Inactivation of the 20S proteasome in *Streptomyces lividans* and its influence on the production of heterologous proteins

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Proteasomes are self-compartmentalizing proteases first discovered in eukaryotes but also occurring in archaea and in bacteria belonging to the order *Actinomycetales*. In bacteria, proteasomes have so far no known function. In order to evaluate the influence of the 20S proteasome on the production of heterologous proteins by *Streptomyces lividans* TK24, the production of a number of heterologous proteins, including soluble human tumour necrosis factor receptor II (shuTNFRII) and salmon calcitonin (sCT), was compared with the wild-type TK24, a proteasome-deficient mutant designated PRO41 and a strain complemented for the disrupted proteasome genes (strain PRO41R). *S. lividans* cells lacking intact proteasome genes are phenotypically indistinguishable from the wild-type or the complemented strain containing functional proteasomes. Using the expression and secretion signals of the subtilisin inhibitor of *Streptomyces venezuelae* CBS762.70 (Vsi) for shuTNFRII and those of tyrosinase of *Streptomyces antibioticus* (MelC1) for the production of sCT, both proteins were secreted in significantly higher amounts in the strain PRO41 than in the wild-type *S. lividans* TK24 or the complemented strain PRO41R. However, the secretion of other heterologous proteins such as shuTNFRI was not enhanced in the proteasome-deficient strain. This suggests that *S. lividans* TK24 can degrade some heterologous proteins in a proteasome-dependent fashion. The proteasome-deficient strain may therefore be useful for the efficient production of these heterologous proteins.

INTRODUCTION

There are many documented examples of eukaryotic proteins successfully secreted by *Streptomyces lividans*, such as soluble CD4 receptor (Fornwald et al., 1993), tumour necrosis factor-α (TNF-α) (Lammertyn et al., 1997) and salmon calcitonin (sCT) (Hong et al., 2003), which show that *Streptomyces* can be an efficient production system for a number of heterologous proteins (Binnie et al., 1997; Van Mellaert & Anné, 2001). The secreted recombinant proteins usually remain in a biologically active form, and the downstream purification is greatly simplified, as validated via the large-scale production of TNF-α (Pozidis et al., 2001). However, depending on the nature of the foreign protein the yield of some proteins is much lower than that of others (Lammertyn, 2000), implying the existence of secretion bottlenecks that can significantly reduce the yield of the desired protein in the supernatant. Such bottlenecks could be related to the characteristics of both the secreted protein and the translocation machinery of the host. Although *S. lividans* is a fairly well-characterized organism from a molecular and genetic viewpoint, the potential bottlenecks relating to the expression and secretion processes in *S. lividans* are presently poorly defined. *S. lividans* is relatively free of endogenous secreted proteases, which often hamper efficient production of heterologous proteins in other Gram-positive bacteria, e.g. *Bacillus subtilis* (Wu et al., 1991). However, N-terminal microheterogeneity of secreted proteins was observed from the earliest reports of heterologous expression in *S. lividans* (Chang & Chang, 1988; Van Mellaert et al., 1994). From then on, degradation of the heterologous proteins by endogenous protease activity of *S. lividans* has been well-documented, and intracellular and

Abbreviations: α-AE, α-amidating enzyme; AMC, 7-amino-4-methylcoumarin; DIG, digoxigenin-11-dUTP; EIA, enzyme immunoassay; sCT, salmon calcitonin; shuTNFRI, soluble human tumour necrosis factor receptor I; shuTNFRII, soluble human tumour necrosis factor receptor II; TNF-α, tumour necrosis factor-α.
extracellular proteases and proteases associated with the mycelium have been described (e.g. Binnie et al., 1995; Butler et al., 1994; Krieger et al., 1994). Recently, the 20S proteasome, a self-compartmentalizing proteolytic system, was identified in Streptomyces (De Mot et al., 1999). So far, no foreign protein degradation has been attributed to the Streptomyces proteasome.

The proteasome represents the major non-lysosomal proteolytic system in eukaryotes (reviewed by Hochstrasser, 1995; Voges et al., 1999). The 20S proteasome forms the catalytic core of the ATP-dependent 26S proteasome that mediates ubiquitin-dependent protein turnover. The proteolytic activity is confined to an inner compartment that is accessible only to unfolded proteins. The counterpart of Table 1.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>E. coli TG1</td>
<td>supE hsdA5 thi Δ(lac-proAB) F' [traD36 proAB+ lacIq lacZAM15]</td>
<td>Sambrook et al. (1989)</td>
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<tr>
<td>S. lividans TK24</td>
<td>str-6 SLP2- SLP3-</td>
<td>Kieser et al. (2000)</td>
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<tr>
<td>PRO41</td>
<td>Proteasome-deficient mutant of strain TK24 with neomycin phosphotransferase-encoding gene aph replacing part of the prcBA operon</td>
<td>This work</td>
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<tr>
<td>PRO41R</td>
<td>Proteasome complemented strain, strain PRO41 transformed with pROAPR</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUC18/19</td>
<td>E. coli general cloning vector with multiple cloning site and amp resistance gene</td>
<td>Sambrook et al. (1989)</td>
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<tr>
<td>pUC18-tsr</td>
<td>pUC18 with BclI fragment of thiostrepton resistance gene (tsr) fragment inserted into BamHI</td>
<td>This work</td>
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<tr>
<td>pGEM-T Easy</td>
<td>E. coli vector for cloning PCR amplified DNA fragments</td>
<td>Promega</td>
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<tr>
<td>pGEM-aph</td>
<td>pGEM-T Easy with the 1.23 kb PCR fragment of aph gene using pFD666 as template</td>
<td>This work</td>
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<td>pBS-CBSS</td>
<td>pBluescriptIKKS derivative containing the vsp promoter, the vsp ribosome-binding site, the vsp signal sequence and a part of the mature vsp gene</td>
<td>Lammertyn et al. (1997)</td>
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<tr>
<td>pCR2.1</td>
<td>E. coli vector for cloning PCR amplified DNA fragments</td>
<td>Invitrogen</td>
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<td>pCBS2TNFRII</td>
<td>pJl486 derivative containing shuTNFRII cDNA fused to the vsp gene with conservation of two codons of the mature vsp gene</td>
<td>This work</td>
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<td>pCBS2TNFRI</td>
<td>pJl486 derivative containing shuTNFRII cDNA fused to the vsp gene with conservation of two codons of the mature vsp gene</td>
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<td>pKC505</td>
<td>Cosmid vector carrying the apramycin resistance gene aac(3)IV</td>
<td>Richardson et al. (1987)</td>
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<td>pHSS19-prcBA</td>
<td>pHSS19 with the 5128 bp KpnI fragment of S. coelicolor A3(2) carrying the prcBA operon</td>
<td>R. De Mot</td>
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<td>pUC19-prcBA</td>
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<td>pUPK2</td>
<td>pUC19-prcBA with the internal 1.26 kb Scal–MluI fragment of the S. coelicolor A3(2) replaced with the 1.23 kb aph fragment</td>
<td>This work</td>
</tr>
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<td>pUTPK</td>
<td>pUC18-tsr with the KpnI fragment of prcBA::aph from pUPK2</td>
<td>This work</td>
</tr>
<tr>
<td>pFD666</td>
<td>Streptomyces shuttle cosmid vector, compatible with vectors derived from pJl101</td>
<td>Denis &amp; Brzezinski (1992)</td>
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<tr>
<td>pFAJ2784</td>
<td>pFD666 derived plasmid carrying the Xhol fragment of prcBA operon of Streptomyces coelicolor A3(2)</td>
<td>R. De Mot</td>
</tr>
<tr>
<td>pPROAPR</td>
<td>pFAJ2784 with aph gene replaced by a PstI–EcoRI fragment of apramycin resistance gene aac(3)IV</td>
<td>This work</td>
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<tr>
<td>pMSA</td>
<td>pJl680-derived plasmid carrying the expression cassettes of sCT-Gly and rat α-amidating enzyme (α-AE) using expression and secretion signals of melC1</td>
<td>Hong et al. (2003)</td>
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the proteasome is ubiquitous in Archaea (Dahlmann et al., 1989), but ubiquitin genes were not found in bacterial genomes sequenced so far. It was therefore unexpected that 20S proteasome homologues were discovered within the genome of Actinomycetes (Nagy et al., 1998), a lineage of high-GC Gram-positive bacteria, including Streptomyces coelicolor A3(2) (Nagy et al., 1998, 2003; De Mot et al., 1999), Mycobacterium leprae and Mycobacterium smegmatis (Knipef & Shrader, 1997), Mycobacterium tuberculosis (Darwin et al., 2003) and Rhodococcus erythropolis (Zuhl et al., 1997). No known function could be attributed to the 20S proteasome in bacteria so far, except for the M. tuberculosis proteasome that has been found to protect the organism against oxidative or nitrosative stress (Darwin et al., 2003). In bacteria, intracellular proteolysis is carried out by at least four other ATP-dependent, compartmentalized proteases of the ClpAP/XP, HslUV, FtsH and Lon families. For example, ClpP in Escherichia coli has been reported to be involved in response to conditions when foreign proteins are overexpressed (Gill et al., 2000). TepA of B. subtilis, showing sequence similarity to protease ClpP, was reported to be required for efficient translocation and processing of secretory proteins (Bolhuis et al., 1999a).

Until now the cellular targets for the bacterial 20S proteasome remain to be identified. In this paper, we report on the inactivation of the 20S proteasome in S. lividans TK24 and its role in the integrity of some secretory heterologous proteins including soluble human tumour necrosis factor receptor II (shuTNFRII) and sCT. Moreover, we also investigated whether a difference in susceptibility to proteasome-related hydrolysis might exist for proteins secreted either through the Sec- (for shuTNFRII and shuTNFRI) or Tat-dependent pathway (for cCT). We also discuss the potential of a proteasome-deficient mutant to increase the secretory production of relevant heterologous proteins in S. lividans.

**METHODS**

**Bacterial strains and culture conditions.** The strains used in this study are described in Table 1. E. coli TG1 was routinely cultured at 37 °C in Luria–Bertani medium, supplemented with 100 μg ampicillin ml⁻¹ when appropriate. For plasmid isolation, S. lividans strains were grown at 27 °C with continuous shaking at 300 r.p.m. in phage medium (Korn et al., 1978). When necessary, thiostrepton (5 μg ml⁻¹) and/or kanamycin (50 μg ml⁻¹) were added. For monitoring heterologous protein expression and secretion, 5 ml primary overnight cultures in phage medium was inoculated into 250 ml conical flasks containing 50 ml casein medium (Hong et al., 2003) or NM medium (Van Mellaert et al., 1994) and grown at 27 °C on a rotary shaker at 300 r.p.m. for several days. Spore isolation, protoplast formation and transformation of S. lividans were carried out as described by Kieser et al. (2000).

**DNA manipulations.** All DNA manipulations used in this work were performed by standard procedures (Sambrook et al., 1989). Restriction endonucleases and other DNA-modifying enzymes were purchased from Roche Diagnostics, Eurogentec and Life Technologies. Oligonucleotides were obtained from Amersham Pharmacia Biotech. PCR was carried out with Pfu polymerase (Stratagene). DNA sequence analysis was performed according to the dideoxy chain-termination method with a Thermo sequence fluorescently labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) on an ALFexpress apparatus (Amersham Biosciences). For Southern blot analysis, relevant DNA fragments were labelled with digoxigenin-11-dUTP (DIG) according to a method described by Engler-Blum et al. (1993). Hybridizing DNA was detected with 0-25 mM CDP-Star as the substrate (Hoeltke et al., 1995). Photo emission was visualized using the CURIX 60 apparatus (Aglä-Gevaert).

**Plasmid constructions.** The plasmids used and constructed in this study are listed in Table 1.

To inactivate the 20S proteasome of S. lividans, a suicide vector for streptomycetes, pUTPK, was constructed using the cloned prcBA operon, encoding β- and -subunits of the 20S proteasome (Fig. 1a) (GenBank accession no. AF086832) of S. coelicolor A3(2), which is closely related to S. lividans (Kawamoto & Ochi, 1998). The prcBA operon was deleted by replacement of 67 % of prcB and 83 % of prcA with the aph (aminoglycoside phosphotransferase) gene from...
Tn5 (prcBA::aph allele, Fig. 1a) conferring resistance to kanamycin. For this purpose, the following steps were carried out. (i) The KpnI fragment of S. coelicolor prcBA operon (5·1 kb) present on pHS19-prcBA was cloned into the pUC19 KpnI site, resulting in plasmid pUC19-prcBA. (ii) The internal 1261 bp Scal–Mfdl fragment, containing part of the prcB and prcC ORFs, was removed by partial digestion with Scal (a site located in pUC19). (iii) Subsequently, it was digested with Mfdl and the protruding ends were filled in using the Klenow polymerase reaction. (iv) The aph gene fragment present in pFD666 (Denis & Brzezinski, 1992) was amplified by PCR using oligonucleotides KAN1 (5′-CAGGGGGGGCGGAGCTGAT-3′) and KAN2 (5′-TACTGCCGCCCGGATCCAGA-3′). (v) The amplified fragment was cloned into pGEM-T Easy, resulting in the plasmid pGEM-aph. (vi) After digestion with EcoRI, the obtained aph fragment (1227 bp) was purified and blunt-ended prior to ligation to the restricted pUC19-prcBA, giving rise to plasmid pUPK2. (vii) Finally, the prcBA::aph fragment from pUPK2 was isolated and cloned into the KpnI site of pUC18-ts, resulting in the suicide plasmid pUPTK. pUPTK also contains a thioesterase resistance gene encoding 23S A1067 rRNA methylase from Streptomyces azureus (Kieser et al., 2000).

To complement the chromosomal deletion in the constructed 20S proteasome knock-put mutant of S. lividans, the complementing plasmid pPROAPR was constructed. Therefore, pFA2784 (kindly provided by R. De Mot, K. U. Leuven, 3001 Heverlee, Belgium), containing the XhoI fragment of the S. coelicolor A3(2) 20S proteasome operon in pFD666, was used. A 1·4 kb PstI–EcoRI fragment containing the apramycin resistance gene aac(3')IV was isolated from pKCS05 (Richardson et al., 1987), blunt-ended and ligated with the 6·9 kb HindIII–ClaI blunt-ended fragment from pFA2784, resulting in plasmid pPROAPR (Fig. 1b).

For the secretory production of shuTNFRII in S. lividans, the expression vector pCBS2TNFRII was made. To this purpose, the DNA fragment encoding shuTNFRII was isolated from pFD666 by PCR using the primers TNFRII-A (5′-GATGCAGCTCCTCCAGTGGCTG4-3′), the underlined sequence indicates the restriction enzyme recognition sequence for BglII) and TNFRII-B1 (5′-AGAAGCCTTCGCCCGAAGCAGGCTG4-3′) the underlined sequence indicates the restriction enzyme recognition sequence for HindIII). The bold characters indicate the TGA stop codon in the complemented reversed sequence) and cloned into pCR2.1 by TA cloning (Invitrogen). The shuTNFRII coding sequence fragment was excised from this plasmid by NsiI. After blunt-ending using T4 DNA polymerase and digestion with HindIII, this fragment was cloned into the DraII-digested, Klenow polymerase blunt-ended and HindIII-digested pBS-CBSS (Lammertyn et al., 1997). As such the shuTNFRII coding sequence could be inserted after the regulatory sequences of Streptomyces venezuelae subtilisin inhibitor (svi) gene consisting of the vsg promoters, ribosome-binding site, signal sequence and two codons of the mature vsg gene. The in-frame fusion was confirmed by DNA sequence analysis using the primer DraII SEQ (5′-CGTGGCAGCTCCTCCAGTGGCTG4-3′). Next, the entire expression cassette excised by HindIII and BamHI digestion was cloned into pIJ482 (Ward et al., 1986), similarly digested, resulting in plasmid pCBS2TNFRII.

Similarly, the expression vector pCBS2TNFRII was constructed for secretory production of soluble human tumour necrosis factor receptor I (shuTNFRI). The expression plasmid pMSA was constructed as described previously (Hong et al., 2003) for the secretion of salmon calcitonin (sCT).

**Growth analysis.** To evaluate the effect of the 20S proteasome on the growth of S. lividans, the wild-type strain TK24, strain PRO41 with a deleted 20S proteasome and strain PRO41R, containing an intact 20S proteasome on a plasmid for complementation of the chromosomally deleted 20S proteasome, were grown in casein medium and NM medium. At indicated time intervals during growth, cells of 5 ml cultures were collected by centrifugation, washed twice with 50 mM Tris/HCl, pH 7·0, and then dried at 100 °C to a constant weight.

Growth was also assessed qualitatively on solid media. A 20 μl volume of spore suspension was spread on the surface of solid minimal and R2YE medium (Kieser et al., 2000), and plates were incubated at 28 and 37 °C for 7 days. The conditions used were essentially those described by Knipfer & Shrader (1997). To analyse the effect of growth under stress conditions, the R2YE medium was supplemented with ethanol (7%, v/v), cadmium chloride (0·1 mM), puromycin (0·1-0·01 mg ml⁻¹) or sodium chloride (6 or 7%, w/v) for the induction of stress.

**Proteasome activity assays.** The 20S proteasome activity was detected by zymography using Suc-LLVY-AMC (succinyl-Leu-Leu-Val-Tyr-7-aminomethylcumarin) as a substrate, as described previously by Pouch et al. (2000). The wild-type strain TK24, deletion mutant PRO41 and the complemented strain PRO41R were cultured for 72 h in NM medium. The mycelium was subsequently collected by centrifugation, washed twice, and resuspended in 5 ml 50 mM Tris/HCl (pH 8·0). The cells were lysed on ice in a sonicator (B. Braun Biotech International Gmbh) and the cell debris was removed by centrifugation at 4 °C. Cell extracts were analysed by native 8% PAGE and a subsequent overlay of the gel with 5 ml 50 mM Tris/HCl (pH 8·0) supplemented with 0·1 mM fluorescent substrate Suc-LLVY-AMC. After incubation for 30 min at 37 °C, the result of the proteasome activity was visualized under UV illumination.

**Western blot analysis.** To assess the secretory production of shuTNFRII and shuTNFRI in the wild-type and proteasome mutants of S. lividans TK24, Western blot analysis was performed. At the indicated time intervals, the culture supernatants of S. lividans containing the specific vector were sampled and immediately stored at −20 °C. To 1 ml of ice-chilled supernatants 0·5 ml ice-cold 60% TCA was added and subsequently thoroughly mixed. After an additional hour on ice, the mixture was centrifuged at 4 °C for 10 min at 12000 g. The pellets were washed in 95% ethanol, dried in vacuum and dissolved in 2× loading buffer. Proteins were separated by 10% SDS-PAGE (Laemmli, 1970) and then blotted onto nitrocellulose membranes using a semi-dry electrophoretic apparatus (Bio-Rad Laboratories). Efficient transfer of the proteins to the membrane was inspected by non-permanent staining with 0·1% Coomassie R-250. The immunoreactive proteins were detected using diluted solution of goat anti-human NFκB and hTNFRI antibodies (R&D Systems) followed by alkaline phosphatase-conjugated rabbit anti-goat antibodies (Sigma).

**Enzyme immunoassay (EIA).** Recombinant sCT secreted by wild-type and proteasome mutants of S. lividans TK24 was quantified by EIA. At the indicated time intervals, the culture supernatants of S. lividans strains containing sCT secretory vector pMSA were sampled and immediately stored at −20 °C. Supernatants were diluted in the assay buffer supplied by the manufacturer and detected with the peptide enzyme immunoassay kit (Peninsula Laboratories) as described by the manufacturer.

**RESULTS**

**Construction of S. lividans TK24 proteasome-deficient mutant and its complemented strain** Using pUPTK, the chromosomal prcBA operon of S. lividans TK24 was replaced by prcBA::aph allele in two steps
A first-round selection identified cells that contained a wild-type prcBA operon and the prcBA::aph allele, separated by the pUC18 and thio-strepton resistance gene sequences. Double homologous recombinants were selected by screening for colonies resistant to kanamycin but sensitive to thiostrepton. Double homologous recombination resulting in the replacement of the ScaI–MluI fragment of the proteasome operon with the aph gene was verified by DNA hybridization experiments using the ScaI–MluIf r a g m e n t o f prcBA operon as a probe (Fig. 2).

One proteasome-negative mutant containing the expected gene disruption was obtained and designated PRO41. In order to restore the proteasome expression in PRO41 and to verify its influence on the production of heterologous proteins, the S. coelicolor A3(2) prcBA operon was reintroduced into the proteasome-negative strain PRO41, using the plasmid pPROAPR (Fig. 1b) carrying the S. coelicolor A3(2) prcBA operon. The construct was a pFD666-based plasmid, which is compatible with vectors containing the pIJ101-ori, often used as the basis of protein expression vectors (Denis & Brzezinski, 1992). In this plasmid, the aph gene was replaced by the apramycin resistance gene aac(3)IV for the selection of transformants from PRO41, which already contains an aph gene in the prcBA site. After introduction of the plasmid pPROAPR into S. lividans PRO41, apramycin-resistant colonies were selected, and the presence of pPROAPR was demonstrated by restriction analysis of isolated plasmids. The resulting complemented strain was designated PRO41R.

To confirm that disruption of the prcBA operon inactivated the 20S proteasome, the proteasomal activity of wild-type and mutant strains was characterized by zymography. Cell lysates prepared from S. lividans TK24, the null mutant (PRO41) and the complemented strain (PRO41R) were compared for their ability to release the fluorophore AMC from the proteasome-specific substrate Suc-LLVY-AMC under the action of the chymotrypsin-like activity of the proteasome (Tamura et al., 1995). The free AMC fluorescence can efficiently be detected at 380 nm excitation and a 460 nm emission filter.

The release of the AMC moiety was detected by zymography and the results (Fig. 3) indicated that replacement of the prcBA operon with the aph gene in PRO41 abolished the 20S proteasomal activity of S. lividans TK24. On the other hand, in PRO41R, an S. lividans strain that contained the plasmid pPROAPR carrying prcBA operon of S. coelicolor A3(2), the proteasome activity was restored. Moreover, the proteasomal activity in PRO41R was higher than in S. lividans TK24, probably due to the reintroduction of the prcBA operon on a high copy number plasmid derived from pFD666.

The 20S proteasome-deficient strain PRO41 was viable and, with respect to growth in different liquid media, indistinguishable from the wild-type strain TK24 and the complemented strain PRO41R (data not shown). On solid media the proteasome mutant and complemented strain sporulated normally and the morphology of their colonies was identical to those of the parental strain. In addition to

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**Fig. 2.** Characterization of the generated mutant PRO41. Southern hybridization using the 1·26 kb Scal–Mlu fragment of prcBA operon to Ncol- (lanes 1 and 2, 2·1 kb) and SmaI- (lanes 3 and 4, 1·8 kb) digested chromosomal DNA from TK24 (lanes 2 and 4) and PRO41 (lanes 1 and 3).

**Phenotypic characterization of the S. lividans proteasome mutants**

To confirm that disruption of the prcBA operon inactivated the 20S proteasome, the proteasomal activity of wild-type and mutant strains was characterized by zymography. Cell lysates prepared from S. lividans TK24, the null mutant (PRO41) and the complemented strain (PRO41R) were compared for their ability to release the fluorophore AMC from the proteasome-specific substrate Suc-LLVY-AMC under the action of the chymotrypsin-like activity of the proteasome (Tamura et al., 1995). The free AMC fluorescence can efficiently be detected at 380 nm excitation and a 460 nm emission filter.

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**Fig. 3.** Zymography of the proteasome activity in the 72 h culture filtrate of S. lividans TK24, PRO41 and PRO41R. The 20S proteasome activity was detected using Suc-LLVY-AMC as a substrate, and the release of the AMC moiety was visualized under UV illumination. The arrow shows the 20S proteasome active bands with fluorescence. Lane 1, S. lividans TK24; lane 2, PRO41; lane 3, PRO41R.
standard growth conditions, the ability of cells of *S. lividans* TK24, PRO41 and PRO41R to grow under different kinds of environmental stress was analysed. Growth of the proteasome-deficient mutant could also not be distinguished from that of the wild-type strain and PRO41R based on sensitivity to ethanol (organic solvent stress), cadmium ions (heavy metal stress), puromycin (generating misfolded puromycyl-containing peptides) or high salt concentration (osmotic stress). Hence, there was no obvious growth- or stress-related phenotype for the proteasome-deficient mutant PRO41 compared to TK24 and PRO41R.

The influence of the 20S proteasome on the yield of secreted shuTNFRII

The heterologous expression cassette of shuTNFRII was constructed by fusion of the coding sequence of the extracellular domain of human TNFRII downstream of the signal peptide and the first 2 aa of the mature *S. venezuelae* subtilisin inhibitor (Vsi) (Van Mellaert *et al.*, 1998). The expression plasmid pCBS2TNFRII constructed from pIJ486, containing a pIJ101 ori, was introduced by transformation into *S. lividans* TK24, PRO41 and PRO41R. The three strains carrying pCBS2TNFRII multiplied at similar growth rates in casein medium, resembling the growth curve of their parent strains (data not shown). The amount of secreted shuTNFRII was detected by Western blot analysis, and the results (Fig. 4) showed that the three strains differed markedly. In *S. lividans* TK24, the secretory production of shuTNFRII was almost undetectable, suggesting some bottlenecks in the expression and/or secretion pathway. When the proteasome-deficient strain PRO41 was used to express shuTNFRII in casein medium, a band with a predicted molecular mass of shuTNFRII (ca. 26 kDa) could be detected by Western blot analysis. A full-length product, but also a specific smaller band (ca. 22 kDa), could be detected through 24–72 h. The latter band was probably a breakdown product of the intact shuTNFRII. When the proteasome operon was reintroduced into the deficient strain resulting in *S. lividans* PRO41R, shuTNFRII was again undetectable. This result indicates that 20S proteasome hampers the extracellular accumulation of shuTNFRII in *S. lividans* TK24.

In NM medium, the production of shuTNFRII could only be observed in the proteasome-deficient strain PRO41. However, some differences in secretion of shuTNFRII by *S. lividans* PRO41 have been detected in NM and casein medium (data not shown). In NM medium, the existence of full-length shuTNFRII (26 kDa) could only be detected at 24 h, but later on it was quickly degraded to small molecular mass fragments as detected by Western blotting.

The influence of proteasome on the yield of secreted sCT

For secretory production of C-terminal amidated sCT, the promoter, ribosome-binding site and signal peptide of *melCl* from *Streptomyces antibioticus* (Bernan *et al.*, 1985) was used to construct the expression cassettes of sCT-Gly and rat α-amidating enzyme (α-AE). The latter enzyme is responsible for the conversion of glycine-extended substrates to the biologically active, C-terminal amidated products. The expression plasmid pMSA (Hong *et al.*, 2003), which contains both expression cassettes, was constructed from the pIJ101-ori containing pIJ680. *S. lividans* TK24,

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**Fig. 4.** Western blot analysis for the detection of shuTNFRII present in the culture filtrates of *S. lividans* TK24 (lanes 1, 4 and 7), PRO41 (lanes 2, 5 and 8) and PRO41R containing the pCBS2TNFRII plasmid (lanes 3, 6 and 9). The strains were cultivated in casein medium for 24 h, (lanes 1–3), 48 h (lanes 4–6) and 72 h (lanes 7–9).

**Fig. 5.** Amount of sCT produced by *S. lividans* TK24, PRO41 and PRO41R each containing pMSA measured by EIA. The strains were cultivated in casein medium (a) and NM medium (b) for 24, 48 and 72 h.
strain PRO41 and PRO41R were each transformed with pMSA, and the three strains carrying pMSA grew at similar growth rates in NM and casein medium, and resembled the growth curve of their parent strains (data not shown). sCT yield was detected by EIA. The results (Fig. 5) showed that, in both NM and casein medium the sCT expression level by the proteasome-deficient strain PRO41 was much higher than that of the wild-type TK24 strain or the complemented proteasome-deficient strain PRO41R. This result indicates that the production of sCT in S. lividans TK24 is also hampered by the 20S proteasome.

The influence of the proteasome on the yield of shuTNFRI

The same construct strategy as for shuTNFRII (fusion of the coding sequence of heterologous proteins after the signal peptide and 2 aa of mature protein of Vsi) was also used to express shuTNFRI. The yield of this protein was compared with the proteasome-deficient PRO41 and the wild-type TK24 strain. Not much difference in yield of shuTNFRI could be observed for the wild-type and the proteasome null mutant in NM medium (Fig. 6). Moreover, when cultured in casein medium, the yield of PRO41 was even lower than that of TK24 (data not shown). Hence, for shuTNFRI the presence of the proteasome does not seem to affect the production of this heterologous protein.

DISCUSSION

The proteasome is a self-compartmentalizing protease first discovered in eukaryotes. Protein turnover in the eukaryotic cytosol is mediated predominantly by the proteasome, which is essential for cell viability (reviewed by Hochstrasser, 1995; Voges et al., 1999). However, much less is known about proteasomes in prokaryotes, where ubiquitin is exceptional (De Mot et al., 1999). In the archaeon Thermoplasma acidophilum, specific inhibition of proteasome activity impaired cell viability following heat shock but not under normal growth conditions (Ruepp et al., 1998). Here, the 20S proteasome of S. lividans TK24 was shown to have no effects on cell growth under normal and stress conditions. This is consistent with previous reports where there was no obvious growth- or stress-related phenotype for several bacteria with a deleted proteasome, e.g. S. coelicolor A3(2) (Nagy et al., 2003) and M. smegmatis (Knipfer & Shrader, 1997). The proteasome of M. tuberculosis is likely to be an exception, because the organism has an evolutionary reduced genome, indicating that non-essential functions have been selectively lost since this bacterium diverged from other mycobacteria (Sassetti et al., 2003), while the 20S proteasome has evolved to resist elimination by the host-immune response (Darwin et al., 2003). In this paper, we described for the first time that 20S proteasome influenced the secretory expression of some heterologous proteins in S. lividans, including shuTNFRII and sCT.

Streptomyces has a naturally high secretion capacity and as a consequence is used as an efficient host for the production of some heterologous proteins (Binnie et al., 1997; Pozidis et al., 2001; Van Mellaert & Anne, 2001). Most proteins are secreted via the Sec-dependent pathway (reviewed by Mori & Ito, 2001), but also the recently detected Tat pathway is functional in Streptomyces (Schaerlaekens et al., 2001). The latter pathway can translocate folded proteins, while the Sec-dependent pathway translocates newly synthesized polypeptides in an unfolded conformation with the help of chaperones. So far, heterologous protein secretion has mostly been investigated using signal peptides that direct the secretion to the Sec pathway, but the Tat pathway can also translocate heterologous proteins, although less efficiently for the proteins tested (Schaerlaekens et al., 2004).

To investigate the possible influence of the proteasome on heterologous protein production, the yields of shuTNFRII, sCT and shuTNFRI were compared with the wild-type S. lividans TK24 strain, a 20S proteasome null mutant obtained by replacement of the 20S proteasome with the aph gene, and with a complemented 20S proteasome deletion strain carrying the 20S proteasome gene on a multicopy plasmid.

Two different types of signal peptide-dependent secretion have been used in the experiments described. The Vsi signal peptide of the S. venezuelae subtilisin inhibitor, which is Sec-dependent, has been successfully used to produce mouse TNF-α in large amounts (Lammertyn et al., 1997). However, when the same construct strategy (fusion of the coding sequence of heterologous proteins after the signal peptide and 2 aa of mature protein of Vsi) was used to express some other eukaryotic proteins including huTNF-α, huTNF-β, shuTNFRI and shuTNFRII, the yield was variable (Lammertyn, 2000). This indicates the existence of bottle-necks in the expression and/or the secretion of a number of heterologous proteins in S. lividans.

sCT was secreted under the control of the melC1 promoter and the Tat-dependent signal peptide of MelC1. The latter is essential for the secretion of the S. antibioticus tyrosinase MelC2 by forming a MelC1–MelC2 complex before export (Schaerlaeken et al., 2001). MelC1 has been successfully used to secrete some heterologous proteins in S. lividans

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**Fig. 6.** Western blot analysis of shuTNFRI in culture filtrates of S. lividans TK24 (lanes 1 and 2) and PRO41 containing the pCBS2TNFRI (lanes 3 and 4). Samples were taken at 24 h (lanes 1 and 3) and at 48 h (lanes 2 and 4).
The yield of shuTNFRII using expression/secretion signals of Vsi and of sCT directed by MelC1 differed significantly for *S. lividans* TK24, the proteasome-deficient strain PRO41 and the 20S proteasome complemented strain PRO41R, with increased levels of heterologous proteins observed only in the 20S proteasome-deficient strain, but not in the wild-type nor in the complemented strain. This difference suggests that the 20S proteasome in *S. lividans* may be important for the production of some heterologous proteins. The reason for this might be that the heterologous proteins may be captured by the 20S proteasome and degraded before being recognized by the secretion machinery of *S. lividans*, including the Sec-dependent as well as the Tat-dependent pathway.

However, the production of another heterologous protein tested, i.e. shuTNFRI, was not affected by the presence of the 20S proteasome. Hence, the 20S proteasome seems to be a secretion bottleneck for some heterologous proteins while not for others, probably depending on the characteristic of the protein to be secreted. This protease vulnerability and the presence of secretion bottlenecks is consistent with what has been reported for *B. subtilis* (Bolhuis et al., 1999b), in which the secretion bottlenecks differed for each heterologous protein tested and the identified secretion bottlenecks were at late stages in the secretion process, such as efficient processing by signal peptidase, passage through the cell wall, proteases secreted into the medium as well as cell-associated proteases, which are responsible for the degradation of secreted heterologous proteins. In *S. lividans*, similar secretory bottlenecks may exist when heterologous proteins are expressed, although our knowledge about this is quite fragmentary. The endogenous proteases produced by *S. lividans* intracellularly (Butler et al., 1994), extracellularly (Lichenstein et al., 1992; Butler et al., 1995; Krieger et al., 1994; Mori & Ito, 2001) or cell wall-associated (Binnie et al., 1995) have been investigated to be bottlenecks for the production of some heterologous proteins. The results obtained here show that the 20S proteasome seems to be a bottleneck for some heterologous proteins produced in *S. lividans* such as shuTNFRII and sCT but not for others as illustrated with shuTNFRI. For the proteins vulnerable for proteasome degradation, the expressed pre-protein may be folded prior to or during secretion, and not efficiently adapted to the Sec- or Tat-dependent pathway apparatus, and as a consequence subjected to the degradation of 20S proteasome.

In conclusion, this report clearly indicates that the proteasome could be an as yet not identified bottleneck in the expression and secretion of some heterologous proteins by *S. lividans*. More importantly, the results presented clearly point out that the optimization of the production of specific heterologous protein(s) is likely to require engineered *S. lividans* strains, in which the proteasome has been removed. With the genetic amenability of this organism and increasing knowledge of the bottlenecks in expression and secretion, it will be possible to engineer the dedicated *S. lividans* host strains for the production of a wide range of heterologous proteins at commercially significant levels.

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of intracellular protein degradation.


