# The secreted signaling protein factor C triggers the A-factor response regulon in Streptomyces griseus: overlapping signaling routes

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# The Secreted Signaling Protein Factor C Triggers the A-factor Response Regulon in *Streptomyces griseus*

OVERLAPPING SIGNALING ROUTES*[S]

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**Members of the prokaryotic genus *Streptomyces* produce over 60% of all known antibiotics and a wide range of industrial enzymes. A leading theme in microbiology is which signals are received and transmitted by these organisms to trigger the onset of morphological differentiation and antibiotic production. The small γ-butyrolactone A-factor is an important autoregulatory signaling molecule in streptomycetes, and A-factor mutants are blocked in development and antibiotic production. In this study we showed that heterologous expression of the 324-amino acid secreted regulatory protein Factor C resulted in restoration of development and enhanced antibiotic production of an A-factor-deficient bald mutant of *Streptomyces griseus*, although the parental strain lacks an *facC* gene. Proteome analysis showed that in the *facC* transformant the production of several secreted proteins that belong to the A-factor regulon was restored. HPLC-MS/MS analysis indicated that this was due to restoration of A-factor production to wild-type levels in the transformant. This indicates a connection between two highly divergent types of signaling molecules and possible interplay between their regulatory networks. *Molecular & Cellular Proteomics 6:1248–1256, 2007.***

Bacteria of the Gram-positive filamentous *Streptomyces* are a well known model system for the study of prokaryotic multicellular differentiation with a complex life cycle culminating in spore formation (1). The onset of development is triggered by nutritional signals (2) and temporally relates to the production of antibiotics and other secondary metabolites (3). Autoregulatory molecules play a key role in controlling both the onset of cellular differentiation and secondary metabolism in these microbes. The best studied autoregulator is A-factor (2-isocapryloyl-(3*R*)-hydroxymethyl-γ-butyrolactone), a small microbial hormone-like molecule (243 Da) that induces both morphological and physiological differentiation in *Streptomyces griseus* (4, 5) and its direct homologue SCB1 that plays an important role in the control of antibiotic production of *Streptomyces coelicolor* (6, 7). In *S. griseus* binding of A-factor to its cellular receptor ArpA derepresses expression of the transcriptional activator AdpA. Although initially identified as the activator of streptomycin production, this protein acts as a central switch, and the AdpA regulon includes several important positive regulators of development, including *ssgA*, *amfR*, and *adsA* (*bldN*) (for a review, see Ref. 8). A-factor-deficient mutants are neither able to sporulate nor able to produce antibiotics (streptomycin).

Another interesting autoregulator is the secreted signaling protein Factor C (molecular mass, 34.555 Da), originally isolated from the culture fluid of "*S. griseus* 45H" but recently shown to be identical to a laboratory strain known as *Streptomyces flavofungini*, itself a member of the *Streptomyces albidoflavus* species group.[1] The Factor C producer strain, like *S. griseus*, readily sporulates in submerged culture (9, 10). Similarly to A-factor, Factor C also plays a key role in cellular communication and cytodifferentiation. Expression of *facC* from a low copy plasmid in a spontaneous A-factor-deficient bald mutant of *S. griseus* NRRL B-2682 restored its aerial mycelium and spore formation on solid medium. This strain does not produce Factor C as shown by immunoblotting (11) and by DNA hybridization studies (12). This suggested that the Factor C response regulon acts independently or has a complex interaction with the A-factor regulon. In preliminary experiments comparing one-dimensional protein gels of the

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[1] Z. Kiss, A. C. Ward, Z. Birkó, K. F. Chater, and S. Biró, unpublished data.

above strains we observed characteristic differences between the extracellular proteomes of the strains that prompted us to perform a detailed analysis. Here we compare the extracellular proteomes of *S. griseus* NRRL B-2682 and its A-factor-non-producing bald mutant with the same strain expressing Factor C from a low copy number vector.

## MATERIALS AND METHODS

*Strains and Preparation of Extracellular Protein Fractions*—Strains of *S. griseus* were grown on R2YE agar plates (13) covered with a polycarbonate track-etched membrane (Poretics; 0.2-$\mu$m pore size). The strains were *S. griseus* NRRL B-2682 (parental strain; in short B2682), its A-factor-non-producing bald mutant *S. griseus* NRRL B-2682 AFN (in short AFN), and a transformant of AFN (designated AFN/pSGF4) that harbors *facC* on the pHJL401-based low copy number plasmid pSGF4 (14). Protein extracts were prepared from spent agar of surface-grown cultures by crumbling the solid medium and passing it through a syringe with frits at 4 °C by centrifugation. Samples of ~300 $\mu$g of protein (measured using the Coomassie protein assay reagent, Pierce) were purified using the ReadyPrep™ 2D[2] cleanup kit (Bio-Rad) according to the instruction manual and dissolved in Rehydration Buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% 100× Bio-Lyte 3/10 (or 4/7) ampholyte, 0.002% bromphenol blue).

*2D Gel Electrophoresis and Image Analysis*—Separation of protein extracts (~300 $\mu$g) in the first dimension was performed by isoelectric focusing using 17-cm-long Immobiline DryStrip gels (IPG) in the pH range of 3–10 or 4–7 (Bio-Rad) on a Protean IEF cell (Bio-Rad). Samples were focused at 250 V for 15 min followed by an increase to 8000 V over 2.5 h and kept at this voltage for 45,000 V-h. Focused strips were separated on the basis of relative molecular weight in the second dimension on 13% SDS-polyacrylamide gels in a Protean II XL vertical gel system (Bio-Rad). For quantitative comparison of extracellular protein profiles gels were stained with colloidal Coomassie G-250 (15). Gels were scanned using a GS-800 imaging densitometer (Bio-Rad), and images were analyzed with PDQuest™ software (Bio-Rad). Histograms comparing spot quantity were generated with this software. 2-fold differences compared with the parental B2682 were considered as significant changes. The data below are from a single representative experiment, but at least two additional biological replicas were performed, and they showed similar results.

*In-gel Digestion*—Gel slices containing 2D PAGE-separated proteins were cut, diced, and then washed with 25 mM $NH_4HCO_3$ in 50% (v/v) acetonitrile/water. After reduction with 10 mM DTT (30 min at 56 °C) and alkylation with 55 mM iodoacetamide (30 min at room temperature in the dark) the proteins were digested with side chain-protected porcine trypsin (Promega, Madison, WI) at 37 °C for 4 h. Tryptic digests were extracted and desalted on $C_{18}$ ZipTips (Millipore, Bedford, MA). Mass spectrometric analysis of the unfractionated tryptic digests was performed in positive ion, reflectron mode on a Reflex III MALDI-TOF mass spectrometer (Bruker, Karlsruhe, Germany) using 2,5-dihydroxybenzoic acid as the matrix. Two-point external calibration was applied; this guarantees a mass accuracy within 200 ppm. The peak lists were generated with flexAnalysis (version 2.0) software: peak detection algorithm, Sophisticated Numerical Annotation Procedure; signal-to-noise threshold, 5; quality factor threshold, 30; sodium adducts; and trypsin autolysis products were deleted from the lists. Masses detected were submitted to a database search with MS-Fit in ProteinProspector version 4.23.4 in the National Center for Biotechnology Information non-redundant (NCBInr) database (July 18, 2006; 3,794,285 sequences).

Search parameters were as follows: mass accuracy, 200 ppm; only tryptic cleavages were permitted; and two missed cleavages were considered. Carbamidomethylation of Cys residues was a fixed modification; methionine oxidation, protein *N*-acetylation and pyroglutamic acid formation from N-terminal Gln residues, and Me esterification of Asp and Glu residues (Coomassie Brilliant Blue-staining side reaction (16) proven by PSD) were the considered variable modifications. Esterified peptides were only accepted when the peptide was also detected without the modification. Protein identification was confirmed by sequence information obtained from MS/MS (post-source decay; data shown in Supplemental Figs. S1–S9) spectra acquired in 10–12 steps, lowering the reflector voltage by 25% in each step, and then stitching the data together. Some MS/MS experiments were performed on an Agilent XCT plus ion trap equipped with an atmospheric pressure MALDI source using 4-OH-$\alpha$-CN-cinnamic acid as the matrix. Search parameters for MS/MS data were 200 ppm for the precursor ion and 1 Da for the fragment ions. Cleavage specificity and covalent modifications were considered as described above. Instead of the accession numbers the locus is listed in our tables thus eliminating the redundancy originated from multiple entries for the same amino acid sequence. When there were multiple entries corresponding to slightly different sequences only the database entry with the most matching peptides was included.

*Measurement of Antibiotic Concentration*—Antibiotic concentration in the supernatants of cultures grown in soybean medium (2% corn-steep liquor, 2% soybean meal, 0.3% NaCl, 0.2% $CaCO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 2% glucose, pH 7.5) was determined by an agar diffusion method. Antibiotic medium (0.5% meat extract, 0.1% $Na_2HPO_4$, 0.01% $KH_2PO_4$, 0.05% peptone) was mixed with *Bacillus subtilis* ATCC 6633 spores. In brief, wells were cut in the agar and filled with either 100 $\mu$l of the concentrated samples, of 10-fold diluted samples, or of a streptomycin standard dilution series and were incubated at 37 °C for 18 h. Diameters of the cleared zones were linear over the range studied (0–10 $\mu$g $ml^{-1}$).

*Prediction of Adp Binding Sites*—Multiple alignments and position weight matrices were generated with the Target Explorer automated tool (17, 18). The weight matrix for AdpA was generated from a set of experimentally validated AdpA binding sites (19).

*Extraction of A-factor from Liquid-grown Cultures*—To assay the production of A-factor, the strains were grown in GYM (glucose, yeast extract, malt extract) medium (20) in submerged cultures in a rotary shaker at 230 rpm at 28 °C for 72 h. The complete culture (400 ml) was extracted with an equal amount of chloroform, and the organic phase was evaporated in a vacuum (Rotadest, Buchi; 60 °C). The concentrated extract was then redissolved in 200 $\mu$l of ethanol.

*Biological Assay of A-factor*—The presence of A-factor in extracts was determined using a modified version of an agar diffusion assay (21). For this, the test strains were streaked on GYM or DM1-agar medium (20), a paper disc containing 20 $\mu$l of extract or authentic A-factor (1 mg/ml in EtOH; Funakoshi Co. Ltd. Code Number KA106) was placed on the agar plates close to the bacteria, and plates were incubated at 28 °C for 96 h. As indicator strains we used *S. griseus* HH1 (22) or *S. griseus* AFN. Restoration of aerial mycelium and spore production were inspected.

*Determination of A-factor by Mass Spectrometry*—MS measurements were obtained on a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Finnigan atmospheric pressure chemical ionization source. The instrument was operated in positive ion mode using selective reaction monitoring and fragment ion scan mode. In selective reaction monitoring mode the first quadrupole was set to select *m/z* 243 ($M + H^+$ of A-factor),

[2] The abbreviations used are: 2D, two-dimensional; Cpase, carboxypeptidase; SGAP, *S. griseus* leucine aminopeptidase; SGPA, Streptogrisin A precursor; Tat, twin arginine translocation; ID, identification; MM, minimal medium.

which was fragmented in the collision cell, and the three most intense fragments (*m/z* 127, 109, and 67) were measured with the last quadrupole. For quantitative determination of A-factor the most intense mass chromatogram (*m/z* 109) was used. The atmospheric pressure chemical ionization needle was adjusted to 4 $\mu$A, and $N_2$ was used as a nebulizer gas. The collision potential was 20 eV. Argon was used as the collision gas, and the pressure in the collision cell region was 2 millitorrs.

Samples were subjected to short gradient HPLC separation before MS analysis. Mobile phases were: solvent A, water and formic acid (99.95:0.05, v/v); solvent B, methanol, water, and formic acid (80:19.95:0.05, v/v/v). The HPLC apparatus was Applied Biosystems 140C. Conditions were as follows: flow rate, 250 $\mu$l $min^{-1}$; gradient, 0–100% B in 10 min; column, home-packed Nucleosil $C_{18}$, 20-$\mu$m-diameter × 2.0-mm length.

*RNA Isolation*—Mycelium was harvested from liquid-grown cultures of *S. griseus* B2682 and its A-factor-non-producing mutant AFN. Strains were grown in TSBS (tryptone, soya broth, sucrose medium) until an $A_{550}$ of 0.6, washed in MM without carbon source, and transferred to MM with 0.5% (w/v) mannitol as the carbon source and 0.1% casamino acids. This elicits production of A-factor among others leading to submerged sporulation. Cultures were incubated at 30 °C, and RNA was isolated after 0, 20, 60, and 180 min. RNA was purified using the Kirby-mix protocol (13). RNA purification columns (RNeasy, Qiagen) and DNase I treatment were used as well as salt precipitation (final concentration, 3 M sodium acetate, pH 4.8) to purify the RNA and fully remove any traces of DNA, respectively. Before use, the RNA preparations were checked for their quality and integrity on an Agilent 2100 Bioanalyzer (Agilent Technologies).

*PCR Amplification and Sequencing of afsA in S. griseus AFN*—PCRs were performed with *Pfu* polymerase (Stratagene) in the presence of 10% (v/v) DMSO. The program used was as follows: 2 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 72 °C. The primers were AfsA-For (5′-ctggaattccggtaaacggcgcggcctgtgag) and AfsA-Rev (5′-agtcagatctatccgcacgggtccggcatccgccag). DNA sequencing directly on the PCR-amplified DNA was performed by BaseClear BV (Leiden, The Netherlands).

*Transcriptional Analysis of afsA by RT-PCR*—Transcriptional analysis of *afsA* was carried out by RT-PCR using the Superscript III one-step RT-PCR system with Platinum® *Taq* DNA polymerase (Invitrogen). For each RT-PCR 100 ng of RNA was used together with 1 $\mu$M final concentration of each primer. The program used was as follows: 45-min cDNA synthesis at 48 °C followed by 2 min at 95 °C and 35 cycles of 45 s at 94 °C, 30 s at 68 °C, and 30 s at 68 °C. The reaction was completed by 5-min incubation at 68 °C. 5 $\mu$l of each sample was visualized by ethidium bromide staining after electrophoresis on a 2% agarose gel in 1× Tris acetate-EDTA buffer. The oligonucleotides were afsA_RTfor (5′-gaagcggtccttccgcacgacca) and afsA_RTrev (5′-gcccgccacttcaggtcggagca). Quantification of the RT-PCRs was done by scanning the gels using the GS-800 imaging densitometer and analysis using Quantity One software (Bio-Rad). For control experiments, to rule out DNA contamination of the RNA samples, the same experiments were repeated under identical conditions but after inactivation of the reverse transcriptase by incubation for 5 min at 95 °C.

## RESULTS AND DISCUSSION

*Extracellular Proteome Analysis*—To study the effect of Factor C on protein expression, we analyzed the extracellular proteomes of *S. griseus* B2682, of its A-factor-non-producing spontaneous mutant (AFN) that had a bald phenotype, and of the AFN strain with restored sporulation due to complementation with a plasmid expressing Factor C (called AFN/pSGF4 (14)). Our choice for the extracellular proteome relates to our focus on signaling and to the fact that many of the AdpA target proteins are extracellularly localized.

To obtain an initial assessment of the protein expression profiles in the different strains, SDS-PAGE was performed, revealing that indeed several protein bands varied strongly in intensity. Considering the low separative resolution of one-dimensional gels the samples were analyzed further by 2D electrophoresis. The vast majority of proteins in the samples collected after 3 and 4 days of growth on R5 solid cultures appeared in the pH range 4–7 when an IPG strip of pH 3–10 was applied. Further separations, using the narrower isoelectric point range pH 4–7 in the first dimension and a molecular mass range between 6.5 and 200 kDa in the second dimension, produced 200–240 detectable spots on each gel after colloidal Coomassie staining (Fig. 1). We focused on proteins whose expression differed at least 2-fold between the studied strains. 50 spots that fulfilled this criterion were isolated and analyzed by MALDI-TOF mass spectrometry. The main question we sought to address was: *what is the response triggered by Factor C that results in the restoration of development to the AFN strain?* Although at the time of this study two complete *Streptomyces* genome sequences (*S. coelicolor* and *Streptomyces avermitilis*) and a nearly complete one (*Streptomyces scabies*) were available, the lack of a complete *S. griseus* database severely hampered our efforts in the identification of the relevant proteins. Nine of 50 spots were unambiguously identified (Tables I and II).

In 72-h samples six protein spots were absent in samples prepared from the AFN mutant but present in samples from the complemented strain AFN/pSGF4 and the parental strain B2682 (Fig. 1, *a*, *b*, and *c*, and Fig. 2*A*, spots 1–6.). These were the trypsin-like protease SprU (spot 1 (23)), the zinc carboxypeptidase precursor Cpase SG (spot 2 (24)), the aminopeptidase SGAP (spot #3 (25)), Streptogrisin A precursor (SGPA; one of five chymotrypsin-type serine proteases in *S. griseus* and encoded by *sprA*; spot 4 (26)), SgmA (a zinc metalloendopeptidase; spot 5 (27)), and StrU (spot 6 (28)), an NAD(P)-dependent oxidoreductase that is part of the streptomycin biosynthesis pathway (Tables I and II). Of these proteins, the latter three (SGPA, SgmA, and StrU) were enhanced in AFN/pSGF4 relative to B2682 (Fig. 2*A*, spots 4–6), whereas for the other three we detected no significant difference in expression (Fig. 2*A*, spots 1–3). Analysis of samples collected after 96 h produced a similar result (Fig. 1, *d*, *e*, and *f*), although some expression was now observed for proteins SprU, CPase, SGAP, and SgmA in AFN (Fig. 2*B*, spots 1–3 and 5), suggesting strongly delayed expression.

Expression levels of the phosphate-binding protein precursor PstS (spot 7) (29) and of two isoforms of the oxidative defense protein SodF (iron,zinc-superoxide dismutase (30); spots 8 and 9) were clearly enhanced in the 72-h samples of the *bld* mutant AFN (Fig. 1, *a*, *b*, and *c*, and Fig. 2*A*, spots 7–9). Both PstS and SodF have been implicated in the control of development. Mutants of *pstS* show accelerated morpholog-

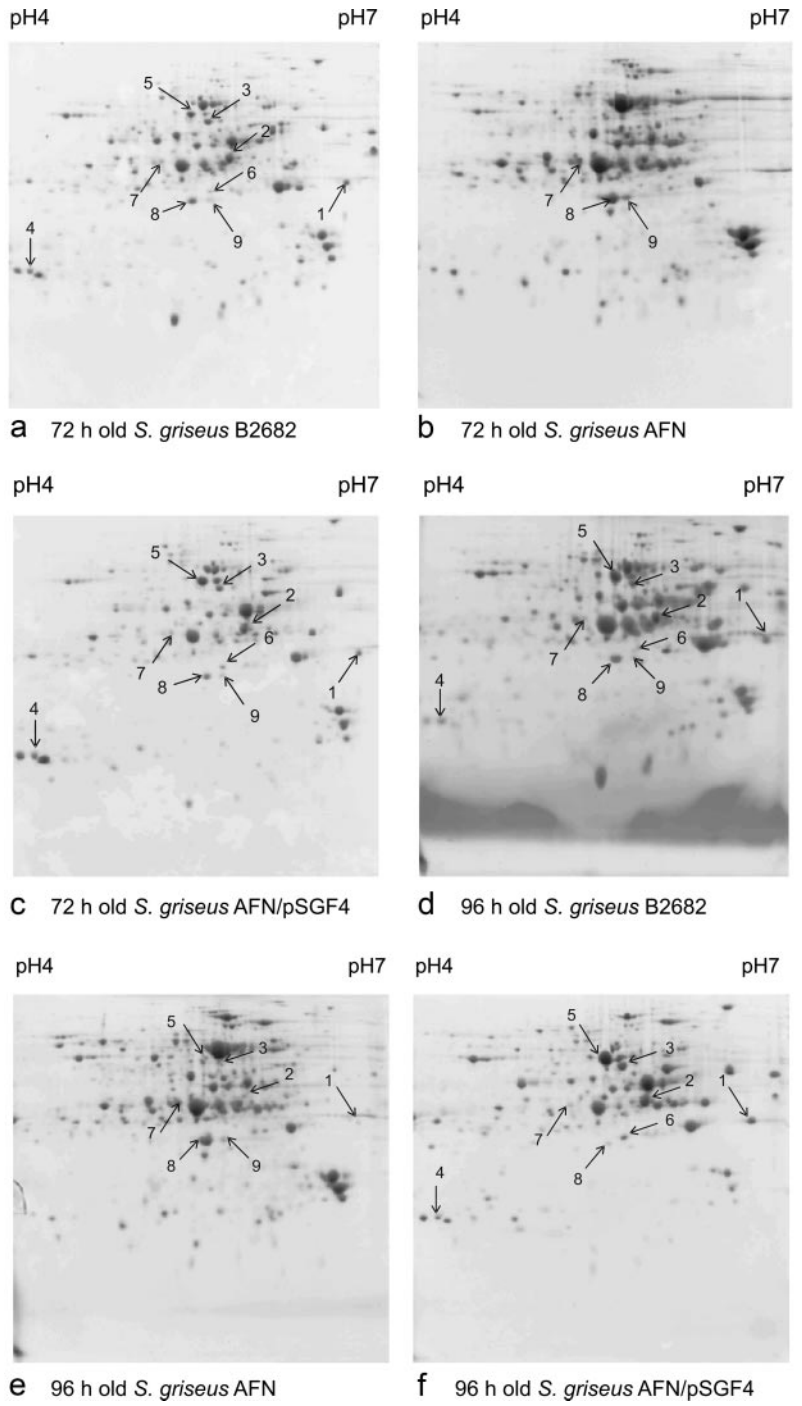


FIG. 1. **2D gel electrophoresis maps of secreted proteins extracted from 72-h-old surface-grown cultures of B2682 (*a*), AFN (*b*), and AFN/pSGF4 (*c*) and from 96-h-old surface-grown cultures of B2682 (*d*), AFN (*e*), and AFN/pSGF4 (*f*).** *Numbers* correspond to the ID numbers of proteins in the tables.

ical differentiation (29), whereas *sodF* is controlled by the A-factor-like γ-butyrolactone SpbR (*Streptomyces pristinaespiralis* butyrolactone-responsive repressor) in *S. pristinaespiralis*, which is also essential for its colony development and antibiotic biosynthesis (30). SodF converts the superoxide anion to hydrogen peroxide and molecular oxygen, catalyzed by cyclic oxidation and reduction of the transition metal in the active site of superoxide dismutases (31). SodF was identified as a cytoplasmic protein in *S. griseus* (32) and as an extracellular enzyme in *S. pristinaespiralis* (30). Because the intracellular and extracellular isoforms have the same molecular weight, SodF may well be autotransported as a leaderless protein (33). Our experiments suggest that at least in *S. griseus* SodF is exported. For SodF we observed a significant difference in expression between the 72- and 96-h cultures. Although its expression decreased significantly in both time points in the *facC*-expressing transformant, expression was typically lower in the 96-h cultures (Fig. 2, *A* and *B*, spots 8 and 9).

TABLE I

*List of the identified differentially expressed Factor C-responsive proteins*

From the NCBI Protein Database.

| ID no. | SSP[a] 72 h | SSP[a] 96 h | Accession no. | Protein | Name | Match[b] | Seq. Cov.[c] | Molecular mass[d] | Sequence confirmed by PSD or CID[e] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | % | *kDa* | |
| 1 | 9301 | 9301 | BAD24662 | SprU | Trypsin-like serine protease | 11/24 | 28 | 26 | $^{103}$VLQAPGYNGTGKDWALIK$^{120}$ |
| 2 | 5403 | 5503 | CAA46635 | Cpase SG | Carboxypeptidase | 27/34 | 41 | 49 | $^{405}$SASGGGFYPPDEVIER$^{420}$ |
| 3 | 4701 | 4603 | BAD67179 | SGAP | Aminopeptidase | 15/28 | 37 | 36 | $^{279}$WGGTAGQAFDR$^{289}$ |
| 4 | 0102 | 0102 | CAA01746 | SGPA | Protease A | 6/21 | 46 | 18 | $^{47}$TGTSFPNNDYGIIR$^{60}$ |
| 5 | 3801 | 4701 | BAC21011 | SgmA | Metalloendopeptidase | 9/17 | 13 | 70 | $^{318}$SGIRGDGVGAYSR$^{330}$ |
| 6 | 4202 | 4302 | CAH94303 | StrU | NAD(P)-dependent oxidoreductase | 13/14 | 31 | 45 | $^{398}$DSVLGYADVTLPPGR$^{412}$ |
| 7 | 2402 | 3501 | CAB65418 | PstS | Phosphate-binding protein | 18/47 | 38 | 39 | $^{353}$ILTDAGYAPIPAEINAK$^{369}$ |
| 8 | 3201 | 4201 | AAD30139 | SodF | Superoxide dismutase | 19/36 | 37 | 23 | $^{121}$AAATTQGSGWGVLAYEPVSGK$^{141}$ |
| 9 | 4201 | 4203 | AAD30139 | SodF | Superoxide dismutase | 13/35 | 34 | 23 | $^{52}$DKEAWGAINGLQK$^{64}$ |

[a] Standard spot numbers are assigned automatically by the PDQuest software and are usually different for the same protein in 72- and 96-h cultures.

[b] Matched/observed masses.

[c] Sequence covered.

[d] Molecular masses calculated from the sequence in the database. Proteins may be post-translationally processed and covalently modified. Thus, the mature forms may feature significantly different molecular masses.

[e] See Supplemental Figs. S1–S9.

TABLE II

*Expression changes of the identified Factor C-responsive proteins*

Detailed results on protein identification are listed in Table I. For each protein the ID numbers are indicated together with changes of the protein levels relative to the level in the parental strain *S. griseus* B2682 in 3- and 4-day-old cultures. Symbols for relative expression levels are as follows: 0, below detection limit; =, similar to parent; ↓, decreased; ↑, increased; ND, not determined. Secr., refers to the secretion pathway that is most likely to be responsible for the export of the protein.

| ID no. | AFN 72 h | AFN 96 h | AFN + pSGF4 72 h | AFN + pSGF4 96 h | Secr. | Cofactor |
|---|---|---|---|---|---|---|
| 1 | 0 | ↓ | = | = | Sec | |
| 2 | 0 | ↓ | = | ↑ | Tat | Zinc |
| 3 | 0 | ↓ | = | ↑ | Tat | Zinc |
| 4 | 0 | 0 | ↑ | ↑ | Tat | |
| 5 | 0 | ↓ | ↑ | = | Tat | Zinc |
| 6 | 0 | 0 | ↑ | ↑ | Tat | NAD |
| 7 | ? | ↓ | = | ↓ | Tat? | |
| 8 | ? | = | = | ↓ | ? | Zinc, iron |
| 9 | ? | = | = | 0 | ? | Zinc, iron |

Several of the proteins whose expression is restored approximately to the original parental levels by Factor C, namely SprU, SGPA, SgmA, and StrU, have been identified previously by Horinouchi and co-workers (8) as members of the AdpA regulon in *S. griseus*. It was also reported that their expression is activated in a growth phase-dependent manner. Cpase SG (24) is also a possible development-related protein as it has significant similarity to the sporulation-related zinc peptidase I in *Bacillus sphaericus* (34). Of the known AdpA-dependent genes, *sprA*, *sgmA*, and *sprU* are all strongly transcribed after ~72 h (23, 26, 27). We could readily identify all gene products in samples prepared from 3–4-day-old mycelia, suggesting possible function in the control of development. The substrate mycelium is lysed during aerial mycelium formation, and a likely function for hydrolytic enzymes such as proteases, nucleases, and lipases is to degrade the cytoplasmic contents and supply material to the newly forming aerial mycelium (35).

Restoration of the expression of StrU by *facC* is particularly interesting as this shows that not only development is restored by Factor C to an A-factor-non-producing *bld* mutant but perhaps also antibiotic production. AdpA transmits the upstream A-factor signal directly to the streptomycin biosynthesis genes via the pathway-specific activator *strR* (28, 36–38). To assess whether indeed streptomycin production was enhanced by Factor C we measured antibiotic production of B2682, AFN, and AFN/pSGF4. Although we failed to detect significant amounts of streptomycin in either B2682 or AFN (*i.e.* below the detection limit of about 1 $\mu$g ml$^{-1}$ for the agar diffusion method used), significant amounts of streptomycin were detected in the spent culture fluid of AFN/pSGF4 with 8.2 $\mu$g ml$^{-1}$ after 48 h and 11 $\mu$g ml$^{-1}$ after 72 h of growth in liquid culture. This strong increase in antibiotic production corresponds well to the observed enhanced expression of StrU relative to that in AFN or B2682.

To further characterize the other targets, we subjected all genes corresponding to the identified proteins (Table I) to an extensive *in silico* analysis using the Target Explorer program (18) with all known AdpA binding sites as input for the production of a reliability matrix. Our predictions (Table III) suggest that all may be controlled by AdpA with statistically reliable sites upstream of *scpD*, *sgaP*, *pstS*, and *sodF.* All of these had not yet been identified as members of the AdpA regulon.

SodF is the only protein whose expression is enhanced in the absence of A-factor in *S. griseus*; this suggests negative control of SodF and therefore its defense against oxidative stress. Our *in silico* predictions revealed two potential AdpA

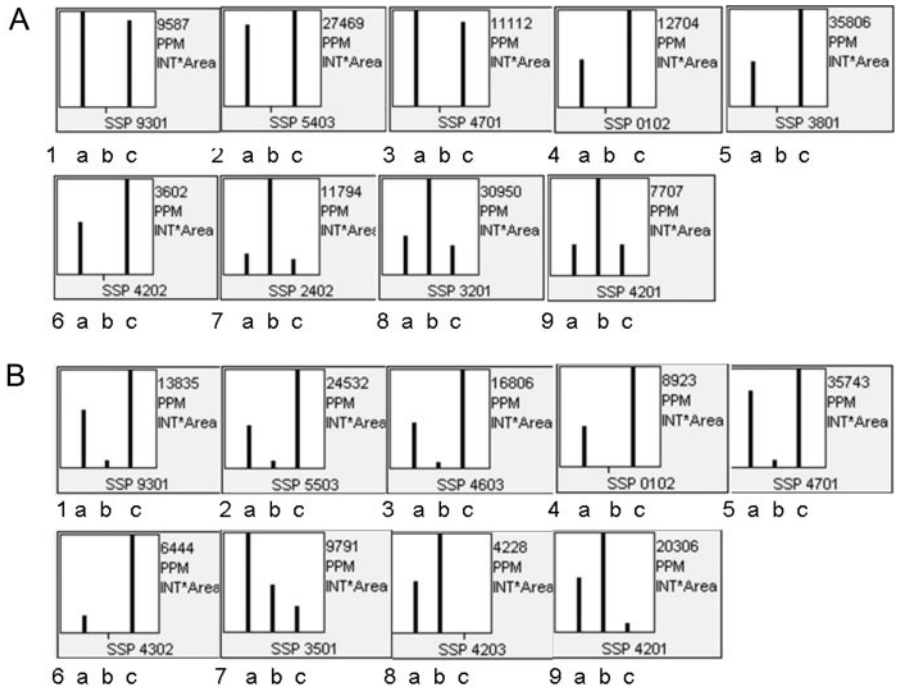


FIG. 2. **Quantitative image analysis of the proteins performed by PDQuest 2D analysis software (Bio-Rad) in 72-h-old cultures (*A*) and 96-h-old cultures (*B*).** *Numbers underneath* each plot correspond to the protein ID numbers in Fig. 1 and Tables I, II, and III. The *bars* represent the relative amount of the different proteins in the strains in the order of B2682 (*a*), AFN (*b*), and AFN/pSGF4 (*c*). *SSP*, standard spot number; INT, intensity.

TABLE III

*Known and predicted AdpA cis*-acting elements upstream of Factor C-responsive target genes

From the NCBI GenBank database. Sec, general secretory pathway.

| ID no. | Accession no. | Gene | Function | *cis* sequence | Position[a] | Score | Validation[b] |
|---|---|---|---|---|---|---|---|
| 1 | AB182576 | *sprU* | Trypsin-like serine protease | TGGCCGAAAA | −300 | 6.84 | Ref. 19 |
| 2 | X65719 | *scpD* | Carboxypeptidase | TGGCCGGAAC | −98 | 8.23 | Predicted |
| 3 | AB125217 | *sgaP* | Aminopeptidase | CGGCCGGATC | −72 | 7.51 | Predicted |
| | | | | TGTCCGGTTT | −171 | 7.23 | |
| 4 | A24972 | *sprA* | Protease A | AGGCCGGATT | −149 | 6.59 | Ref. 22 |
| | | | | TTGCTACCTT | −382 | 0.62 | |
| 5 | AB085745 | *sgmA* | Metalloendopeptidase | TGGCCGGTTT | −100 | 8.89 | Ref. 23 |
| | | | | TGGCCAGATT | −300 | 8.56 | |
| 6 | AJ862840 | *strU*[c] | NAD(P)-dependent oxidoreductase | CGGCTGATTC | −332 | 5.36 | Ref. 24 |
| | | | | TGGCGCGATC | −319 | 7.20 | |
| | | | | TGACCGAAAA | −115 | 4.81 | |
| | | | | TGGCCGTTGC | −89 | 2.79 | |
| 7 | AJ243674 | pstS | Phosphate-binding protein | CGTCCGAAAT | −107 | 4.59 | Predicted |
| | | | | CGGCGGCTTC | −130 | 4.48 | |
| 8, 9 | AF141866 | *sodF* | Superoxide dismutase | TGGCCCGTAA | −89 | 5.79 | Predicted |
| | | | | CGGCCCGAAC | −344 | 5.39 | |

[a] Position refers to the most distal nucleotide position and is relative to the translational start site of the gene (the translational start site was not known for all genes).

[b] Validation refers to either experimental evidence (reference is provided) or *in silico* prediction.

[c] AdpA binding sites refer to those identified upstream of *strR*, which encodes the transcriptional regulator of *strU*.

binding sites in the *sodF* upstream region (Table III). Taken together this suggests that *sodF* may be the first target that is negatively controlled by AdpA and thus that AdpA may act both as activator and as repressor of gene expression. An interesting discrepancy is that although in *S. griseus* the butyrolactone-responsive repressor ArpA negatively controls development by repressing AdpA (8), in *S. pristinaespiralis* SpbR stimulates growth and antibiotic biosynthesis and allows a normal response to oxidative stress (30). The precise control of SodF by butyrolactones in streptomycetes remains to be elucidated.

Importantly the majority of the targets are metal-binding proteins, most of which use zinc ions as cofactor, and carry the twin arginine translocation pathway signature for Tat secreted proteins (Table II). This pathway is responsible for the export of folded proteins together with their cofactors across

the cytoplasmic membrane (39). Factor C itself binds efficiently to a zinc column and has a zinc binding site that shows similarity to that of eukaryotic zinc finger proteins, including zinc finger proteins 275 and 502 of different animal species (40). Like most of the targets discussed in Table I, Factor C also has a secretion signal that conforms to the consensus signal sequence of the twin arginine translocation pathway and is therefore predicted to be exported via the Tat secretion pathway.

*A-factor Production of the Strains*—Restoration of development of AFN by Factor C and the observed high similarity between the extracellular proteomes of AFN/pSGF4 and the wild-type strain B2682 prompted the analysis of A-factor production by B2682, AFN, AFN/pSGF4, and *S. griseus* 45H. Two independent methods were used: a biological assay (21) and mass spectrometry. In the biological assay we used *S. griseus* HH1 (22) and AFN as test strains. Authentic A-factor and extracts prepared from B2682 and AFN/pSGF4 restored aerial mycelium formation of the *S. griseus* HH1 strain, whereas those prepared from AFN (Supplemental Fig. S10) or *S. griseus* 45H (not shown) had no effect. Aerial mycelium and spore formation were also restored by authentic A-factor and by B2682 and AFN/pSGF4 extracts but not by *S. griseus* 45H extracts when AFN was used as test strain (Supplemental Fig. S11). This strongly suggested that expression of *facC* restored A-factor production to the AFN strain.

This observation was further substantiated by the HPLC-MS/MS analysis of the cell extracts by comparison of the MS/MS of the A-factor standard and the cell extracts prepared from B2682, AFN/pSGF4, and AFN (Fig. 3). The mass of the captured fragments was identical in all four cases and in good agreement with the results published recently (41). Quantitative assessment of A-factor production of the different strains based on the measurement of the intensity of the most characteristic peak (109.2 Da) showed that A-factor is produced by B2682 (2.3 mg $ml^{-1}$) and by AFN/pSGF4 (2.0 mg $ml^{-1}$) strains in nearly equal amounts. In contrast, only trace amounts (0.0012 mg $ml^{-1}$, which is 1500–2000 times less than in B2682 and AFN/pSGF4 or in other words pg $ml^{-1}$ in the original culture) of A-factor were produced by AFN, and none was produced by *S. griseus* 45H (Supplemental Table S1 and Figs. S12–S15). Although AFN produces only a very small peak corresponding to A-factor, it is most likely A-factor as the fragmentation pattern is identical, producing the same characteristic peaks of 127, 109, and 67 Da. This implies that the A-factor biosynthetic genes are still functional in AFN and that instead the activation of A-factor biosynthesis has been lost. This makes AFN a very important mutant as uncovering the nature of this mutation would better our understanding of the control of A-factor biosynthesis.

*Sequencing and Transcriptional Analysis of afsA in S. griseus AFN*—The only known A-factor biosynthetic gene is *afsA* (906 bp) (42). To analyze whether the failure of AFN to produce A-factor was due to a defect in *afsA*, the DNA sequence and transcription of *afsA* from B2682 and AFN was compared. To

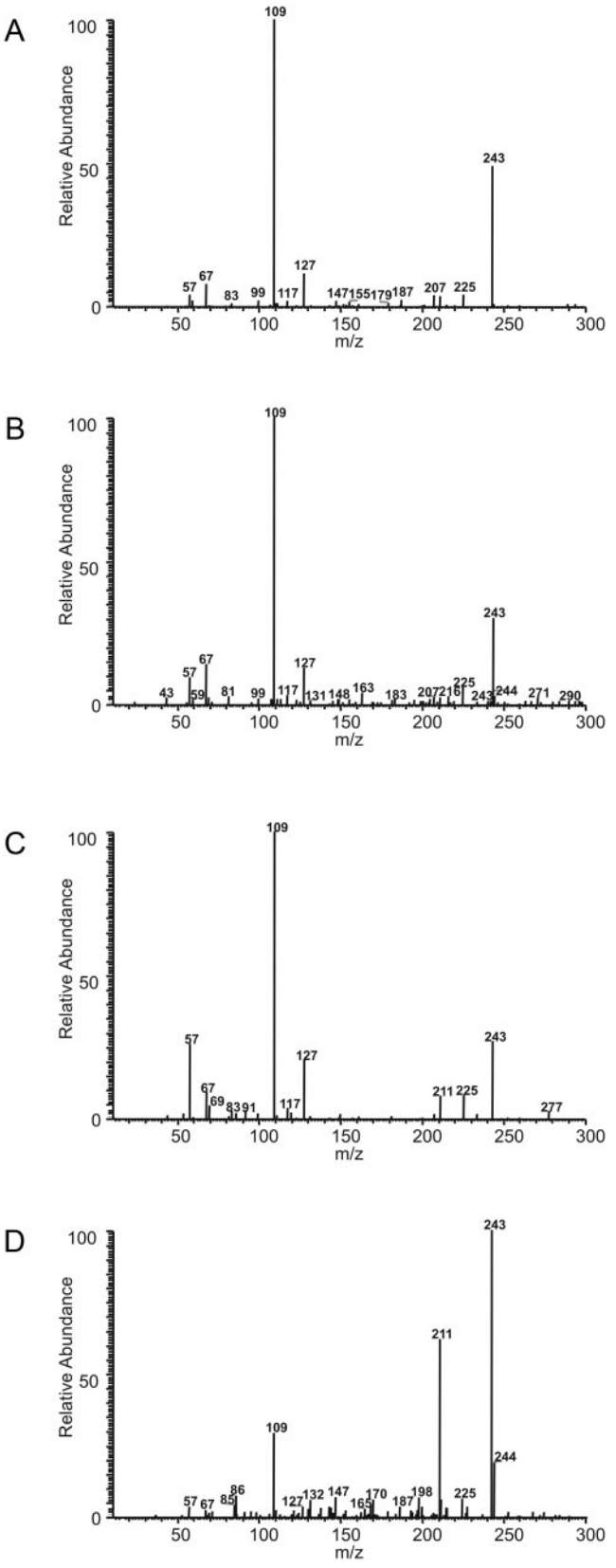


FIG. 3. **Comparison of MS/MS of authentic A-factor (*A*), B2682 (*B*), AFN/pSGF4 (*C*), and AFN (*D*) extracts.**

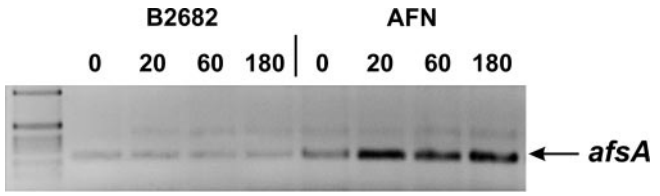


FIG. 4. **Analysis of the transcription of *afsA*.** Semiquantitative RT-PCR was performed on RNA samples isolated from *S. griseus* strains B2682 and its spontaneous bald mutant AFN 0, 20, 60, and 180 min after nutritional shift-down (from rich (TSBS) to MM liquid cultures). Control experiments on 16 S rRNA and without reverse transcriptase showed the integrity of the RNA and the lack of DNA, respectively, in the samples (data not illustrated).

obtain the DNA sequence, *afsA* and 200 bp of upstream sequences were amplified from genomic DNA of AFN by PCR using oligonucleotides afsA-For and afsA-Rev. Comparison with the published sequence of *afsA* (M24250, NCBI GenBank) showed that the predicted *AfsA* from AFN contained a GGT→GGC (Gly) silent mutation in the coding region but otherwise was identical to that of the parental strain B2682. No changes were found in the promoter region (Supplemental Fig. S16).

To establish whether *afsA* transcription was affected in *S. griseus* AFN, RNA was isolated from both strains and analyzed by semiquantitative RT-PCR. A-factor production and the subsequent submerged sporulation by *S. griseus* are triggered by nutritional shift-down, and these conditions were therefore chosen to ascertain significant transcription of *afsA*. Under the conditions chosen, small clublike conidia were formed by the parental strain B2682 at the tips of the hyphae after ~1.5–2 h following nutritional shift-down, whereas submerged sporulation started some 5 h later. Expectedly *bld* mutant AFN did not produce sporogenic submerged hyphae. The RNA was analyzed by semiquantitative RT-PCR using oligonucleotides afsA_RTfor and afsA_RTrev. *afsA* transcription was only slightly induced by the nutritional shift-down; interestingly total transcript levels were significantly higher in the mutant than in the parent (Fig. 4). In a control experiment where reaction conditions and enzymes were identical except that the reverse transcriptase was heat-inactivated, no products were obtained, confirming the absence of DNA contamination. Hence *S. griseus* AFN has a wild-type and actively transcribed *afsA* gene. Considering that AFN is probably able to produce A-factor, this strongly suggests that the defect in AFN lies in another yet unknown gene involved in the control of A-factor biosynthesis. The overexpression of the A-factor biosynthetic gene *afsA* in AFN mutants is best explained as an (unsuccessful) attempt to compensate for the absence of A-factor. Recently we observed a similar effect when we analyzed *S. coelicolor* mutants deficient in the cell division activator gene *ssgA*.[3] SsgA is involved in the localization of septa, and the cell machinery itself is unaffected in *ssgA* mutants; the lack of septal peptidoglycan synthesis prompted strong up-regulation of *ftsI*, which encodes the penicillin-binding protein responsible for synthesis of the septal peptidoglycan. Hence in both cases the genes (*afsA* or *ftsI*) normally responsible for the missing compounds (A-factor and septal peptidoglycan, respectively) are up-regulated. Whether this interesting concept is true for other vital biosynthetic pathways needs further investigation.

In conclusion, our experiments revealed that expression of *facC* in an A-factor-non-producing mutant of *S. griseus* results in significant changes in the expression profiles of at least 50 proteins of the extracellular proteome, restoring their expression to levels similar to that in the parent B2682. Although the genome sequence of *S. griseus* is not available, we positively identified an initial set of nine proteins. Four of these are known members of the A-factor regulon, and in all of the five others we found candidate AdpA binding sites in the promoter regions. The surprising finding that Factor C can fully restore sporulation to a bald A-factor-non-producing strain is explained by its ability to restore A-factor production. How Factor C is able to trigger the production of A-factor is a mystery. Our current research is focused on understanding the molecular basis for this exciting phenomenon.

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*Note Added in Proof*—Recently new insights on A-factor biosynthesis in *S. griseus* were provided by the Horinouchi group (Kato, J., Funa, N., Watanabe, H., Ohnishi, Y., and Horinouchi, S. (2007) *Proc. Nat. Acad. Sci. U.S.A.* **104,** 2378–2383.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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[3] E. Noens, V. Mersinias, C. P. Smith, and G. P. van Wezel, unpublished data.

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