.

WHAT ARE THE RECOMBINANT PROTEINS PRODUCED IN STREPTOMYCES?

50 (out of 94) proteins listed in Table 1 are enzymes with potential industrial/environmental applications (Figure 1A). The most represented class is that of glycosyl hydrolases (23 proteins), including: (i) (hemi)cellulases, for lignocellulose saccharification and biofuel production; (ii) chitinases, for generating value-added chitin-derivatives as chitosan or biopesticides (Berini et al., 2018a); and (iii) amylasses for starch processing. The lipase/esterase group (8 proteins) with applications in detergent, food, and biofuel industries, and the oxidoreductase class (7), including laccases and peroxidases for bioremediation (Berini et al., 2018b), follow. Interesting examples are the phospholipase D from Streptomyces racemochromogenes, for producing phosphatidyl derivatives from lecithin with emulsifying properties for food and cosmetics (Nakazawa et al., 2011), and the cutinase from Thermobifida sp. with polyester-degrading activity in bioplastic recycle (Sinsereekul et al., 2010). Dubé et al. (2008) produced in Streptomyces lividans up to 350 mg/L of Streptomyces coelicolor small laccase, a thermostable enzyme decolorizing synthetic dyes that is considered promising for pollutant degradation in urban or industrial wastewaters. Finally, Table 1 and Figure 1A include transferases (6 proteins) for food processing, proteases/peptidases (5) for feed and detergent industries, and phosphatases (2), including a phytase used as supplement for animal nutrition (Carrillo Rincón et al., 2018). Additionally, Torres-Bacete et al. (2015) expressed a novel Penicillin V acylase for producing semisynthetic penicillins, whereas Rose et al. (2005) a latex clearing protein for bioconversion of rubber wastes. Unfortunately, only few of these studies reported a comparison of protein expression yield between streptomyces and other microbial hosts. Hamed et al. (2017) succeed in producing 90 mg/L of a thermostable cellulase from the bacteroidetes Rhodotermus marinus using S. lividans TK24 as host; the same protein could not be produced in E. coli. Very recently, Snajder et al. (2019) reported the first and so far the only case of expression of an archaeal thermozyme (permisine) in Streptomyces rimosus. The homologous host – the hyperthermophilic Aeropyrum pernix – was uncultivable in industrial fermentation facilities. The protein productivity (10 mg/L) in this case was comparable to that achieved in E. coli, but with the advantage of simplified downstream processes due to protein secretion in the streptomyces (Snajder et al., 2019). Similarly, the Streptomyces halstedii phospholipase expression was approximately 60 and 30 times higher in S. lividans TK24 than in E. coli and Pichia pastoris, respectively (Tao et al., 2019). Sianidis et al. (2006) and Sinsereekul et al. (2010) reported that their attempts to express a xylitol dehydrogenase from Jonesia sp. and a cutinase from Thermobifida sp. failed, respectively, in E. coli and B. subtilis, and E. coli and P. pastoris. Finally, Díaz et al. (2004) produced in S. lividans J66 a xylanase from Aspergillus nidulans with a yield 3- and 19-fold higher than in lactic bacteria and Saccharomyces cerevisiae, respectively. Despite these successes at laboratory level, we are indeed unaware of any further scaling up at industrial level of recombinant enzyme production from streptomyces. We can suppose that this is probably due to an overall limited protein productivity in streptomyces that rarely reaches the g/L production level usually required for industrial application. As reported in Table 1, only in the case of a chitinase (Nguyen-Thi and Doucet, 2016), the protein productivity was more than 1 g/L. These results point out the crucial need to overcome intrinsic bottlenecks in protein productivity in streptomyces, by redesigning their regulatory networks and secretion pathways by system biology, as recently proposed by Kim et al. (2020).

In Table 1, 21 are the recombinant proteins curing human diseases (Figure 1A), including those for treating cancer (interleukin, interferon, Tumor Necrosis Factor Alpha-TNF-α), cardiovascular pathologies (streptokinase, hirudin), and metabolic or auto-immune disorders (glucagon, phenylalanine ammonia-lyase, tendamistat). Recently, S. lividans TK24 was used for producing an Actinoallomurus A8-sourced glutenase, a promising candidate for oral enzymatic management of gluten toxicity (Cavaletti et al., 2019). Streptomyces were also used to express 8 ‘target’ proteins, as antigens from Mycobacterium
| References            | Protein     | Source                                      | Heterologous host | Plasmid | Promoter | Signal peptide | Productivity (up to) | Localization                |
|-----------------------|-------------|---------------------------------------------|-------------------|---------|-----------|----------------|----------------------|-----------------------------|
| Berini et al., 2019   | Chitinase   | Metagenomics                                | S. coelicolor A3(2), S. venezuelae ATCC 10595, S. lividans TK24 | pIJ86   | ermEp⁺    | Absent         | 45 mg/L              | Extracellular              |
| Cavaletti et al., 2019| Glutenase   | Actinoallomurus sp. (Gram +)                | S. lividans TK24   | pIJ86   | ermEp⁺    | Native         | 1.4 × 10⁶ U/L        | Extracellular              |
| Šnajder et al., 2019  | Pernisine   | Aeropyrum pernix (archaeon)                 | S. rimosus M4018   | pVF     | tcp830p   | srT-SP         | 10 mg/L (codon usage optimization, pro-region removal) | Extracellular              |
| Tao et al., 2019      | Phospholipase D | S. halstedii (Gram +)          | S. lividans TK24   | pIJ12739 | Dual promoter (tipA/ermEp⁺) | Native         | 7.1 × 10⁴ U/L        | Extracellular              |
| Carrillo Rincón et al., 2018 | Phytase | Escherichia coli (Gram –)                   | S. rimosus M4018   | pVF, pAB04 | ermEp⁺*, nitA/nitRₚ, tcp830p | Native         | 5 × 10³ U/L in extracellular fraction, < 1 × 10³ U/L in cytoplasm (codon usage optimization) | Extracellular + cytoplasm |
| Daniels et al., 2018  | Cellulase   | Rhodothermus marinus (Gram –)              | S. lividans TK24   | pIJ486   | vsip      | vsi-SP         | 7.5 mg/L              | Extracellular              |
| Noguchi et al., 2018  | Chitobiase  | S. avermitilis (Gram +)                    | S. lividans TK1326 and derivative (expressing a repressor to avoid protein production without inducer) | pIJ350   | xylAp⁺x            | Native         | 1.5 × 10⁶ U/L        | Extracellular              |
| Hamed et al., 2017    | Cellulase   | Rhodothermus marinus (Gram –)              | S. lividans TK24   | pIJ486   | vsip      | vsi-SP         | 90 mg/L (120 mg/g dry cell weight) | Extracellular              |
| Sevillano et al., 2017| α-Amylase  | S. griseus (Gram +)                        | S. lividans ΔTₐ-Tox (pGM160-YefMsl², pALCre²) | pNRoxAnti | pstSp     | NA             | 1.1 × 10⁶ U/L        | Extracellular              |
|                       | Xylanase    | S. halstedii (Gram +)                      | S. lividans ΔTₐ-Tox (pGM160-YefMsl², pALCre²) | pNRoxAnti | pstSp     | NA             | 1.7 × 10⁵ U/L        | Extracellular              |
| Gabarró et al., 2016  | Agarase     | S. coelicolor (Gram +)                      | S. lividans TK21, S. lividans ΔsipY (derivative deficient in the major signal peptidase SipY) | pIJ486   | Native     | NA             | 2.4 × 10⁶ U/L        | Extracellular              |
|                       | Laccase     | S. lividans (Gram +)                        | S. lividans ΔsipY (derivative deficient in the major signal peptidase SipY) | pFD666   | dagp       | NA             | 5.8 U/L               | Extracellular              |
| Liu et al., 2016      | Transglutaminase | S. hygrosopicus (Gram +)                | S. lividans TK24   | pIJ86    | Native (optimized by removal of negative element) | Native         | 5.7 × 10³ U/L        | Extracellular              |
| Nguyen-Thi and Doucet, 2016 | Chitinase | S. coelicolor (Gram +)                      | S. lividans 10-164 | pC109    | NA         | NA             | 1.1 × 10³ mg/L       | Extracellular              |

(Continued)
| References                | Protein          | Source                          | Heterologous host                  | Plasmid                      | Promoter            | Signal peptide                  | Productivity (up to) | Localization |
|---------------------------|------------------|---------------------------------|------------------------------------|-----------------------------|---------------------|----------------------------------|----------------------|--------------|
| Sevillano et al., 2016    | Xylanase         | S. halstedii (Gram +)          | S. lividans 1326, S. lividans GSAL1 (derivative overexpressing the morphogene ssgA) | Derivative of pN702GEM3     | Native, vsip, ermEp, xysAp, pstSp, xylAPnom3, gplQP | Native, amy-SP (as-it-is, or optimized) | 2.5 × 10^5 U/L | Extracellular |
|                           | α-Amylase        | S. griseus (Gram +)            | S. lividans 1326, S. lividans ΔshR, S. lividans ΔshxR (derivatives knocked-out in putative xysA promoter genes) | pHUL401                     | xysAp, pstSp        | xysAp                           | 1.6 × 10^5 U/L | Extracellular |
|                           | Laccase          | S. coelicolor (Gram +)         | S. lividans 1326, S. coelicolor Δhfr (derivatives knocked-out in putative xysA promoter genes) | pHUL401                     | xysAp              | NA                              | 160 U/g dry weight | Extracellular |
| Guan et al., 2015         | Transglutaminase | S. hygroscopicus (Gram +)      | S. lividans TK24, S. griseus, S. lividans 1326, S. hygroscopicus FR008 | plUJ86                      | Native, ermEp        | Native (as-it-is, or optimized) | 687 mg/L (9.6 × 10^3 U/L) | Extracellular |
|                           | Aminopeptidase   | Bacillus subtilis (Gram +)     | S. lividans TK25, xysA p, S. lividans Δhfr (derivatives knocked-out in putative xysA promoter genes) | pI8401                      | tga                 | tga-SP (optimized)              | 2.8 × 10^3 U/L | Extracellular |
|                           | Phenylalanine ammonia-lyase | Rhodotorula glutinis (yeast) | S. lividans TK25, xysA p, S. lividans Δhfr (derivatives knocked-out in putative xysA promoter genes) | pI8401                      | NA                  | NA                              | 2.1 × 10^4 U/L | Extracellular |
| Guifó et al., 2015        | Agarase          | S. coelicolor (Gram +)         | S. lividans TK21, S. lividans ΔsecG, S. lividans ΔtatC (derivatives knocked-out for components of the Sec- or Tat-route respectively) | pAGAs1                      | Native              | Native, aml-SP                  | 60 U/mg dry weight | Extracellular |
| Torres-Bacete et al., 2015| Penicillin V acylase | S. lavendulae (Gram +)        | S. lividans 1326 | pEM4 | ermEp | Native | 11 mg/L (959 U/L) | Extracellular |
| Binda et al., 2013        | D,D-peptidase/D,D-carboxypeptidase | Nonomuraea gerenzanensis (Gram +) | S. venezuelae ATCC 10595, S. coelicolor AS3(2), S. lividans TK24 | plUJ86 | ermEp | Native | 12 mg/L | Cell wall fraction |
| Li et al., 2013           | Endoglucanase    | Thermofructictus (Gram +)      | S. lividans 1326 | pZRI362 | xylAP4m | Native | 173 mg/L (5.8 × 10^3 U/L) | Extracellular |
| Sevillano et al., 2013    | α-Amylase        | S. griseus (Gram +)            | S. lividans pKC796, S. lividans ΔTA-pK796 (pGM160-YefMsp^R), S. lividans ΔTA-pK796-Mx (pGM160-YefMsp^R) | pN702Gem3-Anti | xylAP | NA | NA | Extracellular |
|                           | Xylanase         | S. halstedii (Gram +)          | S. lividans TK24 and derivative (overexpressing phosphoenolpyruvate carboxykinase) | plJ486 | vsip | vsl-SP | 0.9 mg/g dry biomass | Extracellular |
| Lule et al., 2012         | Tumor Necrosis Factor α | Human                          | S. lividans TK24 and derivative (overexpressing phosphoenolpyruvate carboxykinase) | plJ486 | vsip | vsl-SP | 0.9 mg/g dry biomass | Extracellular |
| Dubedout et al., 2011     | Chitosanase      | Kitasatospora sp. N106 (Gram +) | S. lividans TK24, S. lividans ΔcsnR (knocked-out for a negative transcriptional regulator) | Derivative of pHM8a, pFDES | Native (as-it-is or modified), S. ghanaensis phage 119 promoter | NA | 2.4 × 10^4 U/L | Extracellular |
| References          | Protein                  | Source                         | Heterologous host   | Plasmid       | Promoter   | Signal peptide       | Productivity (up to) | Localization       |
|---------------------|--------------------------|-------------------------------|---------------------|---------------|------------|----------------------|----------------------|--------------------|
| Nakazawa et al., 2011 | Phospholipase D          | S. racemochromogenes (Gram +) | S. lividans TK23    | pES           | Native     | NA                   | 3.0 \times 10^4 U/L | Extracellular       |
| Zhu et al., 2011    | Interleukin A            | Human                         | S. lividans TK24    | Derivative of pSGL1 | ermEp* | meIC1-SP, gpp-SP (as-it-is, or optimized) | 0.6 mg/L | Extracellular       |
| Côté and Shareck, 2010 | Lipase                  | Metagenomics                  | S. lividans 10-164  | plAFC109      | NA         | Native               | NA                   | Extracellular       |
| Noda et al., 2010   | Transglutaminase         | Stv. cinnamomeum (Gram +)    | S. lividans 1326    | plU702        | piDp       | piD-SP               | 230 mg/L             | Extracellular       |
| Díaz et al., 2008   | 1, 4-Endoglucanase       | Thermofibida fusca (Gram +)  | S. lividans        | plID-SP       | piD-SP     | piD-SP               | 64 mg/L              | Extracellular       |
| Sinsereekul et al., 2010 | Cutinase               | Thermofibida sp. (Gram +)    | S. rimosus R7       | plU8600       | tipAp      | Native               | 114 mg/L             | Extracellular       |
| Meilleur et al., 2009 | Lipase              | Metagenomics                  | S. lividans IAF10-164 | plAFD95A      | O95Ap      | Native               | 11.3 mg/L            | Extracellular       |
| Díaz et al., 2008   | Alkaline phosphatase    | Thermus thermophiles (Gram +) | S. lividans J66    | plU702        | xysAp      | Native               | 2.7 \times 10^5 U/L | Extracellular       |
| Dubé et al., 2008   | Laccase                 | S. coelicolor (Gram +)        | S. lividans IAF10-164 | plAFD95A      | O95Ap      | Native               | 350 mg/L             | Extracellular       |
| Hatanaka et al., 2008 | Leucine aminopeptidase | S. griseus (Gram +)          | S. lividans 1326    | pTONA5        | sssmp, ermEp*, kibilysin gene promoter | 1.5 \times 10^5 U/L | Extracellular       |
| Proline aminopeptidase | Proline aminopeptidase | Streptomyces sp. (Gram +)    | S. griseus (Gram +) |               |            | Absent               | 5.2 \times 10^5 U/L | Extracellular + cytoplasm |
| Aminopeptidase P    |                          |                               |                     |               |            | Absent               | 3.5 \times 10^4 U/L | Extracellular + cytoplasm |
| Lin et al., 2006, 2008 | Trangetaminase        | S. platensis (Gram +)         | S. lividans JT48    | plU702        | meIC1p     | Native               | 5.8 \times 10^3 U/L | Extracellular       |
| Qi et al., 2008     | Glucagon                | Human                         | S. lividans TK24    | Derivative of plU880 | aphp | meIC1-SP             | 24 mg/L              | Extracellular       |

(Continued)
| References         | Protein                     | Source                  | Heterologous host | Plasmid     | Promoter | Signal peptide | Productivity (up to) | Localization |
|--------------------|-----------------------------|-------------------------|-------------------|-------------|-----------|----------------|----------------------|--------------|
| Ayadi et al., 2007 | α-Integrin A-domain         | Rat                     | S. lividans 1326  | pIJ699      | ermEp     | Long synthetic SP | 8 mg/L               | Extracellular |
| Merkens et al., 2007| Quercetinase                | Streptomyces sp. (Gram +) | S. lividans TK23  | pIJ702      | Native    | Absent         | 5.1 U/mg total protein | Cytoplasm    |
| Pimienta et al., 2007| Streptokinase               | Streptococcus equisimilis (Gram +) | S. lividans TK24  | pUWL-218   | vsIP     | vsi-SP, xhC-SP   | 15 mg/L              | Extracellular |
| Vrancken et al., 2007| Tumor Necrosis Factor α     | Human                   | S. lividans TK24 and derivative (over-expressing the phage-shock protein A homolog) | pSSV05 | vsIP | vsi-SP | 1.1 µg/mg dry weight | Extracellular |
|                   | Enhanced green fluorescent protein | Aequorea victoria (jellyfish) |                     |             |           |                 | 20 mg/L (15.9 U/mg dry weight) | Extracellular |
| Côté et al., 2006  | β-Glucosaminidase           | Amycolatopsis orientalis (Gram +) | S. lividans TK24  | pFD666      | NA       | Native         | 573 U/L              | Extracellular |
|                   | β-Glucosaminidase           | S. avermitilis (Gram +) |                     |             | NA       | NA             | NA                   | Extracellular |
| Sianidis et al., 2006| Xyloglucanase               | Jonesia sp. (Gram +)    | S. lividans TK24  | pIJ486      | vsIP | Native, vsi-SP | 150 mg/L             | Extracellular |
| Vallin et al., 2006| Glycoprotein (antigen)       | Mycobacterium tuberculosis (Gram +) | S. lividans 1326 | pUWL-219   | dagp | dagg-SP | 80 mg/L              | Extracellular |
| Fukatsu et al., 2005| N-substituted formamide deformylase | Arthrobacter pascens (Gram +) | S. lividans TK24, S. coelicolor A3(2) M145, S. avermitilis K139 | pSH19 | nitA/nitRp | NA             | 8.5 U/mg total protein | Extracellular |
| Rose et al., 2005  | Latex clearing protein      | Streptomyces sp. (Gram +) | S. lividans TK23  | pIJ702      | Native    | Native         | NA                   | Extracellular |
| Díaz et al., 2004  | Xylanase                    | Aspergillus nidulans (fungus) | S. lividans J66   | pIJ702      | vsIP | Native, xys1-SP | 1.9 × 10^4 U/L        | Extracellular |
| Lara et al., 2004  | Glycoprotein (antigen)       | Mycobacterium tuberculosis (Gram +) | S. lividans 1326 | pIJ486, pIJ6021 | Native, tipAp | Native | 5 mg/L              | Extracellular |
| Lin et al., 2004   | Transglutaminase            | Stv. ladakanum (Gram +) | S. lividans JT46  | pIJ702      | Native    | Native         | 1.5 × 10^3 U/L        | Extracellular |
| Ogino et al., 2004 | Phospholipase D             | Stv. cinnamoneum (Gram +) | S. lividans 1326  | pJC702      | Native    | Native         | 118 mg/L (5.5 × 10^4 U/L) | Extracellular |
| Schaerlaeken et al., 2004 | Tumor Necrosis Factor α   | Human                   | S. lividans TK24, S. lividans ΔtatB, S. lividans ΔtatC (derivatives knocked-out for components of the Tat pathway) | pIJ486 | vsIP | xhC-SP, mtc1-SP, vsi-SP | 23 mg/L              | Extracellular |
| Zhang et al., 2004 | Interleukin-10              | Human                   | S. lividans TK24  | pSGLgpp     | NA       | gpp-SP         | 166 µg/L             | Extracellular |
|                   | Interleukin-4 receptor       | Human                   | S. lividans TK24  |             |           |                | 10 mg/L              | Extracellular |
| References                  | Protein Source             | Heterologous host | Plasmid | Promoter | Signal peptide (up to) | Productivity (up to) | Localization |
|----------------------------|---------------------------|------------------|---------|----------|------------------------|----------------------|--------------|
| Béki et al., 2003          | β-D-Mannosidase           | Thermobifida fusca (Gram +) | S. lividans TK24 | pIJ899 | Native | Absent | 0.015 U/mg total protein | Cytoplasm |
| Geueke and Hummel, 2003    | l-Amino acid oxidase      | Rhodococcus opacus (Gram +) | S. lividans 1326 | pIJ8021, pIJW1201 | tip4p, ermE* | Native | 18 U/L | Cytoplasm |
| Hong et al., 2003          | Calcitonin (co-expressed with rat α-amidase gene) | Salmon | S. lividans TK54 | pIJ880 | aphp | melC1-SP | 30 mg/L | Extracellular |
| Tremblay et al., 2002      | 19 kDa major lipoprotein antigens | Mycobacterium tuberculosis (Gram +) | S. lividans IA F10-164 | pIJ702 | xhp4p | celA-SP (long) | 200 mg/L | Extracellular |
|                           | 38 kDa major lipoprotein antigens |                     |         |          |                        | 80 mg/L | Extracellular |
| Lammertyn et al., 1997; Pozidis et al., 2001 | Tumor Necrosis Factor α | Mus musculus (Mouse) | S. lividans TK24 | pIJ486 | vsip | vsi-SP (as-it-is or modified) | 300 mg/L | Extracellular |
| Isiegas et al., 1999       | β-Lactamase               | Escherichia coli (Gram −) | S. lividans TK21 | pIJ487 | dagp | dag-SP | 60 U/L | Extracellular |
| Smith et al., 1999         | Alkene monooxygenase      | Rhodococcus rhodochrous (Gram +) | S. lividans TK24 | pIJ6021 | tip4p | NA | 2.2 U/mg total protein | Cytoplasm |
| Lammertyn et al., 1998     | Tumor Necrosis Factor α   | Mus musculus (Mouse) | S. lividans | pIJ486 | vsip | aml-SP5 | 50 mg/L | Extracellular |
| Park and Lee, 1998         | β-Lactamase-inhibitory protein | S. exfoliatus (Gram +) | S. lividans TK24 | pIJ702 | melC1p | Native | 3.0 × 10^4 U/L | Extracellular |
| Binnie et al., 1997        | Extracellular domain of erythropoietin receptor | Human | S. lividans 66 | pCAN46 | aphp | sprtB-SP (modified) | 15 mg/L | Extracellular |
| Motamedi et al., 1996      | 31-O-Demethyl-FK506 methyltransferase | S. hygroscopicus (Gram +) | S. lividans | pIJ943 | NA | Native | NA | Cytoplasm |
| Taguchi et al., 1995       | Transforming Growth Factor α (fused with S. albogriseolus subtilisin inhibitor) | Human | S. lividans 66 | pIJ702 | ssip + melC1p | ssi-SP | 10 mg/L | Extracellular |
| Paradkar et al., 1994      | β-Lactamase inhibitor protein | S. clavuligerus (Gram +) | S. lividans TK24 | pIJ486 | Native | Native | NA | Extracellular |

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TABLE 1 | Continued

| References               | Protein                                      | Source                | Heterologous host | Plasmid     | Promoter              | Signal peptide | Productivity (up to) | Localization   |
|--------------------------|----------------------------------------------|-----------------------|-------------------|-------------|-----------------------|----------------|----------------------|----------------|
| Washizu et al., 1994     | Transglutaminase                            | Stv. mobaraense (Gram +) | S. lividans 3131  | pIJ702      | S. antibioticus tyrosinase promoter | Native         | 0.1 mg/L              | Extracellular  |
| Formwald et al., 1993    | T cell receptor CD4 (as-it-is and derivatives)| Human                | S. lividans 1326  | pLT450      | STI-Ilp, Jlp          | STI-II-SP      | 300 mg/L              | Extracellular  |
| Jung et al., 1993        | Endoglucanase                                | Thermobifida fusca (Gram +) | S. lividans TK24  | Derivatives of pIJ702 | Native | Native | 36 mg/L, (1.9 × 10³ U/L) | Extracellular  |
|                          | Exoglucanase                                |                       |                   |             |                       |                | 17 mg/L (23 U/L)      | Extracellular  |
| Ueda et al., 1993        | Fv domain of monoclonal antibody against hen egg-white lysozyme | Human                | S. lividans 66    | pIJ702      | ssp                  | ssi-SP         | 1 mg/L                | Extracellular  |
| Wolfframm et al., 1993   | Chloroperoxidase                            | Pseudomonas pyrocina (Gram −) | S. lividans TK64  | pIJ486      | Native                | NA             | 11.2 U/g wet weight | Cytoplasm      |
| Hale et al., 1992        | Esterase                                    | S. scabia (Gram +)    | S. lividans 1326  | pIJ486, pIJ702 | NA                  | Native         | 100 mg/L              | Extracellular  |
| Taguchi et al., 1992     | Apidaecin 1b (fused with S. alboplitesol subtilisin inhibitor) | Apis mellifera (Honeybee) | S. lividans 66    | pIJ702      | ssp + melC1p         | ssi-SP         | >200 mg/L             | Extracellular  |
| Jorgensen et al., 1991   | Lipase (co-expressed with a lipase modulator) | Pseudomonas cepacia (Gram −) | S. lividans TK24  | pIJ702      | dagp                 | dag-SP         | Na                   | NA             |
| Bender et al., 1990a     | Hirudin                                      | Hirudo medicinalis (Leech) | S. lividans TK24  | pIJ702      | melC1p               | Ai-SP          | 500 μg/L              | Extracellular  |
| Bender et al., 1990b     | Interleukin-2                                | Human                | S. lividans TK24  | pIJ6800     | NA                   | Ai-SP          | 7.1 × 10⁵ U/L in extracellular fraction, 4.7 × 10⁴ U/L in cytoplasm | Extracellular + cytoplasm |
| Koller and Reiss, 1989   | Human α-amylase inhibitor (tendamistat)     | S. tendae (Gram +)    | S. lividans TK24  | pIJ350, pIJ486, pIJ702 | Native, melC1p or both in tandem | Native         | 700 mg/L              | Extracellular  |
| Swan et al., 1989        | Calcium-binding protein                     | Sac. erythraea (Gram +) | S. lividans TK24  | pIJ702      | Native                | NA             | NA                   | Extracellular  |
### TABLE 1 | Continued

| References                  | Protein                     | Source                              | Heterologous host | Plasmid   | Promoter       | Signal peptide | Productivity (up to) | Localization          |
|-----------------------------|-----------------------------|-------------------------------------|-------------------|-----------|----------------|-------------------|----------------------|-----------------------|
| Lamb et al., 1988           | 65-kilodalton antigen       | Mycobacterium leprae (Gram +)       | S. lividans       | pIJ697    | Native         | NA                | NA                   | Cytoplasm             |
| Lichenstein et al., 1988    | Interleukin-1β              | Human                               | S. lividans 1326  | pIJ350    | βgalp          | βgal-SP          | 3.8 x 10⁶ U/L in extracellular fraction | Extracellular + cytoplasm |
|                            |                             |                                     |                   |           |                |                   | 6.3 x 10⁴ U/L in cytoplasm                    | Cytoplasm             |
| Galaktokinase               |                            | Escherichia coli (Gram –)           | S. lividans 1326, S. lividans galK nucleus (galaktokinase-deficient mutant) |           |                |                   | 345 U/L in extracellular fraction | Extracellular + cytoplasm |
|                            |                             |                                     |                   |           |                |                   | 120 U/L in cytoplasm                      | Cytoplasm             |
| Noack et al., 1988          | Interferon α1               | Human                               | S. lividans TK24  | pIJ487    | saKp           | SAK-SP           | 2.0 x 10⁵ U/L                 | Extracellular         |
| Horinouchi et al., 1987     | Streptothricin acetyltransferase | Human                        | S. lividans TK21  | pIJ41, pIJ702, pIJ487 | saKP, melC1p, Bacillus subtilis cellulase promoter |                   | NA                   | Cytoplasm             |

The list was created by searching PubMed database (accession on 18 December, 2019) with the following query: (((heterologous[Title/Abstract]) AND expression[Title/Abstract]) AND protein[Title/Abstract]) AND streptomyces[Title/Abstract], then manually checked and integrated. Gram +, Gram-positive; Gram −, Gram-negative; NA, data not available; SP, signal peptide; S., Streptomyces; Sac., Saccharopolyspora; Sv., Streptovercillium. Promoters (CO, constitutive; IN, inducible): aphp from S. fradiae aminoglycoside 3'-phosphotransferase (CO); βgalp from S. lividans β-galactosidase (CO); D95Ap from S. coelicolor groEL heat-shock gene (NA); dags from S. coelicolor arabinose (CO); ermEp from Sac. erythraea erythromycin resistance gene (CO); gpp from S. coelicolor glycerophosphoryl diester phosphodiesterase (IN by glycerol-3-phosphate); melC1p from S. antibioticus melanin operon (CO); nitA/nitRp from Rhodococcus rhodochrous nitrilase (IN by -caprolactam); pDp from Stv. cinnamomum phospholipase D (CO); pStSp from S. lividans phosphatase-binding protein (IN by phosphate starvation and carbon sources as fructose, xylitol, or galactose); saKp from Staphylococcus aureus phage 42D staphylokinase (NA); STII-p from S. longisporus protease inhibitor (NA); ssip from S. albogriseolus subtilisin inhibitor (CO); ssmp from S. cinnamoneus metalloendopeptidase (CO in the presence of a rich inorganic phosphate source and glucose); totp830p synthetic promoter from S. coelicolor (IN by tetracycline); tgp from S. hygroscopicus transglutaminase (CO); tAP from S. lividans (IN by thiostrepton); vasp from S. venezuelae subtilisin inhibitor (CO); xAP from S. lividans xylanase A (NA); xylAp from S. avermitilis (xylAp, S. coelicolor (xylAp), or Actinoplanes missouriensis (xyAP, xylase isomerase (IN by xylose); xysAp from S. halstedii xylanase (IN by carbon sources as xylose, xylitol, or fructose). Plasmids (HN, high copy number; MN, moderate copy number; LN, low copy number; SN, single copy number; int, integrative; rep, replicative): pAB04 (LN, int); pAGAs1 (HN, rep); pC109 (HN, rep); pCAN46 (HN, rep); pEM4 (HN, rep); pES (HN, rep); pFD666 (HN, rep); pFDES (HN, rep); pHUL401 (MN, rep); pHM8 (SN, int); pAFC109 (HN, rep); pIL12739 (MN, rep); pIL350 (HN, rep); pIL41 (LN, rep); pIL486 (HN, rep); pIL487 (HN, rep); pIL6021 (HN, rep); pIL61 (LN, rep); pIL680 (HN, rep); pIL689 (HN, rep); pIL702 (HN, rep); pIL86 (HN, rep); pIL8600 (SN, int); pIL943 (LN, rep); pLT450 (HN, rep); pNRoxAnti (HN, rep); pNRoxAnti (HN, rep); pSSG1 (HN, rep); pSSG0 (HN, rep); pSSLov (HN, rep); pSSV05 (HN, rep); pTBNA5 (HN, rep); pUC702 (HN, rep); pUM201 (HN, rep); pUWL218 (HN, rep); pUWL219 (HN, rep); pW12 (HN, rep); pZHR366 (HN, rep). Signal peptide: Al-SP from S. tendae tendamistat (α-amylase inhibitor); almi-SP from S. venezuelae (almi-SP, or S. lividans (almi-SP, α-amylase; amy-SP from S. griseus α-amylase; βgal-SP from S. lividans (β-galactosidase); celA-SP from S. lividans cellulase; dag-SP from S. coelicolor arabinose; gpp-SP from S. globosphorus apoprotein C-1027; lop-SP from S. mimosa lipase; melC1-SP from S. antibioticus melanin operon gene; pD-SP from Stv. cinnamomum phospholipase D; sak-SP from Staphylococcus aureus phage 42D staphylokinase; spnB-SP from S. griseus protease B; tSt-SP from S. mimosa trypsin-like protease; ssl-SP from S. albogriseolus subtilisin inhibitor; STII-SP from S. longisporus protease inhibitor; tgp-SP from S. hygroscopicus transglutaminase; vlsi-SP from S. venezuelae subtilisin inhibitor; xhC-SP from S. lividans xylanase C; xys1-SP from S. halstedii xylanase.}
**tuberculosis** (Vallin et al., 2006) or the α-integrin A-domain for screening ligands for treating inflammatory disorders (Ayadi et al., 2007), and few diagnostic proteins (2) as the T Cell receptor CD4 for diagnosis of HIV infection (Fornwald et al., 1993). Biopharmaceutical production of proteins in streptomycetes is generally acceptable to the Food and Drug Administration and European Medicine Agency since these bacteria have been used for decades in industrial manufacturing of antibiotics, immunomodulating and antitumor drugs, and nutraceuticals (Marinelli and Marcone, 2011). Additionally, these naturally soil-inhabiting bacteria are recognized as useful components of natural ecosystem and they are considered safer than other microorganisms for agricultural use (Berini et al., 2019). Interestingly, besides the proteins listed in Table 1, *S. lividans* was employed by Cangene Corporation (now part of Emergent BioSolutions) for the recombinant production of the macrophage-colony stimulating factor Leucotropin™, a therapeutic agent that successfully completed Phase III trials for treating Hodgkin's and non-Hodgkin's lymphoma (Vrancken and Anné, 2009). To our best knowledge, this is the only reported case of a therapeutic protein production in streptomycetes that reached the clinical phases.

Finally, Table 1 includes 7 proteins without any direct industrial/therapeutic application: they were produced in streptomycetes for studying biochemical/functional properties and/or mode of action, as in case of the novel N-substitute formamide deformylase from *Arthrobacter pascens* involved in the metabolism of isonitriles (Fukatsu et al., 2005). Another example is VanYn, a D,D-dipeptidase/D,D-carboxypeptidase identified as the sole resistant determinant in the glycopeptide producer *Nonomuraea gerenzanensis* (Binda et al., 2013; Dalmastri et al., 2016). VanYn expression in *Streptomyces venezuelae* allowed a higher production than in *E. coli* (Binda et al., 2012), and contributed to elucidating cell wall turnover during antibiotic production (Marcone et al., 2010a, 2014).

**WHERE DO RECOMBINANT PROTEINS EXPRESSED IN STREPTOMYCETES COME FROM?**

71 of the proteins listed in Table 1 derive from prokaryotes and 23 from eukaryotes (Figure 1B). Most of prokaryote-sourced proteins come from Gram-positive bacteria: 49 are from *Streptomyces* or *Streptverticillium* ssp., or other actinomycetes as *Nonomuraea, Kitasatospora*, or *Thermobifida* ssp. This is not surprising, as heterologous expression is facilitated when the host is phylogenetically related to the homologous producer, due to the similar metabolic and genetic background (Binda et al., 2013). Streptomycetes (DNA G + C > 60%) offer an optimized codon usage for high G + C content genes and they represent
a complementary tool versus E. coli (DNA G + C ca. 51%). For instance, chitinases, usually produced by soil-inhabitant actinomycetes, were successfully produced in streptomycetes (Berini et al., 2019). Cloning a S. coelicolor chitinase in S. lividans 10–164 resulted in 486-fold production improvement compared to E. coli, allowing gram-scale production for converting crystalline chitin in N-acetylglicosamine (Nguyen-Thi and Doucet, 2016). 9 additional recombinant proteins derive from the firmicutes Bacillus subtilis and Streptococcus equisimilis, and other 9 from the Gram-negative Escherichia, Thermus, and Pseudomonas spp. (Figure 1B). The thermostable cellulase from the bacteroidetes Rhodotermus marinus (Hamed et al., 2017) and the archaeal thermozyme (pernisine) (Snajder et al., 2019), described above, complete the list of the prokaryote proteins.

Streptomycetes were successfully used for expressing metagenome-sourced bacterial enzymes (Berini et al., 2017). 2 lipases from enriched fed-batch bioreactors (Meilleur et al., 2009; Côté and Shareck, 2010) and 1 chitinase (named 53D1) from agricultural soil (Berini et al., 2019) were produced in different Streptomyces strains. In case of 53D1, the protein was secreted (45 mg/L) into the culture broth by Streptomyces from agricultural soil (Berini et al., 2019) were produced in metagenome-sourced bacterial enzymes (Berini et al., 2017). described above, complete the list of the prokaryote proteins.

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August 2020 | Volume 11 | Article 1958

The heterogeneity of eukaryote sources of the recombinant proteins expressed in streptomycetes confirms their versatility (Table 1 and Figure 1B). The homologous producers of the eukaryote proteins listed in Table 1 span from filamentous fungi or yeasts (2), to invertebrates (insect, leech, and jellyfish; 3) or vertebrates (fish and mammals; 18). Notably, 14 human proteins were produced in these hosts. A chronological analysis indicates that eukaryote protein expression in streptomycetes was more frequent in the 1990s, becoming after that rarer. The last example of eukaryote protein produced in S. lividans TK24 dated back to 2012 (Lule et al., 2012). This is probably due to recent developments in using engineered yeasts, and mammalian and insect cell lines for manufacturing high-value eukaryote proteins, especially those requiring post-translational modifications (Hunter et al., 2019).

WHICH IS THE BEST PROMOTER/VECTORS/HOST SYSTEM FOR RECOMBINANT PROTEIN PRODUCTION IN STREPTOMYCETES?

S. lividans strains are by far the most frequently used heterologous hosts, employed for producing 91 proteins listed in Table 1. 31 proteins were expressed in the parental S. lividans 66 (also named JI66 or 1326), whereas 37 in its derivative TK24, which is a two-plasmid-free mutant carrying streptomycin resistance mutation (str-6, SLP2 −, SLP3 −) (Kieser et al., 2000) (Figure 1C). 1 additional protein was produced in TK64, carrying the same mutations as TK24 plus the pro-2 mutation, and 1 in TK54, characterized by his-2, leu-2, and spc-1 mutations. The use of S. lividans TK24 has the following advantages: (i) low level of extracellular protease activity, (ii) poorly active restriction-modification system of exogenous DNA, (iii) known biochemistry/genetic background due to its high similarity to the model organism S. coelicolor A3(2) (Daniels et al., 2018). Other S. lividans used as hosts were the plasmid-free mutants S. lividans TK23 (for 3 proteins), carrying spectinomycin resistance mutation (spc-1, SLP2 −, SLP3 −), and its derivative JT46 (2 proteins) mutated in rec-46 gene to reduce inter-plasmid recombination (Kieser et al., 2000). 4 proteins were produced in S. lividans TK21, which lacks only SLP2 plasmid. Ad hoc constructed S. lividans hosts were derivatives of S. lividans 66 or TK24, as the pleiotropic mutant S. lividans 10–164 (Hurtubise et al., 1995) defective in cellulbiose and xylose uptake and used for producing a metagenome-sourced lipase (Meilleur et al., 2009; Côté and Shareck, 2010), and S. lividans galK− (galaktokinase-deficient mutant) for the production of E. coli galactokinase (Lichenstein et al., 1988). S. lividans GSAL1, used for the production of a xylanase and a α-amylase, overexpresses the morphogene ssgA, which pleiotropically controls growth and cell division. ssgA overexpression markedly enhances septation in vegetative hyphae, leading to fragmented growth and to wider hyphae, a phenotype that apparently favors protein production and secretion (Sevillano et al., 2016). Other streptomycetes employed as hosts were S. coelicolor A3(2) and its derivative M145 (3 proteins), Streptomyces griseus (3), S. rimosus (3), Streptomyces hygroscopicus (3), S. venezuelae (2), and Streptomyces avermitilis (1) (Table 1 and Figure 1C). Although less frequently used than S. lividans, in certain cases these alternative streptomycetes permitted the production of proteins poorly or not at all expressed in S. lividans (Binda et al., 2013; Berini et al., 2019), thus indicating that expanding the range of streptomycete hosts might be promising.

As regards to vectors, the mostly used are high copy number replicative ones (in 93 cases) (Table 1 and Figure 1D) as for example pIJ702 (25 proteins), pIJ486 (14), and pIJ86 (7 proteins), pIJ702 vector, which carries thiostrepton resistance (tsrR) and tyrosinase production (mel +) markers, is the derivative of pIJ350, a non-conjugative broad host range vector (Kieser et al., 2000). pIJ486 (tsrR) derived from pIJ101, which contains the promoterless neo gene (kanamycin resistance) and lacks both the transfer function and the sti locus that usually confers ‘strong incompatibility’. Removing the sti locus increases the chance that different plasmids can be retained at similar copy numbers (Deng et al., 1988; Kieser et al., 2000). The more recent pIJ86 carries apramycin resistance marker (aprR) and it is a conjugative vector used for the strong constitutive expression of proteins under erythromycin promoter (ermE* promoter) from Saccharopolyspora erythrea. Recent works (Sevillano et al., 2013, 2017) described new replicative high copy number ‘marker-free’ systems, which allowed the production of high levels of proteins without using antibiotics as selection markers. One example is based on the presence of a toxin gene localized in the genome and of an anti-toxin gene located on the expression plasmid of the yeft/McryolB operon from S. lividans (Sevillano et al., 2013). Only for 5 proteins, replicative moderate or low copy number vectors
were used. For instance, the moderate copy number pJJ12739 was constructed for the expression of the phospholipase D from *S. halstedii* in *S. lividans* TK24, following the same approach previously described by Fernández-Martínez and Bibb (2014) to produce a dual-promoter expression vector (Tao et al., 2019). The low copy number pJJ943 was used for producing the 31-

O-demethyl-FK506 methyltransferase in *S. lividans* (Motamedi et al., 1996). For only 3 proteins, integrative vectors were employed such as pAb04 – low copy number plasmid used for producing a phytase (Carrillo Rincón et al., 2018), or pJJ8600 – single copy number vector employed for the expression of the cutinase from the Gram-positive *Thermobifida* sp. in *S. rimosus* R7 (Sinsereekul et al., 2010). Although less explored, integrative vectors might present some advantages. When the integrative single copy number pHM8a plasmid was used for expressing a chitosanase, productivity was comparable to that achieved with replicative multicopy pFDES plasmid, but with the advantage of not requiring antibiotic addition for selection (Dubau et al., 2011). Interestingly, this last work is the only one, among those cited in this mini-review, which allowed a direct comparison on the effect of different vectors on protein yield. Most of the studies were driven by an empirical case-by-case approach to optimize the tools for a specific protein production, making difficult to draw final conclusions on which is the preferable vector system to be used.

In 20 cases (out of 94), the heterologous protein genes were cloned under the control of their native promoters, but more frequently streptomycetes (or other actinomyces) heterologous promoters were used (Table 1 and Figure 1C). The heterologous promoters can be constitutive (e.g., *vsi* from *S. venezuelae*; *dapp* from *S. coelicolor*; *ermE*′p from *S. erythraea*; *sip* from *Streptomyces albogriseolus*; *aphp* from *Streptomyces fradiae*) or inducible (e.g., *xysA* from *S. halstedii*, induced by xylan; *pstSp* from *S. lividans*, by phosphate starvation and different carbon sources; *tcp830p* from *S. coelicolor*, by tetracycline; *tipAp* from *S. lividans*, by thiostrepton). Constitutive promoters were more frequently used than inducible ones (50 vs. 24 cases, respectively). If in *E. coli*, a balance between the vector copy number and the promoter strength is needed for controlling protein production and slowing down inclusion body formation (Adrio and Demain, 2014), in streptomycetes this problem is overcome by protein secretion. On the other hand, in streptomycetes, constitutive expression may cause a growth rate reduction negatively impacting on protein productivity: in these cases, inducible expression could be advantageous, although weak points of an inducible system remain as low level of expression, a narrow host range, and the need of an expensive inducer (Herai et al., 2004). As in the case of vectors, only very few studies systematically compared the effect of different promoters on protein yield. Sevillano et al. (2016) investigated the expression of a xylanase from *S. halstedii* cloning the gene under the control of six strong promoters, including two commonly used (*vsi* and *ermE*′p) and four recently identified. Two belonging to the last group (*xysA* and *pstSp*) performed better than those considered the golden standards, confirming that there is room for developing new tools for improving protein expression in streptomycetes.

In 30 out of the 94 proteins, the presence of native signal peptides (SP) guaranteed secretion in the heterologous hosts, while in 2 cases proteins expressed with their native SP accumulated into the cytoplasm and in 1 case the enzyme was recovered from the cell wall fraction (Table 1). In streptomycetes, the Sec pathway constitutes the main secretion system (Anne et al., 2012). Accordingly, proteins to be secreted have N-terminal hydrophobic SP, followed by a longer hydrophobic H-domain and a C-terminal part containing at the end three amino acids which form the signal peptidase recognition site. Other minor secretion systems were reported, including the twin-arginine dependent translocation (Tat) pathway (Anne et al., 2012). The Tat machinery exports fully folded proteins across the cytoplasmic membrane: SPs that target proteins to this pathway resemble Sec SPs, but contain a conserved twin-arginine motif in the N-region (Valverde et al., 2018). A comparison between the efficiency of these two pathways for recombinant protein production showed that replacing Sec-dependent SP with Tat-dependent SP drastically reduced protein expression (Schaerlaekens et al., 2004). When native SPs were absent or not functional, heterologous genes were fused to SP encoding sequences from genes for highly expressed/secreted endogenous *Streptomyces* proteins (Anne et al., 2016), such as the one from the subtilisin inhibitor (*vsi*) of *S. venezuelae* CBS762.70 (Van Mellaert et al., 1998). Other SP sequences, frequently used in *Streptomyces* expression-systems are also listed in Table 1. They derived from the genes for the trypsin-like protease (*srT*) from *S. rimosus*, for the α-amylase from *Streptomyces tendae*, *S. griseus*, *S. lividans*, or *Streptomyces limosus*, for the melanin operon gene (*melC1*) from *Streptomyces antibioticus*, for the subtilisin inhibitor (*ssi*) from *S. albogriseolus*. The final result is that in 77 out of the 94 proteins listed in Table 1, the recombinant proteins were completely secreted with productivities up to 100s of mg/L (Guan et al., 2015). In the few cases (8) where proteins were accumulated into cytoplasm, their productivity was generally low. 7 proteins were found produced either inside or outside the cells, whereas VanYn was localized in the cell wall fraction where it plays its physiological role in antibiotic resistance (Marcone et al., 2010a; Binda et al., 2012, 2013).

**CONCLUSION**

From the analysis of the literature in the last four decades, it emerges that, although promising, streptomycetes have been used for heterologous protein production less than their potential indicates to do. Notwithstanding their efficient protein secretion machine – which definitively facilitates downstream operations and protein purification – the mycelial lifestyle of these bacteria has probably discouraged scientists to use them more frequently. In liquid media, streptomycetes grow as mycelial pellets consisting of cells in different physiological states, and cultures are not homogenous and might become very viscous. In this regard, combining different specific mutations as *sggA* for improving disperse growth (Sevillano et al., 2016), and *galK* for generating auxotrophic mutants not requiring antibiotic-dependent selection (Lichenstein et al., 1988) might facilitate upstream processes. Additionally, formulation
of novel cultivation media – replacing those used for antibiotic biosynthesis – could facilitate protein downstream (Binda et al., 2013; Berini et al., 2019). Another aspect probably limiting their application is that streptomycetes cannot be genetically manipulated by the methods commonly used for E. coli and S. cerevisiae. They need ad hoc protocols based on intergeneric conjugation or protoplast transformation (Kieser et al., 2000; Marcone et al., 2010b,c). With time, these protocols have become available and, as reported in this review, nowadays we can count on a large variety of vectors, promoters, and SP sequences. What is still missing is the systematic and critical comparison of the available toolkits. Optimization of protein production is still conducted following a case-by-case – and somehow random – approach. Finally, an important issue is the intrinsic low protein productivity of streptomycetes in comparison with the mostly used E. coli and yeasts. Further improvements, in this sense, are urgently needed and may derive from system and synthetic biology approaches, still poorly applied to streptomycetes. Indeed, progresses in system biology and -omics technologies may shed light on the interplay of elements involved in protein expression, thus helping in the rational improvement of both expression platforms and fermentation conditions, finalized at reducing the metabolic burden due to heterologous protein production. A demonstration is present in the pioneering work conducted by Muhamadali et al. (2015) on a S. lividans strain producing the murine TNF-α, where heterologous protein expression determined profound changes in the metabolomics of the streptomycete causing an overflow of organic acids and sugars. In post-genomic era, a further ambitious goal is applying synthetic biology approaches for building a Streptomyces ‘super host’ with metabolic networks rewired to facilitate heterologous protein expression. Synergic application of genome minimization strategies (i.e., systematic removal of those elements – as secondary metabolites or proteases – that can hamper protein production) and engineering of translation and transcription machineries, might help reaching this goal (Kim et al., 2020).

To this end, it is encouraging considering that performing Streptomyces ‘super hosts’ have been already constructed for the heterologous production of antibiotics (Gomez-Escribano and Bibb, 2011; Myronovskyi et al., 2018). We believe that integrating these tools could help in improving streptomycetes as robust producers of recombinant proteins, increasing their competitiveness to other platforms and stimulating their large-scale application as cell factories.

**AUTHOR CONTRIBUTIONS**

FB and EB collected the data and analyzed them. FB, FM, and EB co-wrote the review. FB prepared the figure and the table. EB coordinated the work. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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