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Original article

The stringent response is strongly activated in the antibiotic producing strain, *Streptomyces coelicolor*

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ABSTRACT

S. lividans and *S. coelicolor* are phylogenetically closely related strains with different abilities to produce the same specialized metabolites. Previous studies revealed that the strong antibiotic producer, *S. coelicolor*, had a lower ability to assimilate nitrogen and phosphate than the weak producer, *Streptomyces lividans*, and this resulted into a lower growth rate. A comparative proteomic dataset was used to establish the consequences of these nutritional stresses on the abundance of proteins of the translational apparatus of these strains, grown in low and high phosphate availability. Our study revealed that most proteins of the translational apparatus were less abundant in *S. coelicolor* than in *S. lividans* whereas it was the opposite for ET-Tu 3 and a TrmA-like methyltransferase. The expression of the latter being known to be under the positive control of the stringent response whereas that of the other ribosomal proteins is under its negative control, this indicated the occurrence of a strong activation of the stringent response in *S. coelicolor*. Furthermore, in *S. lividans*, ribosomal proteins were more abundant in phosphate proficiency than in phosphate limitation suggesting that a limitation in phosphate, that was also shown to trigger RelA expression, contributes to the induction of the stringent response.

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1. Introduction

S. coelicolor (SC) and *S. lividans* (SL) are phylogenetically closely related model strains possessing the same specialized metabolite biosynthetic pathways but these are mainly highly expressed in SC [1]. Consequently, the comparative study of these two strains is useful to get a better understanding of what triggers antibiotic biosynthesis in *Streptomyces* species. Previous comparative studies revealed that SC has a lower growth rate than SL [2,3]. This might be due, at least in part, to the fact that SC has a lower ability than SL to assimilate nitrogen (N) as well as phosphate (P) and this results into nutritional stress [1,3,4]. In most microorganisms, N stress is known to trigger the stringent response that leads to the inhibition of the synthesis of ribosomal components [5]. Indeed, N limitation

leads to amino acid starvation and thus to the depletion of aminoacylated (charged) tRNA pools, resulting in the accumulation of ribosomes stalled at the ribosomal site A with non-aminoacylated (uncharged) tRNA [6,7]. The ppGpp synthetase RelA is then recruited to stalled ribosomes and activated to synthesize (p) ppGpp from GTP [8,9]. ppGpp is the mediator of the stringent response and controls at the transcriptional and translational levels a broad range of metabolic processes that allows the adaptation of the bacteria to nutritional limitation and is correlated with a reduction of growth rate.

In *Escherichia coli*, as in other microorganisms, the expression of genes involved in r-RNA and r-protein biosynthesis is down-regulated in condition of stringent control. Indeed, ribosome biogenesis and assembly involve the transcription, processing, folding, and modification of r-RNA and r-proteins and the binding of the latter to r-RNAs. The completion of functional ribosomes is facilitated by a broad range of proteins: GTPases, RNA helicases, endonucleases, modification enzymes, molecular chaperones etc ... that assist proper r-RNAs and r-proteins folding and proper r-proteins-r-RNA interactions [10]. Subsequently, the translational process itself that involves initiation [11], elongation [12], termination

Abbreviations: SC, *Streptomyces coelicolor*; SL, *Streptomyces lividans*.

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steps as well as the recycling of ribosomal components, requires the interaction and correct positioning of the ribosomal particles on mRNA (initiation), the synthesis of tRNAs, their charging by a specific amino acid by aminoacyl-tRNA synthetases [13] and their possible modification. The elongation step requires a correct codon–anticodon interaction and involves the peptidyl transferase center of the ribosome as well as elongation factors for the synthesis of peptidyl-tRNA. At last, when a stop codon is encountered, termination factors hydrolyze the ester bond of the peptidyl-tRNA and release the nascent protein that has to be correctly folded [14]. In *E. coli*, the synthesis of most ribosomal protein (r-protein) is coordinated with that of rRNA by mechanisms acting both at the transcriptional and translational levels [15–18]. ppGpp binds directly to the RNA polymerase [19] and in association with DksA regulates the transcriptional activity of r-proteins promoters [20]. Furthermore, the 5' region of the r-protein mRNA is often the target for translational feedback regulation [15]. ppGpp also inhibits the activity of several enzymes involved at key stages of mRNA translation including initiation, elongation and termination [21] and alters the formation of active 70S ribosomes by inhibiting small GTPases involved in ribosome maturation [22–24]. Altogether, these effects lead to a drastic reduction of the translation rate and thus of protein synthesis resulting into a reduced growth rate [25,26].

In Actinobacteria, as in other Gram+, the regulation of the stringent response slightly differs from that of *E. coli*. In Gram+ bacteria, the signal molecules ppGpp and c-di-GMP that regulate morphological and metabolic differentiation, both originate from GTP [27]. The lowering of intracellular GTP levels, linked to the biosynthesis of these molecules, might lead to a reduction of the transcription of genes requiring GTP as the initiating nucleotide, such as rRNA genes [27]. Furthermore, the biosynthesis of ppGpp was shown to be necessary for the production of antibiotics in *SC* as well as in other *Streptomyces* species [28–31]. Indeed, the inactivation of the two genes encoding the enzymes known to modulate the levels of ppGpp in *SC*, *sco1513* encoding the ppGpp synthetase RelA and *sco5794* encoding the ppGpp synthetase/hydrolase RshA/SpoT, abolishes the synthesis of ppGpp as well as that of the blue polyketide antibiotic, actinorhodin (ACT) under nitrogen-limiting conditions [32–34]. The production of these metabolites could thus be considered as part of the stringent response and the nature of the link between these two processes is discussed.

In order to determine whether the stringent response was more activated in *SC* than in *SL*, data corresponding to proteins playing a pivotal role in the triggering of the stringent response (RelA, RshA/SpoT and DksA) as well as to those of the translational apparatus were extracted from a previous proteomic dataset of *SC* and *SL*, grown in condition of low and high phosphate availability (dataset identifier PXD029263 and 10.6019/PXD029263). This analysis revealed that most proteins of the translational apparatus, whose expression is known to be under the negative control of the stringent response, were less abundant in *SC* than in *SL* with the exception of two proteins, ET-Tu 3 (SCO1321) [35] and the TrmA-like methyltransferase (SCO5901) [36], whose expression is known to be under the positive control of the stringent response. These proteins were more abundant in *SC* than in *SL*. This suggested the occurrence of a strong activation of the stringent response in *SC*. Interestingly our data also revealed the up-regulation of RelA expression in condition of Pi limitation and consistently the abundance of most proteins of the translational apparatus was down-regulated in Pi limitation or in other terms up-regulated in condition of Pi proficiency in *S. lividans*. However such regulation did not occur in *S. coelicolor*. These regulatory differences between these two strains are discussed and an explanatory hypothesis are proposed.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

Spores of *S. coelicolor* M145 (*SC*) [37] and *S. lividans* TK24 (*SL*) [38] used in this analysis were prepared from solid Soya Flour Mannitol (SFM) medium [39]. The 2 strains were grown, in quadruplets, on solid modified R2YE medium, with no sucrose added, on 5 cm diameter Petri dishes. The modified R2YE medium [40] contained glucose (50 mM) as major carbon source and was supplemented or not with K₂HPO₄. R2YE not supplemented with K₂HPO₄ contained 1 mM free Pi originating from the constituents of the growth medium (Pi limitation) and that supplemented with K₂HPO₄ contains 5 mM Pi (Pi proficiency), as determined with a PiBlue phosphate assay kit (Gentaur, France). Spores (10⁶) of the strains were plated on the surface of cellophane disks (Focus Packaging & Design Ltd, Louth, UK) laid down on the top of agar plates and incubated at 28 °C in darkness for 48 h or 60 h. The time points of 48 and 60 h were chosen as they correspond to the beginning of the production of the blue pigmented polyketide antibiotic, actinorhodin (ACT), in *SC* [6]. Mycelial lawns of the 4 independent biological replicates of each strain were collected with a spatula, washed twice with deionized water, lyophilized and weighted.

2.2. Proteins sample digestions

The 32 protein samples (2 strains x 2 media x 2 culture times x 4 biological replicates) compared in this analysis were prepared as described previously [4] for an in-depth shotgun proteomic analysis and label-free relative quantification. Briefly each protein sample was alkylated, digested using Lysyl-Endopeptidase (Wako Chemicals USA) and sequencing-grade-modified trypsin (Promega). After pre-cleaning, proteolytic peptides were concentrated under vacuum and stored.

2.3. Mass spectrometry-based protein identification and relative protein quantification

The mass spectrometry proteomics data reanalyzed in this paper are included in to the dataset identifier PXD029263 and 10.6019/PXD029263 deposited to the ProteomeXchange Consortium via the PRIDE partner repository [41,42]. These data were obtained from 1 µg of proteolytic peptides using nanoLC-MS/MS (liquid chromatography tandem mass spectrometry) with a nano-Elute liquid chromatography system (Bruker) coupled to a timsTOF Pro mass spectrometer (Bruker) and included the conversion of raw MS and MS/MS data into mgf files as described previously [43]. Protein identifications against *SC* and *SL* protein database from UniprotKB (15012020) using the MASCOT search engine (Matrix science, London, United Kingdom) and changes in protein abundance by label-free mass spectrometry-based quantification using spectral counts or MS1 ion intensities named XIC (for extracted ion current) were performed as described previously [1,4].

2.4. Protein abundance changes and statistical analysis of proteins involved in translation

The data corresponding to the two strains, *SC* and *SL*, were extracted from the large dataset 10.6019/PXD029263 deposited to the ProteomeXchange. Statistical analyses of protein abundance changes in these two strains was performed in R Studio (version 1.4.17.17) using the homemade R script as described previously [1]. The present analysis thus consisted in a pairwise comparison of 28 combinations and focuses exclusively on proteins involved in the

translational process identified using the UniprotKB (15012020) protein database and the annotations of the KEGG pathway. For each of the identified proteins, a significant difference was set to 10 for spectral count and 1 Log₂ fold change for label-free quantitation (LFQ) values. Additionally a threshold of 4 significant pairs and an adjusted p-value of 0.05 was set to consider a protein abundance as significantly variable. The selected proteins were used for heat-map representation.

3. Results

3.1. Proteins involved in the stringent response

Data corresponding to proteins playing a role in the stringent response were extracted from our previous a proteomic dataset of SC and SL, grown in condition of low and high phosphate availability (dataset identifier PXD029263 and 10.6019/PXD029263). DksA (SCO2075) [44] was not detected in our study but SpoT/RshA (SCO5794) [45] and RelA (SCO1513) were identified. The abundance of SpoT/RshA (SCO5794) was similar in all the conditions tested whereas an up-regulation (1.7 fold) of RelA abundance was noticed at 48 h and 60 h in SL and SC, respectively, in condition of Pi limitation. This triggering was correlated, as expected, with the down-regulation of most r-proteins in SL in Pi limitation whereas the abundance of r-proteins remained low in both Pi conditions in SC. The up-regulation of RelA abundance in Pi limitation suggested that its expression might be under the positive control of the two components system PhoR/PhoP (SCO4229/SCO4230) and thus belongs to the Pho regulon [46]. A close inspection of the *relA* promoter region indeed revealed the presence of a rather well conserved Pho box (Fig. S1). This suggests that a Pi limitation contributes to the triggering of the stringent response via the up-regulation of RelA abundance in Pi limitation. A contribution of phosphate limitation in the triggering of the stringent response was previously reported in another Actinobacteria, *Mycobacterium tuberculosis* [47].

3.2. Proteins involved in rRNA modification and ribosome assembly

The 26 proteins, detected in our study that are involved in rRNA modifications, fall into 2 groups (A and B) as shown in Fig. 1:

- Group A includes 7 proteins that had a similar abundance in the 2 strains and whose abundance did not vary significantly with Pi availability nor with time point. This group includes 2 GTPases RsgA-like (SCO5211 and SCO6149) involved in the late steps of the maturation of the functional core of the 30S ribosomal subunit [48] as well as the GTPase HflX (SCO5796) that acts as a ribosome splitting factor that disassembles the 70S ribosomes into its subunits [49]; a methyltransferase type G (SCO3885) of the 16S rRNA that in *Bacillus subtilis* specifically methylates the N7 position of guanine in position 527 of 16S rRNA [50]; a methylase of polypeptide chain release factors (SCO5361) that is required for normal translation termination *in vivo* [51], the ribosome maturation factor RimM (SCO5593) involved in the late assembly stage of the 30S ribosomal subunit [52] and the ribosome silencing factor RsfS (SCO2577) that slows down cell growth by inhibiting protein synthesis during periods of reduced nutrient availability [53].
- Group B includes 19 proteins. Most of these proteins were more abundant in SL than in SC in both Pi conditions and were up-regulated in Pi proficiency in SL but not in SC. One noticeable exception was the TrmA-like methyltransferase (SCO5901) that catalyzes the S-adenosylmethionine-dependent methylation of the uracil in position 54 in all *E. coli* tRNAs and is essential for viability [54], whose abundance was higher in SC than in SL.

Otherwise this group includes 9 methyltransferases [55], 3 GTPases [56], the methylthiotransferase RimO (SCO5752) involved in the methylthiolation of the ribosomal protein S12 [57], 2 proteins involved in the maturation of the 30S sub-unit RimP (SCO5703) [52] and RbfA (SCO5708) [58], the ribosome associated translation inhibitor RaiA (SCO3009) that may play a role in translation fidelity [59] and the ribosome binding ATPase YchF (SCO5627), involved in ribosome recycling [60]. Interestingly the absence of YchF, was shown to enhance the translation of leaderless mRNA in stressful conditions in *E. coli* [61]. The 9 methyltransferases that might be involved in the fine tuning of ribosomal decoding center [62] include 4 putative tRNA/rRNA methyltransferases of the SpoU family (SCO3836, SCO1552, SCO1597 and SCO4236) [63], 4 methyl transferases of the small subunit of the rRNA: RsmC-like (SCO1041) [64], RsmE-like (SCO2552) [65], RsmH-like (SCO2092) [66] and RsmI-like (SCO3253) [67] as well as a rRNA adenine dimethylase of the KsgA family (SCO3149) [68]. The 3 GTPases involved in ribosome assembly and homeostasis [69] belong to the Era (SCO2539) [70], Der/EngA (SCO1758) [71] or Obg (SCO2595) [72] families. Interestingly, in condition of over-expression Obg has a negative impact on aerial mycelium formation in SC [73].

3.3. Ribosomal proteins

Ribosomes are made of several rRNA molecules and of 50–80 proteins that play a role in assembly, structure and function of the ribosome [74,75]. The 58 ribosomal proteins detected in our study fall into 4 groups (A to D) as shown in Fig. 2:

- Group A includes 15 proteins (25,8% of total ribosomal proteins) that were more abundant in SL than in SC especially in Pi proficiency at both time points as well as in Pi limitation at 48 h. This group includes 7 and 8 proteins belonging to the 50S (L4, L5, L10, L19, L23, L27, L28) and 30S (S1, S2, S4, S5, S7, S13, S16, S17) subunits, respectively. Interestingly, the S1 protein was far more abundant in Pi limitation than in Pi proficiency at 48 h in SL. In Pi limitation, this protein might play a role in mRNA decay besides its role in translation [76,77].
- Group B includes 22 proteins (37,9%) that were up-regulated in Pi proficiency in SL but not in SC. These include 16 and 7 proteins belonging to the 50S (L1, L2, L3, L6, L9, L13, L14, L15, L16, L18, L20, L21, L22, L24, L35) and 30S (S3, S8, S9, S11, S12, S17, S19) subunits, respectively.
- Group C includes 8 proteins (13,7%) that were up-regulated in Pi proficiency in a similar way in both strains. These includes 3 and 5 proteins belonging to the 30S (S6, S14 and S18) and 50S (L7/12, L28, L31, L32.2 and L33.1) subunits, respectively.
- Group D includes 11 proteins (18,96%) that had a similar abundance in the two strains and their abundance did not significantly vary with Pi availability. These includes 5 and 6 proteins belonging to the 30S (S8, S10, S15, S18 and S20) and 50S (L11, L25, L28, L29, L30, L33.2) subunits, respectively. The expression pattern of L31 (SCO5359) did not fit into any of the 4 groups. L31 was far more abundant in SC than in SL in Pi limitation, at both time points. L31 is thought to contribute to sub-units association [78] and to enhance initiation of translation efficiency as well as to reduce frameshifting and miscoding [79]. In SC the expression of L31 was shown to be under the positive control of Sigma R [80] that controls the expression of genes involved in the resistance to oxidative/disulfide stress [81]. So the high expression of L31 in SC is likely to be due to high oxidative stress linked to the previously reported strong activation of the oxidative metabolism of this strain [1,2,82].

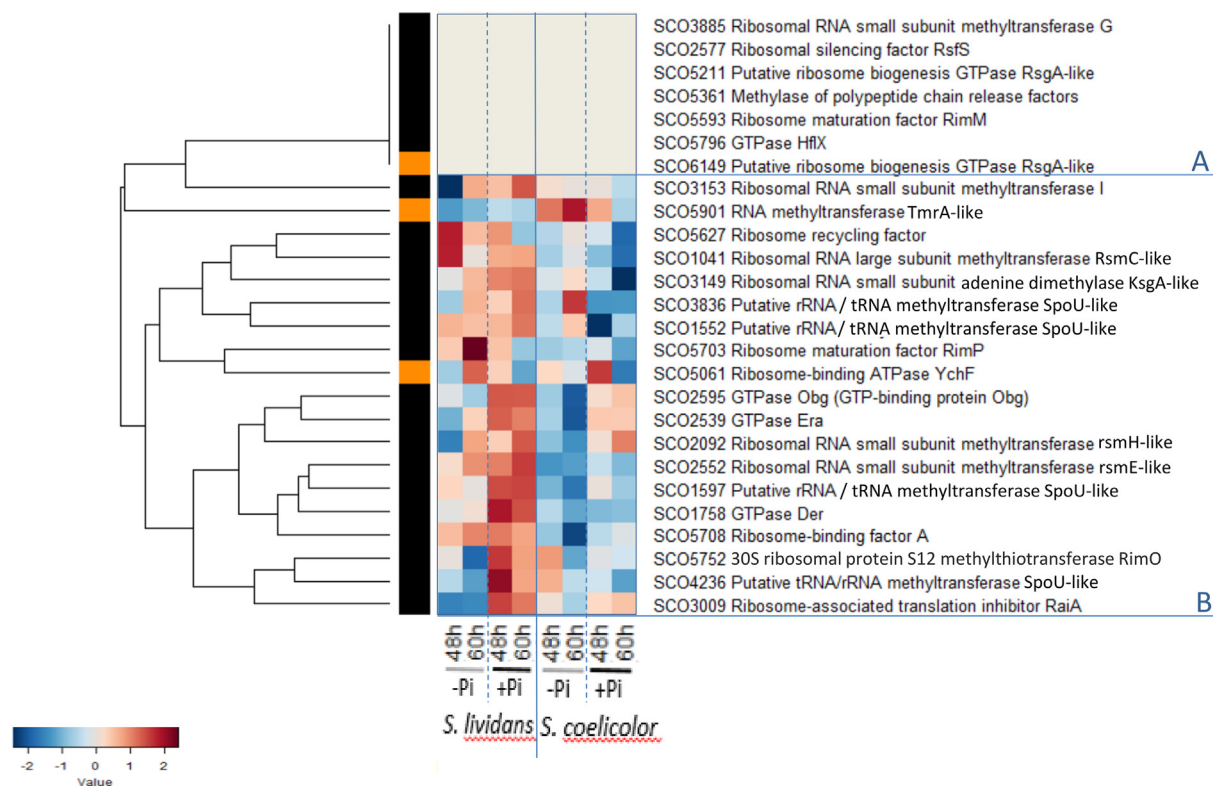


Fig. 1. Heatmap representation of the abundance of proteins involved in rRNA modification of SL and SC grown for 48 h or 60 h in R2YE medium either limited (-Pi, 1 mM) or proficient (+Pi, 5 mM) in phosphate. This heatmap is divided in 2 sub-clusters A and B. Cluster A includes 7 proteins that have a similar abundance in the two strains, conditions and time points. Cluster B includes 19 proteins more abundant in SL than in SC and up-regulated in Pi proficiency in SL but not in SC.

The abundance of proteins of group A and B (65.5% of total ribosomal proteins) was up-regulated in condition of Pi proficiency in SL but not in SC. A similar absence of up-regulation in Pi proficiency was previously reported for some proteins belonging to C and N metabolism in SC [1,3,4]. However, obviously proteins of groups C and D escape this regulation and these might constitute a minimal ribosome machinery abundant in SC that would be able to translate a subset of proteins involved in the alleviation of the multiple stresses this bacteria is suffering from.

3.4. Enzymes involved in tRNA synthesis or modification

The 48 proteins, detected in our study that are involved in tRNA synthesis or modification, fall into 6 groups (A to F) as shown in Fig. 3:

- Group A includes 6 proteins that were mainly expressed in SC in both Pi conditions and at both time points. This group includes Cys, Arg, Phe, Lys and Val tRNA synthetases, enzymes that catalyze the esterification of the corresponding amino acid on the 3' end of tRNA [83], as well as the Glu-tRNA^{Gln} amidotransferase subunit B (SCO5501) that allows the formation of correctly charged Gln-tRNA(Gln) through the transamidation of misacylated Glu-tRNA(Gln) when glutamyl-tRNA synthetase is missing [84].
- Group B includes 4 proteins mainly expressed in SC in Pi proficiency at both time points as well as in SL in Pi limitation but mainly at 60 h. Its includes the Glu-tRNA^{Gln} amidotransferase subunit C (SCO5498), the deacylase YkaK-like (SCO1311) involved in the tRNA editing of mis-charged tRNAs [85] as well as 2 tRNA-specific adenosine deaminases (SCO4038 and

SCO4039). These enzymes catalyze the editing of adenosine into inosine at the wobble position of tRNA^{Arg} in prokaryotes [86–88]. This modification is necessary to allow a single tRNA specie to recognize synonymous codons when tRNA specie recognizing the precise codon is poorly available [89].

- Group C includes 7 proteins with a similar abundance in the two strains, Pi conditions and time points. This group includes Thr, Arg, Met and Ileu tRNA synthetases as well as the following tRNA modifying enzymes: tRNA N6-adenosine threonylcarbamoyl-transferase (SCO4752) [90], tRNA (guanine-N(7))-methyltransferase (SCO4111) [91], Met-tRNA formyl transferase (SCO1473) [92] and methionyl-tRNA formyltransferase (SCO5791) [93].
- Group D includes 9 proteins strongly up-regulated in Pi deficiency in SL but not in SC. It includes Tyr and Lys tRNA synthetases [94], putative archaeal-like Glu-tRNA^{Gln} amidotransferase subunit E (SCO3581) and the following tRNA modifying enzymes: the pseudouridine tRNA synthase (SCO1768) that catalyzes a post-translational modification of uridine in some tRNA that might stabilize the correct anticodon-codon pairing during translation [95], tRNA threonylcarbamoyl adenosine modification protein YjeE-like (SCO4747) [90], tRNA (guanine-N1)-methyltransferase (SCO5594) which may which prevent frameshifting [96], ribosomal RNA large subunit methyltransferase N (SCO5645) [97] and tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase (SCO5488) [98].
- Group E includes 13 proteins far more abundant in SL than in SC in both Pi conditions and at both time points. This group includes Pro, Thr, 2 Trp, Lys, Gly, Leu, Ala and Met tRNA-synthetases as well as the following tRNA modifying enzymes: pseudouridine tRNA-synthase (SCO5709) [95], D -tyrosyl-tRNA(Tyr) deacylase (SCO4182) that removes some D -amino

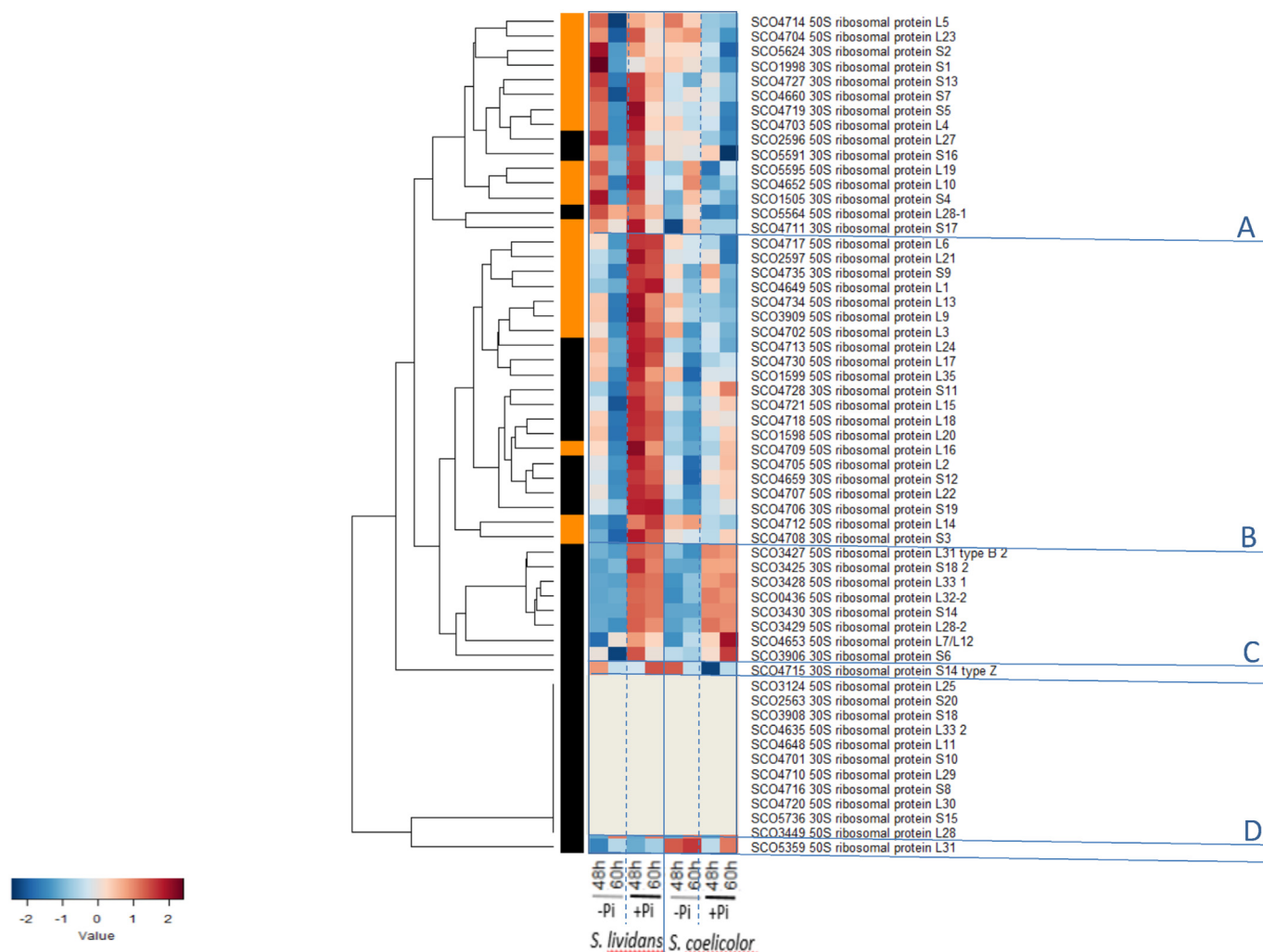


Fig. 2. Heatmap representation of the abundance of ribosomal proteins of *SL* and *SC* grown for 48 h or 60 h in R2YE medium either limited ($-Pi$, 1 mM) or proficient ($+Pi$, 5 mM) in phosphate. This heatmap is divided in 4 sub-clusters. Cluster A includes 15 proteins more abundant in *SL* than in *SC* and especially abundant in *SL* at 48 h and 60 h in Pi limitation and proficiency, respectively. Cluster B includes 21 proteins more abundant in *SL* than in *SC* and up-regulated in Pi proficiency in *SL* but not in *SC*. Cluster C includes 8 proteins similarly abundant in *SL* than in *SC* and up-regulated in Pi proficiency in both strains. Cluster D includes 11 proteins that have a similar abundance in the two strains, conditions and time points. Two proteins S14 type Z (SCO4715) and L31 (SCO5359) do not fit in any of these clusters.

acids from mischarged tRNAs allowing their replacement by the L form used in translation [99,100] and a dimethylallyl adenosine tRNA methyltransferase MiaB (SCO5787) thought to be involved in improvement of reading frame maintenance [79,80]. Mutation in *miaB* were shown to affect morphological differentiation and secondary metabolism in *Streptomyces* [81–84].

- Group F includes 6 proteins more abundant in Pi limitation than in Pi proficiency in both strains and more abundant at 48 h and 60 h in *SL* and *SC*, respectively. These proteins were also more abundant in Pi proficiency in *SL* than in *SC*. This group includes Ser, Phe, Glu, Asp, His tRNA synthetases and the pseudouridine synthase (SCO4731) [95].

The regulation of the expression of aminoacyl-tRNA synthetases in prokaryotes proved to be complex and diverse [101]. Our results indicate that tRNA synthetases of Fig. 3/groups D and E were more abundant in *SL* than in *SC* but in contrast tRNA synthetases of Fig. 3/group A (cys-tRNA ligase (SCO4235), the-tRNA ligase beta subunit (SCO1594), val-tRNA ligase (SCO2615), arg-tRNA ligase (SCO3304) and the Glu-tRNA_{Gln} amidotransferase (SCO5501) were more abundant in *SC* than in *SL*.

Even if the expression of most aminoacyl-tRNA ligases/synthetases is coordinated with the synthesis/availability of their cognate amino acids [102] and increases with growth rate [101], up-regulation of tRNA synthetases abundance has been reported in *B. subtilis* when the availability of their substrates is low [101]. In *SC*, a similar regulation might occur indicating the low availability of some amino acids due to the reduced ability of this strain to up-take amino acids present in the R2YE medium.

3.5. Proteins involved in translational initiation, elongation and termination processes

The 13 proteins, detected in our study that are involved in translational initiation, elongation and termination, fall into 2 groups (A and B) as shown in Fig. 4:

- The 2 proteins of group A had a similar abundance in the two strains and their abundance did not vary with Pi availability nor with time point. This group includes the translational initiation factors IF1 [103] and IF3 [104,105].

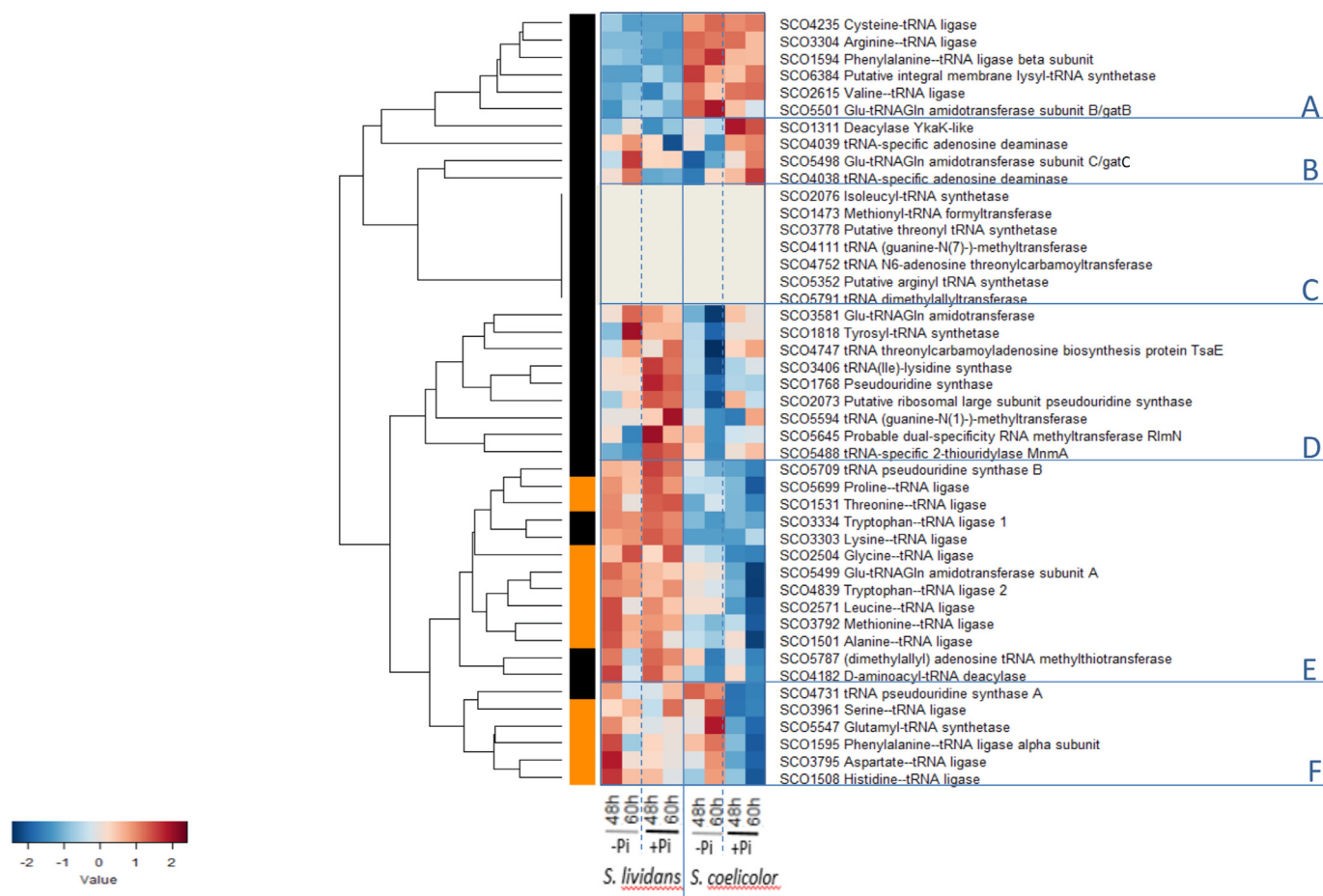


Fig. 3. Heatmap representation of the abundance of enzymes involved in tRNA synthesis or modification of *SL* and *SC* grown for 48 h or 60 h in R2YE medium either limited (-Pi, 1 mM) or proficient (+Pi, 5 mM) in phosphate. This heatmap is divided in 6 sub-clusters A to F. Cluster A includes 6 proteins more abundant in *SC* than in *SL* in both Pi conditions and at both time points. Cluster B includes 4 proteins more abundant in *SC* than in *SL* and up-regulated in *SC* but not in *SL*. Cluster C includes 7 proteins that have a similar abundance in the two strains, conditions and time points. Cluster D includes 9 proteins more abundant in *SL* than in *SC* and up-regulated in Pi proficiency in *SL* but not in *SC*. Cluster E includes 13 proteins more abundant in *SL* than in *SC* in both Pi conditions and at both time points. Cluster F includes 6 proteins more abundant in *SL* than in *SC* and up-regulated in Pi limitation in both strains.

- Most of the 11 proteins of group B were more abundant in *SL* than in *SC* in both Pi conditions. This group includes the translation initiation factor IF2 [106], 7 elongation factors [12], 2 peptide chain release factors [107] and one peptidyl tRNA hydrolase [108]. However, the elongation factor EF-Tu3 (SCO1321) escapes this rule since that it was far more abundant in *SC* than in *SL* in Pi limitation at both time points.

3.6. Proteins involved in protein folding and modification

The 20 proteins, detected in our study and involved in protein folding and modification, fall into 5 groups (A to E) as shown in Fig. 5:

- Group A includes two chaperones DnaJ2 (SCO2554) and HtpG (SCO7516) [109,110] and a peptidyl-prolyl cis-trans isomerase (PPIase, SCO1639) [111] that were up-regulated in Pi limitation in both strains. PPIases assist protein folding via the catalysis of cis/trans isomerization of peptide bonds preceding prolyl residues [112].
- Group B includes 4 proteins, the chaperone (SCO4761), two Heat Shock Proteins (HSP) HspR (SCO3668) [113] and SCO1405 (Hsp90 family) [114] and a peptide deformylase (SCO0883)

[115]. These proteins were up-regulated in Pi limitation in both strains but were far more abundant in *SC* than in *SL*. Peptide deformylases remove the formyl group from the formylmethionine present at the N-terminus end of all nascent peptides [116]. These proteins are thought to contribute to the correct folding of membrane proteins and thus to maintain proper structure and function of the membrane [116]. The over-expression of SCO0883 in *SC* might contribute to fight membrane stress that occurs in *SC* [1,4].

- Group C includes 6 proteins, two chaperones, DnaK (SCO3671) [117] and the ATP-dependent chaperon ClpB (SCO3661) [118], three PPIases (SCO3856, SCO1638 and SCO7510) and a peptide deformylase (SCO5221). These proteins were up-regulated in *SL* in Pi limitation but not in *SC*.
- Group D includes 4 proteins, two chaperones, DnaJ (SCO3669) and SCO2899, the heat shock protein GrpE-like (SCO3670) [119] and a second PPIase (SCO1510). These proteins had a similar abundance in the two strains and their abundance did not vary with Pi availability nor time points.
- Group E includes 3 proteins, the heat shock protein GrpE-like (SCO1771) and two 60 KDA chaperones, GroEL2 (SCO4296) and GroEL1 (SCO4762) [120]. These proteins were up-regulated in *SC* in Pi proficiency at both time points but not in *SL*.

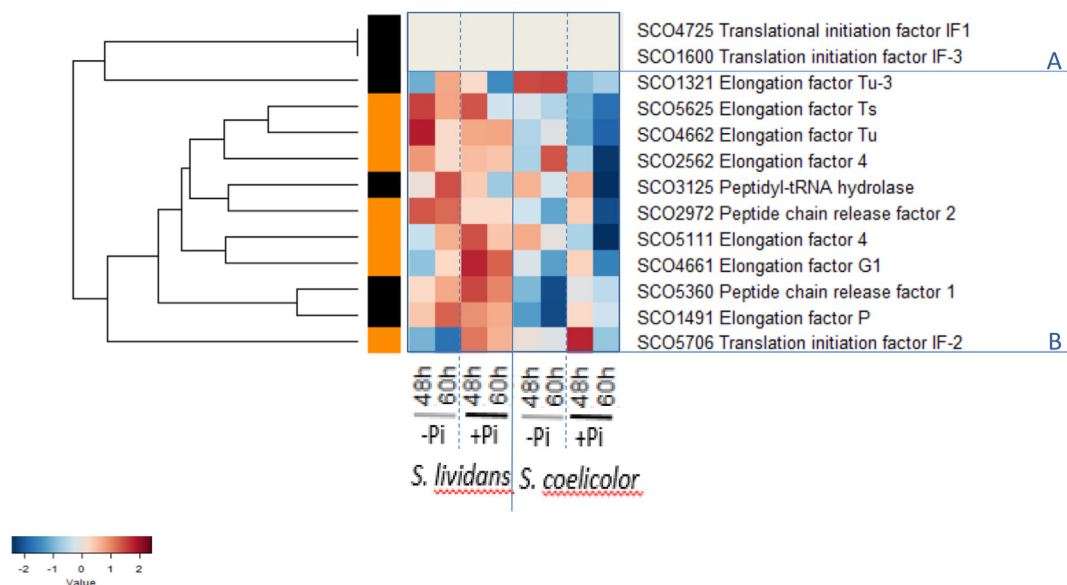


Fig. 4. Heatmap representation of the abundance of proteins involved in translation initiation, elongation and termination processes of *SL* and *SC* grown for 48 h or 60 h in R2YE medium either limited (-Pi, 1 mM) or proficient (+Pi, 5 mM) in phosphate. This heatmap is divided in 2 sub-clusters A and B. Cluster A includes 2 proteins that have a similar abundance in the two strains, conditions and time points. Cluster B includes 11 proteins more abundant in *SL* than in *SC* in both Pi conditions and at the two time points.

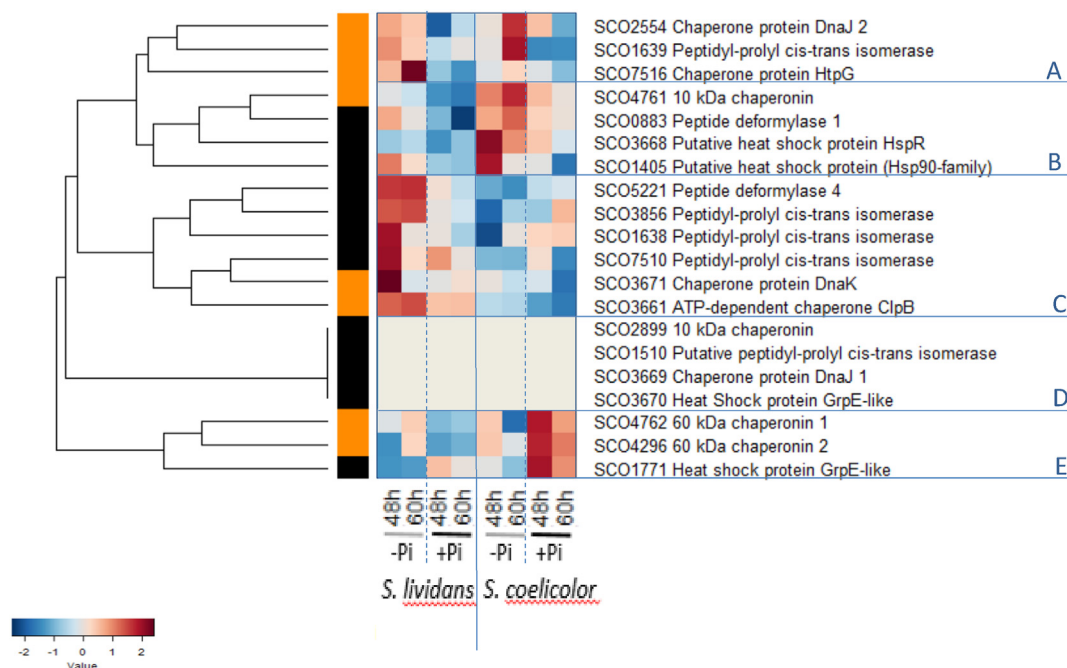


Fig. 5. Heatmap representation of the abundance of proteins involved in proteins folding and modification enzymes of *SL* and *SC* grown for 48 h or 60 h in R2YE medium either limited (-Pi, 1 mM) or proficient (+Pi, 5 mM) in phosphate. This heatmap is divided in 5 sub-clusters A to E. Cluster A includes 3 proteins more abundant in *SL* than in *SC* and up-regulated in Pi limitation. Cluster B includes 4 proteins more abundant in *SC* than in *SL* and up-regulated in Pi limitation in *SC*. Cluster C includes 6 proteins more abundant in *SL* than in *SC* and up-regulated in Pi limitation in *SL* but not in *SC*. Cluster D includes 4 proteins that have a similar abundance in the two strains, conditions and time points. Cluster E includes 3 proteins up-regulated in Pi proficiency in *SC* but not in *SL*.

The higher abundance of proteins of groups A and B in both strains or of proteins of group C in *SL*, in Pi limitation, suggest that these proteins may belong to the Pho regulon.

3.7. Relative abundance of proteins translated from leaderless RNA in *S. coelicolor* and *S. lividans*

Our data revealed that a most elements of the translational apparatus were less abundant in *SC* than in *SL* whereas a few were similarly abundant in the two strains. These few proteins

might thus constitute the core of minimal ribosomal particles that would be more abundant in *SC* than in *SL*. Since some reports in the literature mention that minimal ribosomal particles might preferentially translates leaderless mRNAs (l-mRNAs) [121–123], we uploaded the list of *SC*'s l-mRNAs from Jeong et al. [124] in order to determine whether they would be more efficiently translated in *SC* than in *SL*. Among the 713 l-mRNAs mentioned in Jeong et al. [124], 368 were detected in our study. These l-mRNAs could be classified into 6 groups (Figure S2A–F).

Proteins translated from lmrRNAs of groups A1 (9.5%), A3 (5.1%) and A4 (4.6%) were more abundant in Pi limitation than in Pi proficiency, especially in SC and proteins of group A5 (3.8%) were more abundant in SC than in SL in Pi proficiency. Proteins of group A2 (2.17%) were similarly up-regulated in Pi limitation in both strains. Group A2 includes proteins belonging to the Pho regulon [46,125]: the sensor kinase PhoR (SCO4229), the polyphosphate kinase Ppk (SCO4145) [126] as well as 6 genes encoding proteins involved in the biosynthesis of teichulosonic acid cell wall glycopolymers (SCO4877 to SCO4882) [127,128]. Phosphate free teichulosonic and teichuronic acids cell wall glycopolymers are known to replace phosphate rich teichoic acids in Gram + bacteria in condition of Pi limitation [129].

Proteins translated from lmrRNAs of groups B and C (35,6% of total lmrRNAs) were clearly more abundant in SC than in SL. Proteins of group B were more abundant in SC than in SL in both Pi conditions whereas proteins of group C were mainly up-regulated in Pi limitation in SC.

Proteins translated from lmrRNAs of group D1 (5.1%) were more abundant in Pi proficiency than in Pi limitation in both strains but rather more abundant in SL than in SC. Proteins of group D2 (2.4%) were similarly up-regulated in Pi proficiency in both strains. Proteins of group D3 (9.78%) were also up-regulated in Pi proficiency but mainly in SC.

In contrast proteins belonging to groups E and F (21,46% of total lmrRNAs) were clearly more abundant in SL than in SC. Proteins of group E (16%) were more abundant in SL than in SC in both Pi conditions. The 8 proteins of group F1 (2.17%) were clearly up-regulated in Pi proficiency in SL but not in SC and the 12 proteins of group F2 (3.26%) were up-regulated in Pi limitation in SL but not in SC. Group F1 includes 3 genes encoding large (SCO2486 and SCO2487) and small (SCO2488) subunits of the nitrite reductase, the heme peroxidase (SCO6042), two transcriptional regulators (SCO3810 and SCO4766) and the phosphoribosyltransferase (SCO3677) that belongs to the purine salvage pathway that is usually up-regulated during the stringent response [130]. Group F2 includes the response regulators SCO3013, located downstream the translation initiation factor SCO3014 and PhoP (SCO4230) that governs adaptation to Pi limitation, the inositol monophosphatase (SCO6445) that might be involved in a phosphatidylinositol signaling pathway controlling cell growth in *Streptomyces* [131] and the ribokinase (SCO2158) that is involved in the biosynthesis of ribose 5 phosphate, a metabolite proposed to link nucleotide and amino acids metabolism in condition of stringent control [132].

Our data indicate that only 7% of total proteins belonging to groups A2 (up-regulated in Pi limitation), D1 and D2 (up-regulated in Pi proficiency) were similarly regulated in both strains whereas 54.6% of the proteins translated from lmrRNAs were more abundant in SC than in SL (72 and 59 proteins from groups B and C, 33 proteins from groups A3/A5 and 37 proteins of group D3) when only 27,4 % of the proteins translated from lmrRNAs were more abundant in SL than in SC (59 and 20 proteins of groups E and F and 14 and 8 proteins of groups D1 and D3). Proteins translated from lmrRNAs are thus two fold more abundant in SC than in SL but it cannot be concluded with certainty whether this is due to higher transcriptional level of lmrRNAs or to higher translational rate of the latter's, that might be linked to the higher abundance of minimal ribosomal particles, in SC than in SL.

4. Discussion

The regulatory process called "stringent response" that is aimed at the adaptation of bacterial growth to nutrient limitation is known to be highly conserved throughout the bacterial domain of life [17]. Stringent control is known to regulate negatively the

expression of most ribosomal proteins [25,26]. Our data indicated that the majority of ribosomal proteins (Fig. 2, groups A and B), of proteins involved in rRNA modifications (Fig. 1, group B), of enzymes involved in tRNA synthesis and modification (Fig. 3, groups D and E) and of proteins involved in translation initiation, elongation and termination processes (Fig. 4, group B) were less abundant in SC than in SL. In consequence, the reduced abundance of these proteins in SC is proposed to be due to the strong activation of the stringent response in this strain that is most likely linked to the reduced ability of this strain to assimilate nitrogen as well as phosphate, to a lesser extent [1,3,4]. This hypothesis is supported by the observation that two proteins whose expression is known to be under the positive control of stringent response in other bacteria were over-expressed in SC compared to SL. These are the tRNA^{m5}(U34) methyltransferase TrmA-like (SCO5901) (Fig. 1) [36] and the elongation factor EF-Tu3 (SCO1321) (Fig. 4) [35]. The protein L31 (SCO5359) was also found more abundant in SC than in SL at 48 h and 60 h in Pi limitation. The expression of this protein was shown to be under the positive control of Sigma R that controls the expression of genes involved in the resistance to oxidative/disulfide stress in *Streptomyces* {Paget, 1998 #1191} [80]. So the high expression of L31 in SC ought to be related to high oxidative stress linked to the previously reported strong activation of the oxidative metabolism of this strain [1,2,82]. Interestingly, it was recently demonstrated that ppGpp is also involved in the response to oxidative [133] and heat stresses [134]. ppGpp might thus be involved in the response to any stresses that would lead to a growth slow down mediated by a downregulation of the translational process. Such regulation would also reduce the load on the protein quality control system (including numerous chaperones and proteases) that is induced by these stresses.

Interestingly, the abundance of 31 proteins, 8.4% of total proteins detected (Fig. 1, 7 of group A; Fig. 2, 11 of group D; Fig. 3, 7 of group C; Fig. 4, 2 of group A and Fig. 5, 4 of group D), was similar in the two strains in both Pi conditions. These proteins might thus constitute the core of a minimal translational apparatus that would be relatively more abundant in SC than in SL and might be responsible for the more efficient translation of lmrRNAs in SC than in SL.

Furthermore, the abundance of most ribosomal proteins (Fig. 2, group B), of proteins involved in rRNA modifications (Fig. 1, group B), of enzymes involved in tRNA synthesis and modification (Fig. 3, groups D and E) and of proteins involved in translation initiation, elongation and termination processes (Fig. 4, group B) was up-regulated in Pi proficiency in SL but such up-regulation did not occur in SC except for group C of ribosomal proteins (Fig. 2). These observations indicate that high Pi availability reduces the intensity of the stringent response in SL or in other terms that Pi limitation contributes to the stringent response. However this regulatory process does not occur in SC since the abundance of most proteins of the translational apparatus remains similarly low in both Pi conditions. We propose that the absence in SC of this phosphate-mediated up-regulation of ribosomal proteins in Pi proficiency might be due a default in external phosphate sensing and/or uptake. This default itself might be due to the strong activation of the stringent response occurring in this strain that would control negatively phosphate assimilation and phosphate-mediated regulation. Indeed, the assimilation of nitrogen (N), phosphate (P) and carbon (C), ought to be coordinated in *Streptomyces* [135], as in all living organisms. The poor ability of SC to assimilate N is correlated with the reduced abundance of proteins playing a role in C up-take and assimilation such as the glucose permease (SCO5578) (Fig. 16B of [1]) and all glycolytic enzymes in SC compared to SL (Fig. 2 of [1]) as well as of proteins of the Pho regulon [3]. The low affinity phosphate transporter Pih1 (SCO4138) is also poorly expressed in

Pi proficiency in SC compared to SL (Group D of Fig. 6 of [1]) and consequently some proteins involved in Pi re-cycling such as the nucleases SCO4152 and SCO1908, the phospholipases SCO6691 and SCO1048, the phosphodiesterases SCO1968 and SCO1565 and the phosphatase SCO2068 were up-regulated in SC compared to SL, in condition of Pi proficiency (Groups A and C of Fig. 6 of [1]). This suggests that despite the abundance of Pi in the growth medium, SC does not up-take it efficiently. SC is thus limited in phosphate, even when Pi is abundant in the growth medium, and is forced to degrade its phosphate-rich constituents in order to recycle phosphate. Altogether our data suggest that nitrogen and phosphate limitation both contribute to the triggering of the stringent response that in turn controls negatively nitrogen, phosphate as well as carbon metabolism to achieve growth reduction in SC. In contrast the stringent response obviously controls positively the expression of some specialized metabolites biosynthetic pathways [28]. Similar regulations were shown to occur in *Pseudomonas protegens* [136].

The production of these specialized metabolites could thus be considered as part of the stringent response. Since the main aim of the stringent response is to reduce growth when essential nutrients, such as N or P, are poorly available, we can consider that some specialized metabolites also contribute to growth slow down. Interestingly ACT, as well as other specialized metabolites of the polyketide family, were proposed to be able to capture electrons of ROS/NOS as well as of the respiratory chain, thanks to their quinone groups [137]. This ability would confer them anti-oxidant and anti-respiratory properties leading to a reduction of ATP generation [137]. Consistently, the onset of ACT production was shown to coincide with an abrupt drop in the intracellular ATP concentration [2] that might contribute to growth slow down.

Further studies are needed to elucidate the complex regulatory network governed by ppGpp in *Streptomyces* but it should be stressed that these studies have great fundamental as well as practical interest since the stringent response has a strong positive impact on the production of bio-active specialized metabolites that have considerable utility in the medical and agricultural fields.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.resmic.2023.104177>.

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