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Appl Microbiol Biotechnol (2012) 96:1301–1312
DOI 10.1007/s00253-012-4490-5

GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach

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Received: 10 September 2012 / Revised: 30 September 2012 / Accepted: 1 October 2012 / Published online: 16 October 2012

Abstract Streptomycetes are proficient producers of enzymes and antibiotics. When grown in bioreactors, these filamentous microorganisms form mycelial pellets that consist of interconnected hyphae. We here employed a flow cytometry approach designed for large particles (COPAS) and demonstrate that liquid-grown *Streptomyces* cultures consist of two distinct populations of pellets. One population consists of mycelia with a constant mean diameter of approximately 260 μm, whereas the other population contains larger mycelia whose diameter depends on the strain, the age of the culture, and medium composition. Quantitative proteomics analysis revealed that 37 proteins differed in abundance between the two populations of pellets. Stress-related proteins and biosynthetic proteins for production of the calcium-dependent antibiotic were more abundant in the population of large mycelia, while proteins involved in DNA topology, modification, or degradation were overrepresented in the population of small mycelia. Deletion of genes for the cellulose synthase-like protein CslA and the chaplins affected the average size of the population of large pellets but not that of small pellets. Considering the fact that the production of enzymes and metabolites depends on pellet size, these results provide new leads toward rational strain design of *Streptomyces* strains tailored for industrial fermentations.

Keywords Heterogeneity · Fermentation · COPAS · Cell wall · Proteomics

Introduction

Streptomycetes are Gram-positive mycelial soil bacteria that are commercially very attractive for the production of a wide range of natural products such as antibiotics, anticancer agents, and immunosuppressants (Hopwood 2007).

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4490-5) contains supplementary material, which is available to authorized users.

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Moreover, streptomycetes produce a plethora of enzymes that allow them to utilize almost any naturally occurring polymer, such as cellulose, mannan, chitin, xylan, starch, glycan, and agar. Many of these enzymes are industrially important for the conversion of renewable energy sources (Vrancken and Anné 2009). Unlike unicellular bacteria, which grow exponentially by binary fission with a constant generation time (Errington et al. 2003), streptomycetes grow by hyphal extension, with branching adding the exponential component. During growth, vegetative hyphae are divided into compartments by cross-walls (Chater and Losick 1997; Jakimowicz and van Wezel 2012). The reproductive phase is initiated by the erection of sporogenic structures called aerial hyphae, which differentiate following a complex cell division event whereby the multigenomic hyphae are converted into chains of unigenomic spores. Aerial hyphae are formed only on solid-grown cultures, giving the colonies their characteristic white and fluffy appearance (Kelemen and Buttner 1998; Flärdh and Buttner 2009). The transition from vegetative to aerial growth is accompanied by the production of an extracellular protein layer, composed of chaplins and rodlins, which makes aerial hyphae hydrophobic (Claessen et al. 2002, 2003, 2004; Elliot et al. 2003). During the formation of aerial hyphae, numerous secondary metabolites are produced, including many antibiotics (van Wezel and McDowall 2011).

Industrial-scale production with streptomycetes occurs in large bioreactors. Under these conditions, pellets are formed that consist of interconnected hyphae (Celler et al. 2012). Such mycelial pellets have very different sizes. Elongation and branching of hyphae, as well as fragmentation and lysis are regarded as factors controlling the size and morphology of these multicellular structures (Nielsen et al. 1995; Nielsen 1996). Moreover, the cell wall plays an important role in pellet architecture. Overexpression of the actinomycete-specific cell division protein SsgA, which controls septum-site localization (Willemse et al. 2011) and several aspects of cell wall homeostasis (Noens et al. 2007), leads to increased fragmentation and hence smaller pellets. Interestingly, this is accompanied by a strongly increased enzyme production (van Wezel et al. 2006). Likewise, the absence of the cellulose synthase-like protein CslA in *Streptomyces coelicolor* (Xu et al. 2008; de Jong et al. 2009b) has a profound effect on mycelial growth, leading to a significant decrease in pellet size. CslA produces a polysaccharide polymer at the hyphal tip, which has been suggested to be cellulose (Xu et al. 2008; de Jong et al. 2009b). This secreted polysaccharide is thought to maintain the integrity of the hyphal tip that is subject to constant remodeling due to ongoing cell wall synthesis orchestrated by DivIVA (Flärdh 2003). Taken together, these observations show the importance of the cell wall in the establishment and stability of pellet architecture.

Flow cytometry has been used to study populations of single-celled organisms (Hutter and Eipel 1979; Phillips and Martin 1983). This method could not be used for the multicellular pellets of *Streptomyces* that are simply too large to pass the nozzle. Recently, the COPAS (Complex Object Parametric Analyzer and Sorter) platform has been used to analyze pellets of the fungus *Aspergillus niger* with sizes ranging from 30 to 750 microns (de Bekker et al. 2011). We here, for the first time, use this platform to analyze bacterial multicellular structures. Our work demonstrates that *Streptomyces* liquid cultures consist of two distinct populations of pellets that differ in size. Stress-related proteins are more abundant in the population of large pellets, while proteins involved in DNA topology, modification, or degradation are more abundant in the population of small pellets. The size of the larger pellets, but not that of the smaller pellets, was species- and medium-dependent.

Materials and methods

Strains and culture conditions

The strains used in this study are shown in Table S1. Media were prepared as described (Kieser et al. 2000). *Streptomyces* species were grown at 30° on solid R5 or MS agar plates, or as shaken cultures (180 rpm) in 250-ml flasks equipped with coil springs containing 100 ml YEME, TSBS, R5 with glucose, or NMMP-defined medium containing glucose (Kieser et al. 2000). Liquid media were inoculated with 10^6 spores ml^{-1}.

Flow cytometry using the COPAS Plus

Pellets were harvested and fixed with 4 % formaldehyde for 30 min on ice. They were washed twice with phosphate-buffered saline (PBS) and stored at −20 °C until further use. Pellets were analyzed based on size (time-of-flight [TOF]) using a COPAS Plus profiler equipped with a 1-mm nozzle (Union Biometrica, Holliston, MA). The flow design of the COPAS Plus is such that pellets pass the laser beam over their longitudinal axis. All data points with an extinction [EXT]≥25 (thus excluding single hyphae and debris) were used for subsequent analysis.

Statistical analysis

The statistical analyses were performed as described by van Veluw et al. (2012). Briefly, the log-transformed datasets were fit by a probability distribution assuming two normal distributions (Vinck et al. 2005). This model determines five parameters: the participation fraction (p), two means (μ_1; μ_2), and two standard deviations (σ_1; σ_2). For each parameter, the 95 % confidence interval (CI) estimate was obtained by refitting with the model after bootstrapping (1,000 replicates) using the open source Scilab language. Datasets with non-overlapping CIs of

the mean and a $0.025<p<0.975$ were considered to be derived from a culture with two populations of pellets.

Proteomics analysis

Mycelia from 48-h-old YEME-grown cultures were washed in PBS and sorted using the COPAS Plus. Proteomics experiments were carried out as detailed in Gubbens et al. (submitted). Briefly, size-fractionated mycelia were sonicated for 5 min at 12 W output power using 5:5 s on/off intervals in 100 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, and 5 mM dithiothreitol, after which the debris was removed by centrifugation (16,000×*g* for 10 min at 4 °C). The 110 μg of total protein per sample was precipitated using chloroform/methanol (Wessel and Flügge 1984). Protein samples were digested with trypsin as described (Piette et al. 2005) and dried in a vacuum concentrator. Samples were then labeled using stable isotope dimethyl labeling on-column using Sep-Pak C18 200 mg columns (Waters) as described (Boersema et al. 2009). Labeled peptides were mixed 1:1, dried, and dissolved in ~1 mL 5 % formic acid. The mixture was fractionated by cationic exchange (SCX) using a polysulfoethyl A column (PolyLC, 100×2.1 mm; particle size, 5 μm; average pore size, 200 Å; column volume, 0.346 ml) and 0.5 ml sample loaded on the column. Mobile phases were SCX A (10 mM KH_2PO_4, 20 % (*v*/*v*) acetonitrile, pH 3) and SCX B (10 mM KH_2PO_4, 20 % (*v*/*v*) acetonitrile, 0.5 M KCl, pH 3). Peptides were fractioned at a flow rate of 250 μl/min with a gradient of 0–18 % SCX B in 18 CV, 18–30 % SCX B in 6 CV, and 30–100 % SCX B in 5 CV. In total, 24 peptide fractions were collected for liquid chromatography mass spectrometry analysis on an LTQ-Orbitrap setup. Data analysis was performed using MaxQuant 1.2.2.5 (Cox and Mann 2008). Tandem mass spectra were searched against the UniProt *S. coelicolor* reference proteome set (organism 100226, excluding SCP1 plasmid proteins, version 2012_06) with a false discovery rate of 1 % for both proteins and peptides, and an additional second peptide search was performed (Cox et al. 2011). Low-scoring versions of already identified peptides were retained for quantification, and the minimum peptide ratio count for quantification was set to 3. For all expression ratios, a B significance (Cox and Mann 2008) was calculated using Perseus 1.3.0.4 (part of the MaxQuant suite) and results filtered based on a Benjamini–Hochberg false discovery rate of 5 %. Subsequently, proteins with a significant expression ratio that was based on only three or four quantification events and a variability of >150 % were removed, since these quantifications could be regarded as dubious. Protein descriptions were taken from StrepDB (The *Streptomyces* Annotation Server, http://strepdb.streptomyces.org.uk).

Results

Application of large-particle flow cytometry for size fractionation of mycelia

S. coelicolor forms pellets of variable size in liquid-grown cultures (Fig. 1a). To analyze the size distribution, large-particle flow cytometry was deployed using a COPAS Plus. *S. coelicolor* pellets were analyzed on the basis of their TOF in milliseconds (Fig. 1b). To relate the pellet diameter to TOF, 27 pellets were measured by microscopy (Fig. 1c). This revealed a relationship between TOF and pellet diameter whereby the diameter equals 0.57 X TOF+159 μm.

To analyze the size distribution of the *S. coelicolor* pellets, the TOF of the events with an extinction≥25 were divided by the mean TOF of the population. Mathematical modeling of the pooled data of biological triplicates showed that the pellet size in liquid-grown cultures was not normally distributed when YEME, NMMP, R5, or TSBS were used as growth medium. Instead, the size distribution could be fitted assuming the existence of two normally distributed populations (Fig. 2; Table 1). These two populations were observed between day 1 and day 7 (Fig. 2; Table 1). The average diameter (μ_1) of small pellets was 266 μm (±10 %) regardless of the medium that was used or the age of the cultures. In contrast, the average diameter (μ_2) of large pellets varied in time (e.g., varying between 454 and 728 μm in TSBS). A

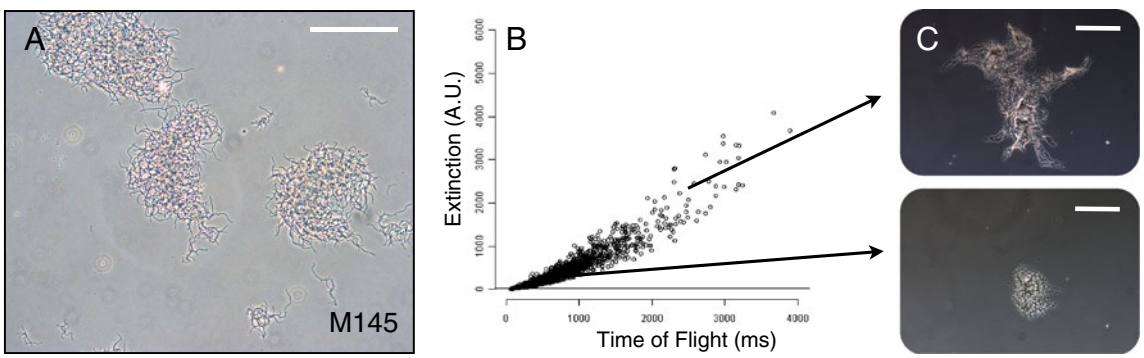

Fig. 1 Heterogeneity in pellet size in liquid-grown YEME cultures of *S. coelicolor*. Size distribution of pellets (**a**) was analyzed by COPAS (**b**). The COPAS technology allows separation of colonies according to size (**c**). *Bars* represent 400 (**a**) and 300 μm (**c**)

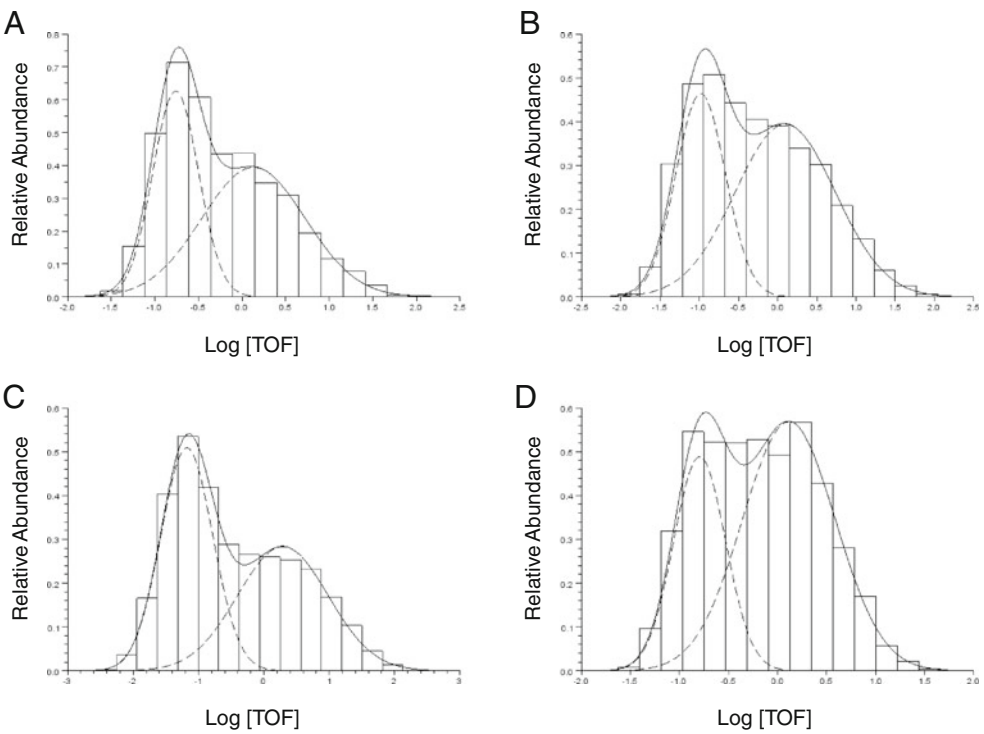

Fig. 2 Relation of medium and size distribution of pellets in liquid-grown cultures of *S. coelicolor*. *S. coelicolor* was grown for 24 h in NMMP with glucose (**a**), R5 (**b**), TSBS (**c**), or YEME (**d**). Two populations of pellets that differ in size were detected in all media (see also Table 1)

difference in the diameter of large pellets was also observed between different media (varying from 457 μm in case of NMMP to 728 μm in case of TSBS medium; Table 1). The populations of large pellets formed in R5- and YEME-grown cultures reached their maximal size (521 μm in case of R5 and 474 μm in case of YEME medium; Table 1) after 48 h of

Table 1 Heterogeneity between pellets in liquid-grown cultures of *S. coelicolor*

Medium	Time, h	μ_1, μm	95 % CI (μ_1, μm)	μ_2, μm	95 % CI (μ_2, μm)	PF1	95 % CI PF1
NMMP	24	260	258–262	405	399–413	0.413	0.391–0.437
	48	285	284–287	347	331–365	0.791	0.711–0.850
	72	281	278–284	364	358–373	0.551	0.507–0.608
	96	285	282–289	347	333–363	0.630	0.520–0.725
	168	278	275–281	457	443–477	0.585	0.550–0.626
R5	24	261	257–264	458	446–475	0.368	0.334–0.409
	48	256	254–259	521	507–537	0.363	0.340–0.391
	72	259	256–261	450	438–465	0.550	0.522–0.579
	96	256	253–260	398	387–410	0.511	0.469–0.553
	168	251	249–254	346	338–354	0.483	0.437–0.531
TSBS	24	290	287–294	728	693–761	0.507	0.478–0.536
	48	285	281–290	629	609–650	0.457	0.427–0.486
	72	281	276–285	581	559–603	0.489	0.449–0.523
	96	274	270–279	477	463–493	0.456	0.416–0.496
	168	271	268–275	454	440–469	0.506	0.467–0.546
YEME	24	246	243–250	376	364–388	0.324	0.272–0.377
	48	257	253–261	474	458–494	0.316	0.276–0.362
	72	254	250–259	408	395–425	0.360	0.306–0.423
	96	248	246–251	366	357–376	0.383	0.344–0.427
	168	242	238–245	304	289–324	0.703	0.592–0.805

Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean diameter of both populations (μ_1 and μ_2) and a confidence interval of the participation fraction (PF) between 0.025 and 0.975

growth, after which their size decreased to 346 and 304 μm, respectively. In contrast, in TSBS medium, the pellets decreased in size after 24 h of growth. Unlike in other media, in minimal medium (NMMP), the diameter of the large pellets increased, after an initial decrease (Table 1). The largest pellets in NMMP-grown cultures (457 μm) were detected after 7 days of growth. Taken together, these data show that the size of the large pellets formed by *S. coelicolor* is dynamic in time and depends on the medium composition.

Two populations of pellets could also be distinguished in liquid-grown cultures of *Streptomyces lividans* and the more phylogenetically distant species *Streptomyces scabies* and *Streptomyces griseus* (Fig. 3; Table 2). The average diameter of small pellets (μ_1) remained constant in time and was similar for all streptomycetes (averaging 256 μm±10 %). In contrast, the average size of the large pellets (μ_2) fluctuated in case of *S. lividans* and *S. scabies* (Fig. 4; Table 2). Less variation was observed for *S. griseus*. This again shows that streptomycetes form two different populations of pellets in liquid-grown cultures, with a dynamic size distribution for large pellets, while that of the small pellets is constant.

Synchronous spore germination and pellet heterogeneity

To exclude that differences in spore germination could contribute to culture heterogeneity, we inoculated *S. coelicolor* cultures with spores that had been pre-germinated by 10 min heat shock at 50 °C followed by pre-incubation in rich 2×YT media (Kieser et al. 2000). Phase contrast microscopy showed that indeed the pre-germination procedure resulted in near-synchronous spore germination (not shown). The size distribution of pellets after 24 h of growth again revealed two distinct populations of pellets (Fig. 5), strongly suggesting that germination efficiency did not play a role in culture heterogeneity. Consistent with this idea, a bimodal distribution was also observed in a Δ*nepA* mutant strain (Fig. 5c) that germinates synchronously (de Jong et al. 2009a). Two populations of pellets were also detected when mycelium rather than spores was used as the inoculum (data not shown). Taken together, these data show that pellets are heterogeneous in size regardless of the inoculum.

Deletion of genes for cell surface-related proteins affects the average diameter of large pellets

A *cslA* mutant strain was previously shown to form smaller pellets (Xu et al. 2008). Yet, again, two populations were detected in this strain throughout growth (Table 2). The average size of the small pellets was about 250 μm, similar to that observed for the parental wild-type *S. coelicolor* strain. In contrast, sizes of the large pellets were different from the parent. After 24 h, the average size of the large *cslA*

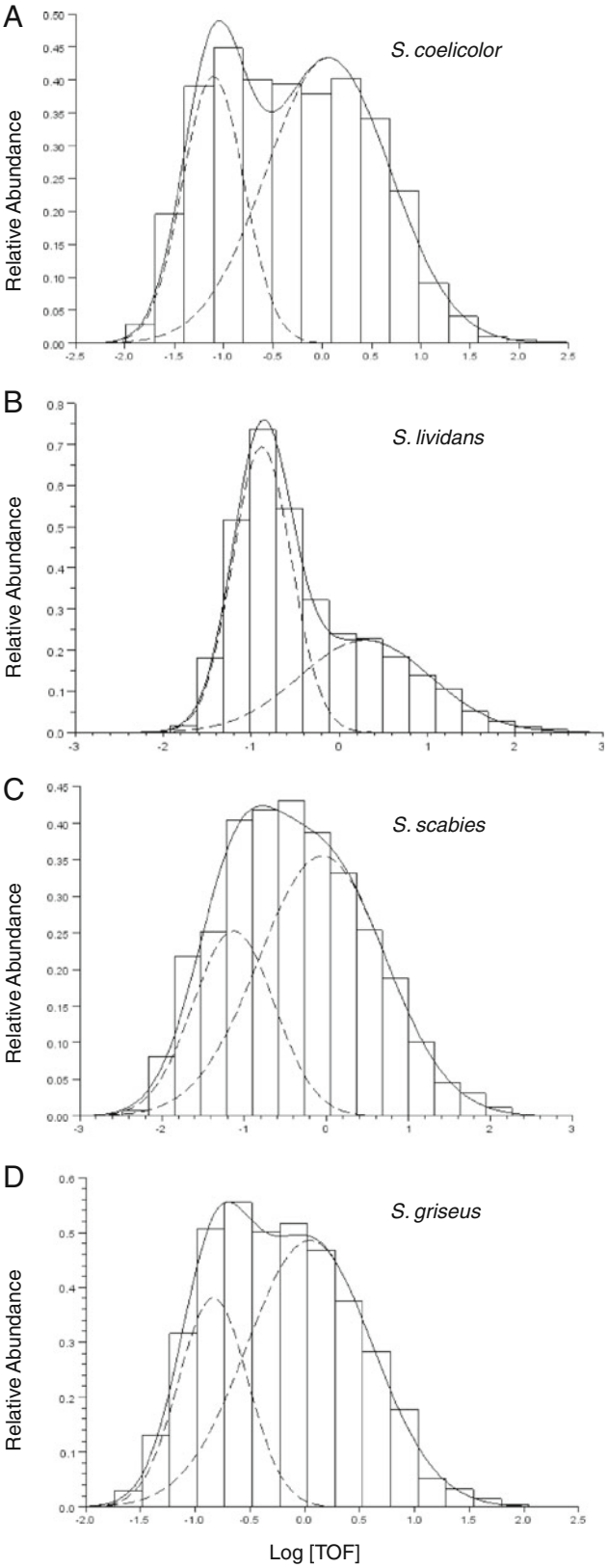

Fig. 3 Size distributions of pellets of liquid-grown cultures of *S. coelicolor* (**a**), *S. lividans* (**b**), *S. scabies* (**c**), and *S. griseus* (**d**) grown for 48 h in YEME medium. Two populations of pellets that differ in size are detected in all strains (see also Table 2)

Table 2 Heterogeneity between pellets in liquid-grown YEME cultures of different *Streptomyces* strains

Strain	Time, h	μ_1, μm	95 % CI (μ_1, μm)	μ_2, μm	95 % CI (μ_2, μm)	PF1	95 % CI PF1
S. coelicolor	24	246	243–250	376	364–388	0.324	0.272–0.377
	48	257	253–261	474	458–494	0.316	0.276–0.362
	72	254	250–259	408	395–425	0.360	0.306–0.423
	96	248	246–251	366	357–376	0.383	0.344–0.427
	168	242	238–245	304	289–324	0.703	0.592–0.805
S. lividans	24	244	233–266	302	285–399	0.457	0.238–0.916
	48	262	260–264	492	475–511	0.582	0.558–0.609
	72	276	269–284	412	396–446	0.446	0.369–0.553
	96	267	263–272	374	365–385	0.520	0.465–0.577
	168	264	258–272	376	365–390	0.431	0.360–0.518
S. scabies	24	251	247–258	332	320–356	0.564	0.479–0.694
	48	271	258–286	489	462–525	0.313	0.235–0.410
	72	279	267–298	557	499–715	0.526	0.418–0.692
	96	252	247–258	426	410–446	0.349	0.298–0.408
	168	259	253–265	400	383–423	0.491	0.422–0.567
S. griseus	24	247	215–281	385	293–1971	0.629	0.163–0.967
	48	252	248–256	384	374–398	0.302	0.256–0.363
	72	249	247–252	370	363–378	0.424	0.387–0.461
	96	253	248–259	374	360–389	0.397	0.324–0.471
	168	239	236–243	288	270–316	0.749	0.588–0.871
ΔcslA	24	238	235–244	271	263–300	0.603	0.455–0.871
	48	244	242–248	312	307–318	0.328	0.278–0.396
	72	255	251–258	378	369–388	0.391	0.349–0.436
	96	255	250–259	328	320–338	0.466	0.388–0.542
	168	245	243–248	310	296–329	0.760	0.677–0.827
ΔchpABCDEFGH	24	259	247–269	344	306–462	0.743	0.490–0.921
	48	268	261–276	404	384–429	0.531	0.450–0.618
	72	266	261–388	388	372–409	0.406	0.329–0.500
	96	260	256–355	355	343–368	0.484	0.411–0.558
	168	270	262–281	379	360–417	0.553	0.439–0.720

Heterogeneity is defined as non-overlapping confidence intervals (CI) of the mean diameter of both populations (μ_1 and μ_2) and a confidence interval of the participation fraction (PF) between 0.025 and 0.975

pellets was 271 μm compared with 376 μm for the parental strain (Table 2). A comparable difference was detected after 48 h with 312 μm for the *cslA* mutant and 474 μm for the parent. At later time points, the differences decreased until the sizes were almost identical after 168 h (310 μm for the *cslA* mutant and 304 μm for the parent).

Pellets of the Δ*chpABCDEFGH* strain were analyzed to investigate the role of chaplins in the size of pellets in liquid-grown cultures (Table 2). Like the parent *S. coelicolor* M145, two distinct populations of pellets could be distinguished. The small pellets had a diameter of approximately 265 μm irrespective of culture age, and this was similar to that of parental strain (Table 2). In contrast, the large pellets formed by the Δ*chpABCDEFGH* mutants were 404 μm in diameter, which is significantly smaller than the 474 μm observed for the parent strain after 48 h (Table 2). However, the size difference was less pronounced than observed for the *cslA* mutant.

Comparative proteomics of size-fractionated mycelia

One important application of the cell sorting technology described above is cytomics, in other words: the application of -omics technology on different cell types (i.e., small and large pellets). As proof of concept, and to determine protein profiles in the different populations, we applied quantitative proteomics using stable isotope dimethyl labeling on size-fractionated mycelial pellets from YEME liquid cultures. Using this approach, relative abundance ratios of 1,149 proteins could be determined with at least three independent quantification events. Interestingly, only 37 proteins (3.2 %) were found to be significantly different between the two populations; 17 were significantly overrepresented in the large pellets relative to the small pellets, and 20 were significantly underrepresented (Table 3).

Many of the proteins that were either overrepresented or underrepresented in the large versus the small mycelial

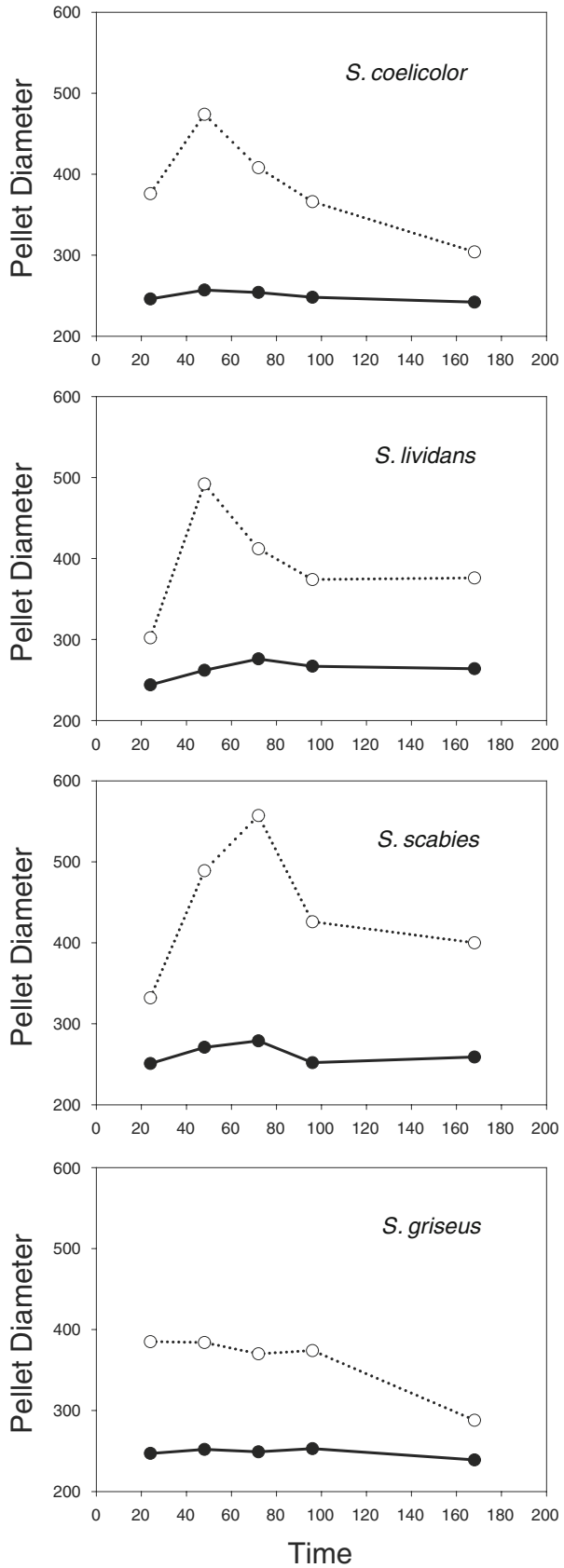

Fig. 4 Dynamics of pellet size in time in liquid-grown YEME cultures of *S. coelicolor* (**a**), *S. lividans* (**b**), *S. scabies* (**c**), and *S. griseus* (**d**)

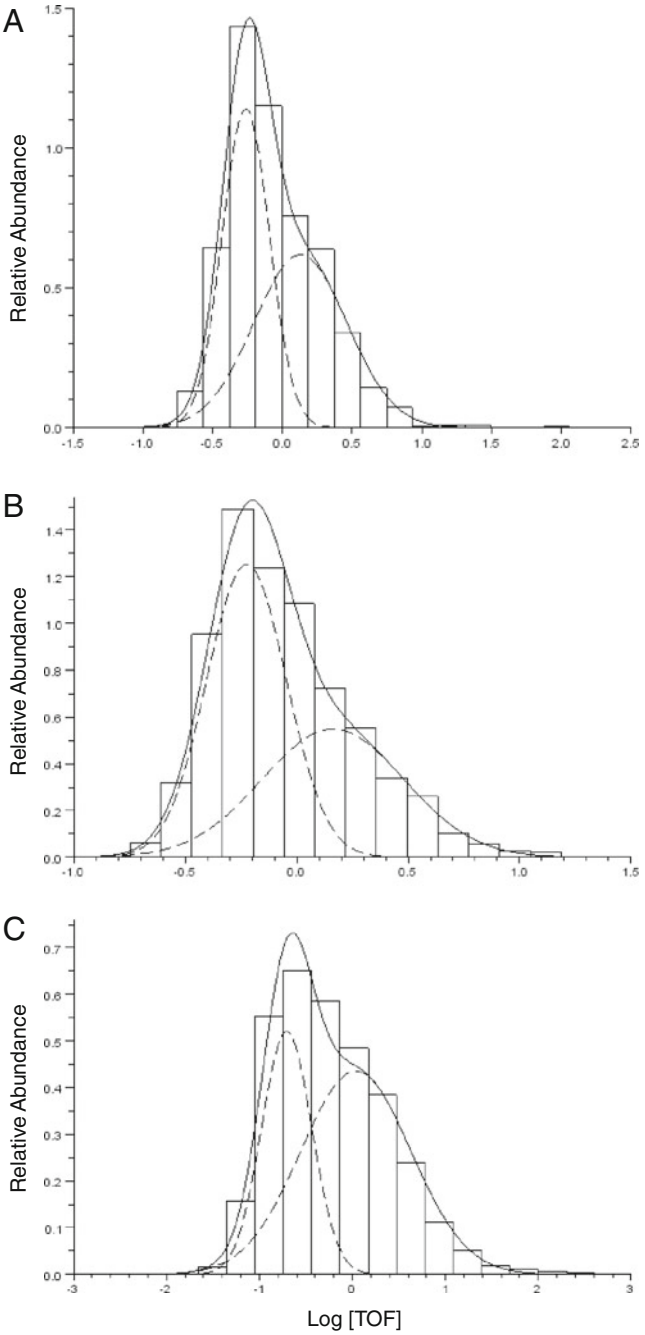

Fig. 5 The effect of synchronous spore germination on the size distribution of *S. coelicolor* pellets in YEME medium. Two populations of pellets were detected after 24 h using untreated (**a**) or heat-shocked (**b**) spores. Two populations that differ in size are also detected in the *nepA* mutant whose spores germinate more synchronously (**c**)

pellets could be tentatively assigned to specific functional classes. Several stress-related proteins are overrepresented in the list of targets that are more abundant in the population of large pellets, which may be explained by reduced oxygen availability. The protein that is most strongly (around 30-fold) enhanced in the larger pellets is SCO0913 or EgtD. The *egtD* gene is the last gene of the *egtABCD* operon,

Table 3 Proteins under- and overrepresented in the large mycelia relative to the small mycelia

Fold change (^{2}log value)	Independent quantification events	SCO number	Description
Proteins underrepresented in the population of large mycelia			
−4.8	3	SCO0917	Putative oxygenase
−4.4	7	SCO5289	CvnA5 putative two-component sensor kinase
−3.7	18	SCO4860	Putative secreted hydrolase
−3.5	5	SCO2157	Putative aminotransferase
−2.3	3	SCO3686	Putative uncharacterized protein
−2.0	13	SCO1551	Putative eukaryotic-type protein kinase
−2.0	6	SCO5104	Putative uncharacterized protein
−1.9	10	SCO6762	Putative phytoene dehydrogenase
−1.8	5	SCO2613	Putative membrane protein
−1.7	5	SCO4091	BldC putative DNA-binding protein
−1.6	3	SCO2236	YoeB toxin
−1.4	6	SCO6811	Putative secreted protein
−1.1	9	SCO5803	LexA repressor
−0.9	5	SCO4092	HrpA ATP-dependent helicase
−0.9	13	SCO5497	Putative uncharacterized protein
−0.9	4	SCO5698	Putative uncharacterized protein
−0.9	4	SCO7507	Putative dioxygenase
−0.8	4	SCO3748	F40 cold-shock protein
−0.8	9	SCO0436	RpmF2 50S ribosomal protein L32-2
−0.8	13	SCO4711	RpsQ 30S ribosomal protein S17
Proteins overrepresented in the population of large mycelia			
0.8	56	SCO0200	Universal stress protein (USP)
0.8	18	SCO0179	Putative zinc-containing dehydrogenase
0.9	5	SCO1384	Putative uncharacterized protein
1.0	17	SCO0617	Probable phosphoketolase
1.0	7	SCO0174	Putative DNA-binding protein
1.0	6	SCO3236	AsnO L-asparagine oxygenase
1.0	5	SCO5389	Putative uncharacterized protein
1.0	3	SCO5869	Putative uncharacterized protein
1.1	17	SCO3230	CdaPSI CDA peptide synthetase I
1.2	12	SCO0201	Putative integral membrane protein
1.4	7	SCO3945	CydA putative cytochrome oxidase subunit I
1.4	4	SCO3231	CdaPS2 CDA peptide synthetase II
1.5	4	SCO6273	Putative type I polyketide synthase
1.7	26	SCO7511	Gap2 glyceraldehyde 3-phosphate dehydrogenase
3.4	6	SCO4252	Putative uncharacterized protein
4.4	4	SCO4060	Putative uncharacterized protein
4.9	5	SCO0913	Putative uncharacterized protein

which is involved in the biosynthesis of the rare amino acid ergothioneine. Only a few microorganisms can synthesize this molecule, notably actinobacteria (including mycobacteria) and filamentous fungi (Seebeck 2010). The role of ergothioneine in microbes is unclear, but the molecule has antioxidant properties (Cheah and Halliwell 2012). Other stress-related proteins include those encoded by genes in the region around the response regulator gene SCO0204 (Table S2). Our recent studies revealed that SCO0204 controls development and oxidative stress (van Rossum et al., manuscript in preparation). SCO0204 probably has a similar regulon as DosR, which is the oxygen-sensitive dormancy response regulator in *Mycobacterium tuberculosis* (Chauhan et al. 2011; Gerasimova et al. 2011). One important target that is significantly more abundant in large pellets and part of the SCO0204 regulon is the universal stress protein (USP) (SCO0200).

Interestingly, also proteins for biosynthesis of the glycopeptide calcium-dependent antibiotic (CDA) were more abundant in the population of large pellets, consistent with the observation that antibiotics are preferentially produced in larger pellets (Wardell et al. 2002). These proteins included the CDA peptide synthase I and II (SCO3230 and SCO3231) and a secreted hydrolase (SCO3233) that is also included in the CDA biosynthetic machinery (Table S3; Bentley et al. 2002). The biosynthetic proteins for the antibiotics actinorhodin and undecylprodigiosin were not detected.

Several of the hits in the list of less abundant proteins in large pellets are related to DNA topology, modification, or degradation. These include the SOS response regulator LexA (SCO5803; Kelley 2006) and the DNA helicase HrpA (SCO4092). The list further contained the cysteine desulfurase DndA (SCO2157), the pyridine nucleotide-disulphide oxidoreductase SCO6811, and the top hit SCO0917 (30-fold less abundant in larger pellets). The latter encodes a luciferase-type flavin monooxygenase, the gene for which is immediately upstream of and probably coregulated with the *uvrA*-like gene SCO0918. UvrA is part of the bacterial nucleotide excision repair system (Sancar 1996). Besides HrpA (SCO4092), the genetically adjacent BldC (SCO4091) was also identified as a protein underrepresented in the larger pellets. BldC is a developmental control protein required for the onset of morphological differentiation of streptomycetes, via a yet unknown mechanism (Hunt et al. 2005). Interestingly, three sensory kinases were less abundant in the population of large pellets, including SCO1551, SCO5289, and SCO5104, which is a multidomain protein that carries a histidine kinase domain found in, among others, DNA gyrases and topoisomerases, several GAF, and PAS sensory domains and a domain found in sigma factors. Taken together, these data show significant differences in protein abundance in different pellet populations.

Discussion

A major complicating factor in the use of streptomycetes for industrial fermentation is that, like filamentous fungi, these microorganisms grow as intricate networks of branched hyphae, producing characteristic filamentous multicellular structures referred to as pellets. This results in high viscosity of the fermentation broth, and the concomitant low yield per unit of time is a major bottleneck for industrial applications. Understanding how morphology correlates to the production of natural products and enzymes is of great importance for industry-scale production. Large pellets typically produce antibiotics (Wardell et al. 2002), while small fragments optimally produce enzymes (van Wezel et al. 2006). These results imply that changing the relative abundance of smaller or larger pellets, as appropriate, might improve yield. Heterogeneity of cultures is a common trait in microbial communities, perhaps because heterogeneous populations have increased fitness as compared with homogeneous populations. One of the best-studied examples is the sporulation process in *Bacillus subtilis* (Errington 2003). Sporulation is an irreversible process that is initiated when nutrients become limiting. When sporulation starts, not all cells enter this process, leading to two different populations of cells: sporulating and non-sporulating cells (Smits et al. 2006). The relevance of sporulation is evident for individual cells: These structures are highly resistant to harsh environmental conditions, thereby ensuring maintenance of the species. However, the non-sporulating cells can continue to grow on nutrients that are released during cell lysis and sporulate later, or resume growth when new nutrients become available (Veening et al. 2008). As such, this diversification in two populations of cells benefits the entire population. Heterogeneity is also evident in solid-grown sporulating cultures of streptomycetes. For instance, a vertical cross-section through an individual colony reveals that only the vegetative mycelium in the central part of the colony forms the red-pigmented antibiotic undecylprodigiosin (Chater 1998). The presence of (sporulating) aerial hyphae on top of such colonies introduces another form of heterogeneity (Chater 1998). We here show that heterogeneity is also present in liquid-grown cultures, where it is characterized by the presence of two distinct populations of pellets.

The presence of two populations of pellets in liquid cultures of streptomycetes was observed irrespective of media composition and culture age. The pellet diameter in the population of small pellets was similar between the tested streptomycetes and was also not affected by culture age (i.e., between day 1 and day 7) and medium composition. In contrast, the mean size of the population of large pellets did vary. Pellet size is affected by parameters such as the geometry of the flask or bioreactor, the composition of the growth medium, pH, temperature, and the stirring speed (Tough and Prosser 1996; Cui et al. 1998; Celler et al. 2012). Our data suggest that these parameters would impact in particular the population consisting of large pellets and not that of the small ones. Deletion of genes for the cellulose synthase-like protein CslA and for the chaplin cell surface proteins affected only the size of the large pellets. The observed decrease in their size implies that the cell surface has a critical role in establishing or maintaining pellet architecture. Taken together, these data suggest that the population of small pellets is an intrinsic property of growth of streptomycetes not influenced by environmental conditions and cell wall components. In contrast, the diameter of large pellets is determined by such factors and also differs between *Streptomyces* species.

So far, it is not clear how the heterogeneity in pellet size is established. The size of fungal pellets is influenced by aggregation of spores (primary aggregation) and of germlings (secondary aggregation; Lin et al. 2008). This implies that the pellet size depends on the surface properties of both spores and hyphae (van Veluw et al. 2012). Significantly, in *Streptomyces*, two populations of pellets were observed irrespective whether spores or mycelium were used as an inoculum. This implies that heterogeneity is not the result of spore aggregation. The large pellets might result from the aggregation of small pellets. Heterogeneity in surface properties of the small pellets may result in distinct populations. This is consistent with the important role that cell surface proteins have on pellet size.

In terms of application, we demonstrate here that the COPAS technology allows, among others, a comparison between different bacterial cell types (i.e., small and large pellets), also known as cytomics. Cytomics is a promising technology applied frequently in the eukaryotic field (Kumar and Borth 2012), and we feel this may form an important asset in the design of producer strains with improved productivity. Application of quantitative proteomics demonstrated relatively small differences between small and large pellets. Out of the over 1,100 proteins that could be quantified in the mycelial fractions, the abundance of only 37 was significantly different (17 were more abundant and 20 were less abundant in large mycelia relative to small mycelia). The production of antibiotics and proteins is affected by pellet size, but it is yet unknown what the molecular basis for this phenomenon is. For instance, the production of erythromycin by *Saccharopolyspora erythraea* (closely related to *Streptomyces*) requires larger pellets for optimal yields (Wardell et al. 2002). We indeed showed that biosynthetic proteins for the production of the antibiotic CDA were enhanced in the large pellets. Several oxidative stress-related proteins were also overrepresented in the population of large pellets. This is most likely explained by the enhanced oxygen stress in large mycelial pellets due to mass transfer problems (Celler et al. 2012). Notably, several proteins encoded by genes in the chromosomal region between SCO0168-0208, most of which belong to the regulon of the response regulator SCO0204, were also enhanced in larger pellets. This includes the important stress protein USP, and we anticipate that this protein may play an important role in mycelial stress management. SCO0204 itself is an oxygen sensory protein (van Rossum et al., manuscript in preparation), and together with the sensory kinase SCO0203, it forms a two-component system that is orthologous to the DevS-DevR two-component system controlling dormancy in *Mycobacterium* species (Chao et al. 2010).

Many of the proteins that were less abundant in large pellets related to DNA topology, modification, or degradation, including the SOS response regulator LexA (Kelley 2006) and the DNA helicase HrpA. While the fact that BldC (SCO4092), encoded by a gene adjacent to that for HrpA, was also less abundant is perhaps suggestive, it is yet unknown if this regulator of morphogenesis is functionally related to *hrpA*. Other DNA metabolism-related proteins were the cysteine desulfurase DndA and the pyridine nucleotide-disulphide oxidoreductase SCO6811. The relatively high abundance of these proteins in small mycelia may be explained by the generally faster growth in small pellets, which presumably requires more intensive DNA replication, folding/unfolding, and quality control.

Summarizing, we successfully applied the COPAS technology to size-fractionate liquid-grown mycelia of streptomycetes, which revealed that *Streptomyces* cultures consist of two different mycelial types. This allowed us to identify differences in protein abundance depending on mycelium size. The initial data set obtained in this work provides insight into proteins that may play a role in growth and stress management of liquid-grown mycelia. This also provides strong validation for the applicability of our approach. The efficiency and reproducibility of the sorting process was highlighted by the highly similar protein profiles obtained from biologically independent replicate experiments. Besides size fractionation, flow cytometry also allows fractionation of mycelia based on fluorescence intensity or a combination of fluorescence intensity and size (de Bekker et al. 2011). Our work therefore opens new avenues for the cell biological research on streptomycetes.

Acknowledgments MLCP and DC were appointed at IBL from the award to Prof. Dr. P.J.J. Hooykaas as Academy Professor. Bogdan Florea and Hermen Overkleeft are thanked for help with mass spectrometry. HABW and GPvW gratefully acknowledge the Dutch Applied Research Council (STW) for continuing financial support

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