Phosphorylation of a Novel Cytoskeletal Protein (RsmP) Regulates Rod-shaped Morphology in Corynebacterium glutamicum*

Received for publication, June 14, 2010, and in revised form, July 7, 2010 Published, JBC Papers in Press, July 9, 2010 DOI 10.1074/jbc.M110.154427

Maria Fiuza1, Michael Letek‡, Jade Leiba1, Almudena F. Villadangos‡, José Vaquera1, Isabelle Zanella-Cléon‡, Luis M. Mateos2, Virginie Molle1,2, and José A. Gil3

From the 1Departamento de Biología Molecular, Área de Microbiología, Facultad de Biología, Universidad de León, León 24071, Spain and the 2Institut de Biologie et Chimie des Protéines (UMR 5086), CNRS, Université Lyon1, IFR128 BioSciences, Lyon-Gerland, 7 Passage du Vercors, 69367 Lyon Cedex 07, France

Corynebacteria grow by wall extension at the cell poles, with DivIVA being an essential protein orchestrating cell elongation and morphogenesis. DivIVA is considered a scaffolding protein able to recruit other proteins and enzymes involved in polar peptidoglycan biosynthesis. Partial depletion of DivIVA induced overexpression of cg3264, a previously uncharacterized gene that encodes a novel coiled coil-rich protein specific for corynebacteria and a few other actinomycetes. By partial depletion and overexpression of Cg3264, we demonstrated that this protein is an essential cytoskeletal element needed for maintenance of the rod-shaped morphology of Corynebacterium glutamicum, and it was therefore renamed RsmP (rod-shaped morphology protein). RsmP forms long polymers in vitro in the absence of any cofactors, thus resembling eukaryotic intermediate filaments. We also investigated whether RsmP could be regulated post-translationally by phosphorylation, like eukaryotic intermediate filaments. RsmP was phosphorylated in vitro by the PknA protein kinase and to a lesser extent by PknL. A mass spectrometric analysis indicated that phosphorylation exclusively occurred on a serine (Ser-6) and two threonine (Thr-168 and Thr-211) residues. We confirmed that mutagenesis to alanine (phosphoablative protein) totally abolished PknA-dependent phosphorylation of RsmP. Interestingly, when the three residues were converted to aspartic acid, the phosphomimetic protein accumulated at the cell poles instead of making filaments along the cell, as observed for the native or phosphoablative RsmP proteins, indicating that phosphorylation of RsmP is necessary for directing cell growth at the cell poles.

In virtually all bacteria, the peptidoglycan (PG) cell wall maintains the shape and integrity of the cell. However, it is less clear how the shape of the cell wall “sacculus,” and thereby the cell itself, is genetically determined to yield a specific morphology. Recently, cytoskeletal proteins in the cytoplasm have been shown to be critical in determining bacterial cell shape and in controlling the two distinct modes of cell wall assembly, cell division and cell elongation, in time and space (for reviews see Refs. 1–3). Septa are organized and positioned at midcell by the tubulin homologue FtsZ, which forms a cytokinetic ring at the division site and recruits the various proteins (FtsA, FtsW, PBPs, etc.) involved in positioning the division septum at this site (4). Each division event gives rise to two daughter cells that undergo cell elongation. In most of the well established rod-shaped models such as Escherichia coli (5), Bacillus subtilis (6), and Caulobacter crescentus (7), cell elongation occurs by intercalation of new PG into the lateral wall along most of its length, and the poles remain largely inert (8). This elongation requires the actin homologue MreB, which-assembles into a helical cytoskeleton along the cell (9). Daniel and Errington (10) used fluorescently labeled vancomycin (Van-FL) staining to visualize active sites of PG assembly and showed that elongation of the lateral wall occurred by insertion of new cell wall material in a helical pattern along the length of the cell and that this pattern was dependent on the MreB isoform Mbl. Together, these observations led to a model in which the helical MreB cytoskeleton plays a role in organizing or localizing enzymes involved in cell wall assembly during elongation of the lateral wall, presumably by being linked to the cell wall synthetic machinery via MreC, MreD, and RodA (1, 11–13).

Although most rod-shaped or filamentous bacteria possess mreB genes, a second MreB-independent mode of cell elongation and acquisition of rod shape is present in actinobacteria, like Corynebacterium glutamicum, Streptomyces coelicolor, and Mycobacterium tuberculosis. In agreement with a previous report in Corynebacterium diphtheriae (14), staining with Van-FL revealed in C. glutamicum assembly of PG primarily at the cell poles instead of along the lateral wall (10). Because corynebacterial genomes lack mreB homologues (15–18), this polar cell wall elongation must be MreB-independent. Thus, in the simplest model for polarization of PG assembly in C. glutamicum, components of the cell division machinery would be sufficient to recruit the enzymes for wall elongation to the new cell pole. Interestingly, recent reports in C. glutamicum, S. coelicolor, and M. tuberculosis demonstrated that DivIVA is involved in apical growth and cell shape determination in these...
Characterization and Phosphorylation of *C. glutamicum* RsmP

**TABLE 1**

| Strains or plasmids | Genotype or description | Source or Ref. |
|---------------------|-------------------------|----------------|
| **E. coli** TOP10   | F' ΔmcrA Δ(mrr-hsdRMSC-mcrBC) φ80lacZ Δ(ara-leu)7697 galL galK rpsL endA1 napG; used for general cloning | Invitrogen |
| E. coli S17-1       | Mobilizing donor strain, pro recA, with an RP4 derivative integrated into the chromosome | 71 |
| E. coli BL21(DE3)Star | F2 ompT hsdS(rB2 mB2) gal dcm (DE3); used to express recombinant proteins in *E. coli* | Stratagene ATCC |
| C. glutamicum ATCC 13869 | Wild-type control strain | 72 |
| C. glutamicum R31   | C. glutamicum ATCC 13869; derivative used as recipient in conjugations | 23 |
| C. glutamicum LAC3264 | C. glutamicum R31 derivative carrying a chromosomal copy of divIVA under the control of Plac and a plasmid carrying lacI | This work |
| pOJ260              | Mobilizable plasmid containing an *E. coli* origin of replication and the apramycin resistance gene (*am*) | 42 |
| pOJ3264int          | pOJ260 derivative carrying an internal 419-bp fragment of the cg3264/rsmp gene from *C. glutamicum* | This work |
| pOJ3264             | pOJ260 derivative carrying the 5’ end (667-bp) of the cg3264/rsmp gene from *C. glutamicum* under the control of the Plac promoter | This work |
| pEAG6               | Mobilizable plasmid able to replicate in *E. coli* and *C. glutamicum* containing the Pdiv promoter, egfp2, and the kanamycin resistance gene (*kan*) | 23 |
| pE_RsmP-GFP         | pEAG6 derivative carrying the wild-type rsmp gene and expressing the wild-type RsmP protein fused to GFP | This work |
| pE_RsmPA-GFP        | pEAG6 derivative carrying an *in vitro* mutated rsmp gene and expressing a RsmP protein with S6A/T168A/T211A and fused to GFP | This work |
| pE_RsmPD-GFP        | pEAG6 derivative carrying an *in vitro* mutated rsmp gene and expressing a RsmP protein with S6D/T168D/T211D and fused to GFP | This work |

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Conjugal Plasmid Transfer from *E. coli* to *C. glutamicum*—Bacterial strains and plasmids are described in Table 1. Strains used for cloning and expression of recombinant proteins were *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3)Star (Stratagene), respectively. *E. coli* was grown and maintained at 37 °C in LB medium...
supplemented with 100 µg/ml ampicillin, 50 µg/ml apramycin, or 50 µg/ml kanamycin when required. C. glutamicum was grown at 30 °C in trypticase soy broth (Oxoid) or trypticase soy broth containing 2% agar medium supplemented with 12.5 µg/ml apramycin and/or 12.5 µg/ml kanamycin when needed. Plasmids to be transferred by conjugation from E. coli to corynebacteria were introduced by transformation into the donor strain E. coli S17-1. Mobilization of plasmids from E. coli S17-1 to C. glutamicum R31 was accomplished as described previously (32).

Two-dimensional Gel Electrophoresis—Wild-type C. glutamicum R31 and LACID strains (Table 1) were grown to early stationary phase. Cells were harvested, washed twice with 10 mM Tris-HCl, pH 8, and resuspended in lysis buffer (10 mM Tris-HCl, pH 8, 5 mM EDTA, and 100 mM DTT), and antiprotease mixture (Roche Applied Science). C. glutamicum was disrupted using Fast PROTEIN Blue Lysing Matrix (Qbiogene, Carlsbad, CA, USA) and the BIO101 Thermo Savant FastPrep (Bio-Rad, pH 4–7) and electrophoresed in a Protean IEF cell in the first dimension and on a 10% SDS-PAGE in the second dimension. Proteins were identified by mass spectrometry using an Ultraspec III MALDI-TOF (Bruker Daltonics) and the software Flex-Analysis and Biotools (Bruker Daltonics).

Construction of Plasmids for C. glutamicum Manipulations—An internal fragment of cg3264 was amplified from the C. glutamicum ATCC 13869 chromosome using primers cg3264int1/cg3264int2 (Table 2). The amplified 419-bp fragment was EcoRI/HindIII-digested and subcloned in the suicide plasmid pOJ260, yielding pOJ3264int (Table 1). To reduce expression of cg3264 in C. glutamicum, a plasmid was designed to place the copy of cg3264 under the control of the Plac promoter. The first 667 bp of cg3264 were amplified by PCR using 3264lac1/3264int2 primers (Table 2), digested with EcoRI and HindIII, and subcloned in the pOJ260 plasmid, yielding pOJ3264 (Table 1). Genetic constructs of C. glutamicum transconjugant strains were confirmed by PCR and Southern blot hybridization, using probes obtained by PCR amplification and labeled with digoxigenin according to the manufacturer’s instructions (Roche Applied Science). To study localization of the Cg3264/RsmP protein in C. glutamicum, cg3264 mutated gene copies were PCR-amplified without stop codons using the cg3264-1/cg3264-2ns primer pair. The PCR product was NdeI-digested and cloned into the bifunctional mobilizable vector pEAG6, yielding the vectors pE_RsmP-GFP, pE_RsmPA-GFP, and pE_RsmPD-GFP (Table 1). The pEAG6 vector was used to clone any gene under control of the Pdiv promoter and fused to the egfp2 gene (encoding an enhanced green fluorescent protein) (23).

Cloning, Expression, and Purification of Cg3264 Proteins—First, cg3264 was cloned to generate a recombinant protein expressed in E. coli. Then cg3264 was amplified by PCR using C. glutamicum ATCC 13869 genomic DNA as a template and the primer pair cg3264-1/cg3264-2 (Table 2), containing NdeI and BamHI restriction sites, respectively. The 876-bp amplified product was digested with NdeI and BamHI and ligated with the pETPhos vector generating pPhosRsmP (Table 1). E. coli BL21(DE3) Star cells transformed with this construct were used for expression and purification of His6-tagged RsmP under native conditions with nickel-nitrilotriacetic acid resin (Qiagen) as described previously (33, 34). To purify the protein under denaturing conditions, 8 M urea was used as described in the Qiagen user manual.

Production of Antibodies against RsmP and Protein Quantification—The native His-tagged RsmP was used to immunize male rabbits (Speedy 28-day Rabbit Programme, Eurogentec) for the production of polyclonal anti-RsmP antibodies. The resulting polyclonal antibodies specifically recognized a protein of 43 kDa, the expected size of RsmP. To quantify RsmP, total proteins from C. glutamicum were quantified by Bradford. Proteins (1 µg) were separated by SDS-PAGE and stained with Coomassie Blue or electroblotted onto polyvinylidene difluoride membranes (Millipore); the latter was immunostained with a 1:10,000 dilution of rabbit polyclonal antibodies raised against His tags (Santa Cruz Biotechnology) and with polyclonal antibodies raised against purified His-tagged RsmP (see above). Anti-rabbit immunoglobulin G-alkaline phosphatase
Characterization and Phosphorylation of C. glutamicum RsmP

(Santa Cruz Biotechnology) was used as the secondary antibody at a 1:10,000 dilution.

In Vitro Filament Formation and Electron Microscopy—In vitro polymerization experiments were performed using the cross-linking agent dimethyl 3,3′-dithiobispropionimidate (DTBP) (Pierce). The proteins purified under native conditions in 50 mM NaH₂PO₄ and 300 mM NaCl buffer, were incubated with 10 times molar excess of DTBP for 15 or 30 min at room temperature. The polymerization reaction is reversible by the addition of 1 mM dithiothreitol at 37 °C for 30 min. To stop the reaction, 1 M Tris-HCl, pH 8.0, was added. Proteins were separated on a 12% SDS gel, electroblotted onto polyvinylidene difluoride membranes (PVDF, Millipore), and detected using anti-His antibodies at 1:10,000. Alkaline phosphatase antibody-conjugated anti-rabbit was used as a secondary antibody at a 1:10,000 dilution. To induce filament formation, protein samples purified under denaturing conditions were dialyzed against a buffer containing 10 mM Tris-HCl with 150 mM NaCl at pH 7.0, overnight at 4 °C. The dialyzed samples were applied to carbon-coated grids, stained with 1% uranyl acetate, and observed by transmission electron microscopy.

In Vitro Kinase Assays—In vitro phosphorylation was performed with 2 μg of RsmP in 20 μl of buffer P (25 mM Tris-HCl, pH 7.1, 1 mM dithiothreitol, 5 mM MgCl₂, 1 mM EDTA) with 200 μCi/ml [γ-³²P]ATP corresponding to 65 nM (PerkinElmer Life Sciences, 3000 Ci/mmol) and 0.5 μg of kinase. Plasmids pGEXA, pGEXB, and pGEXL (Table 1) were used for expression and purification in E. coli of the recombinant STPKs from C. glutamicum as described previously (33). After a 15-min incubation, the reaction was stopped by adding sample buffer and heating the mixture at 100 °C for 5 min. The reaction mixtures were analyzed by SDS-PAGE. After electrophoresis, gels were soaked in 20% trichloroacetic acid for 10 min at 90 °C, stained with Coomassie Blue, and dried. Radioactive proteins were visualized by autoradiography using direct exposure films.

Mass Spectrometry Analysis—Purified wild-type and mutant RsmP proteins were subjected to in vitro phosphorylation by GST-tagged PknA as described above, except that [γ-³²P]ATP was replaced with 5 μM ATP. Subsequent analyses using nanoLC/nanospray/tandem mass spectrometry (LC-ESI/MS/MS) were performed as described previously (33).

Site-directed Mutagenesis—The three residues from C. glutamicum RsmP identified by mass spectrometry after in vitro phosphorylation with GST-tagged PknA were independently replaced by alanine residues by site-directed mutagenesis using inverse PCR amplification. A first PCR was carried out using pPhosRsmP (Table 1) as a template with the primer pairs N-RsmP168A/C-RsmP168A and N-RsmP168D/C-RsmP168D (Table 2) to generate pPhosRsmP_S6A and pPhosRsmP_S6D (Table 2). These mutants were used as a template in another PCR using the primer pairs N-RsmP211A/C-RsmP211A and N-RsmP211D/C-RsmP211D (Table 2), yielding pPhosRsmP_3A (S6A/T168A/T211A) and pPhosRsmP_3D (S6D/T168D/T211D), respectively. All the resulting constructs were verified by DNA sequencing. The different His-tagged mutant proteins were overexpressed and purified as described above.

Microscopy—Living C. glutamicum or stained cells with fluorescent dyes were observed in a Nikon E400 fluorescence microscope. Pictures were taken with a DN100 Nikon digital camera. Vancomycin BODIPY FL (Van-FL, Molecular Probes) staining was performed by adding an equal portion of unlabelled vancomycin and Van-FL to growing cultures at a final concentration of 1 μg/ml (10). The culture was then incubated for 5 min to allow adsorption of the antibiotic, after which the cells were viewed directly by fluorescence microscopy. Staining with 4’,6-diamino-2-phenylindole (DAPI) was performed as described previously (35).

RESULTS

Coccoid C. glutamicum DivIVA-depleted Cells Showed Increased Expression of a Novel Coiled-coil Protein—Previously, we constructed a C. glutamicum LACID strain, expressing low levels of DivIVA (23). This strain presented a coccoid phenotype that was morphologically different from the rod-shaped cells of the parental strain C. glutamicum R31. The total cytoplasmic proteins synthesized by C. glutamicum R31 (parent strain) and C. glutamicum LACID (DivIVA partially depleted C. glutamicum, Table 1) were characterized by two-dimensional gel electrophoresis; representative gels depicting a consistent pattern of the protein profiles are shown in Fig. 1A. Fifteen proteins of various molecular sizes appeared to be over- or underexpressed in C. glutamicum LACID compared with the R31 parental strain (Fig. 1B).

Among the 13 underexpressed proteins, 10 could be identified by peptide mass mapping technique as alkyl hydroperoxide-dase (Cg2674), isocitrate lyase (Cg2560), CoA-transferase (Cg2623), N-acetylglutamate synthase (Cg2382), fructose-biphosphate aldolase (Cg3068), phosphoglyceromutase (Cg0482), thioredoxin domain-containing protein (Cg0792), phosphoglycerate kinase (Cg3264), and probably the mraZ gene product (Cg2782). Apart from MraZ, which is encoded by the first gene of the bacterial dcv (division and cell wall biosynthesis) cluster with its promoter (Pmra) responsible for the transcription of nine genes of the cluster (36), the remaining identified proteins were involved in central metabolic pathways. These results indicate that partial depletion of DivIVA in C. glutamicum represses expression of mraZ and probably the expression of downstream genes involved in cell division and PG biosynthesis, which were not detected/identified in our two-dimensional gel analysis.

In contrast, three proteins appeared to be overexpressed in C. glutamicum LACID, but only one (Cg3264) was positively identified as a protein similar to the phage shock protein A (PspA, Cg2151). Antibodies raised against Cg3264 confirmed that this...
protein was overexpressed in the LACID mutant (Fig. 1C). Both PspA and Cg3264 belong to the Pfam/InterPro family of PspA/IM30 (PF04012/IPR007157) proteins that putatively suppress $\alpha$54-dependent transcription (37) and could play a role in maintaining cytoplasmic membrane integrity and/or the proton-motive force (38). PspA and the PspA-like protein Cg3264 correspond to 2 of the 37 coiled-coil proteins present in the genome of C. glutamicum. Exhaustive analysis of the PspA/IM30 protein family in actinomycetes revealed authentic PspA proteins in all the sequenced actinomycete genomes, although Cg3264 homologues were found exclusively in corynebacterial genomes (Fig. 2) and not in those of Mycobacterium species. Only a few Streptomyces species (Streptomyces sp. Mg1, Streptomyces griseus, and Streptomyces pristinae) contained a Cg3264 homologue.

Interestingly, Cg3264 has a secondary structure rich in $\alpha$-helical regions according to the program PSIPRED (39), which are predicted to contain a central rod domain of two coiled-coil segments (containing several heptad repeat regions) as well as a linker lacking a coiled-coil structure (Fig. 3). Moreover, Cg3264 contains two stutters (41), where coiled-coil 2 is clearly interrupted for a few amino acids at the beginning and end of the coiled coil, and the rod domain is flanked by head and tail domains lacking the coiled-coil region and $\alpha$-helical structures. Therefore, based on the facts that the Cg3264 protein is characteristic of corynebacte-
Cg3264 Is Essential and Involved in C. glutamicum Polar Growth—To investigate whether cg3264 was necessary for the growth and viability of C. glutamicum, we attempted gene disruption experiments using an internal fragment of cg3264 cloned into the conjugative suicide plasmid pOJ260 (42) yielding the pOJ-3264int vector (Table 1). This plasmid was introduced into E. coli S17-1 and the transformant mated with C. glutamicum R31. Apramycin-resistant transconjugants could not be obtained with pOJ-3264int after many attempts, suggesting that cg3264 is essential for the viability of C. glutamicum. Because the genes located immediately downstream and upstream of cg3264 are transcribed in the opposite direction to cg3264, a possible polar effect on adjacent gene expression by insertion of pOJ-3264int was discounted. We therefore constructed a conditional gene expression strain using the Plac promoter, which allows regulation of the expression of essential genes in C. glutamicum (43). C. glutamicum was transformed with the suicide plasmid pOJ3264 (Plac-cg3264) (Table 1), which, when introduced into C. glutamicum, could integrate by homologous recombination into the chromosomal cg3264 locus, thus disrupting the cg3264 gene under its natural promoter and creating a full-length copy under control of the Plac promoter. Southern blot analysis of DNA from the transconjugant strain C. glutamicum LAC3264 (Table 1) showed the expected pattern for Campbell-type integration of pOJ3264 at the chromosomal cg3264 locus (data not shown).

The phenotype of this conditional mutant was investigated to define the function of the Cg3264 protein. Interestingly, the coccoid morphology of this mutant seems to be similar to the one obtained when the level of the essential protein DivIVA was depleted (Fig. 4A) (23), and the typical polar and septal Van-FL staining shown by the wild-type C. glutamicum R31 (23) was lost in the C. glutamicum LAC3264 strain (data not shown). On the other hand, antibodies raised against Cg3264 confirmed that this protein was underexpressed in C. glutamicum LAC3264 (Fig. 4B). Therefore, Cg3264, previously of unknown function, was renamed RsmP for rod-shaped morphology protein.

RsmP Forms Filamentous Structures in Vivo—RsmP is a protein predicted to form coiled coils (44), and coiled-coil proteins are the main structural elements of many fibrous proteins in eukaryotes (41, 45) and prokaryotes (27). Therefore, based on the evolutionary relationships of PspA- and RsmP-like proteins in 14 complete corynebacterial genomes. The evolutionary history was inferred using the Neighbor-Joining method (68). The optimal tree with the sum of branch lengths = 4.00209249 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (69) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 218 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (70).
the conditional mutant phenotype showing that RsmP (Cg3264) is involved in polar growth or rod-shaped determination, we hypothesized that RsmP could support cell shape by forming intracellular filaments or a bacterial cytoskeleton like CreS, FilP, or Ccrp (29, 30, 46). To test this hypothesis, we formed intracellular filaments or a bacterial cytoskeleton like CreS, FilP, or Ccrp (29, 30, 46). To test this hypothesis, we cloned rsmP into the E. coli/corynebacterial shuttle plasmid pEAG6 designed to easily clone and overexpress any promoterless gene under the control of the Pdiv promoter and fused to egfp2 (23), thus generating pE-RsmP-GFP. Then, when this plasmid was introduced into C. glutamicum, the cells showed a club-shaped morphology (Fig. 4A) similar to that in DivIVA-overexpressing strains (19, 23) and RsmP-GFP proteins localized as long filaments along the cell (Fig. 5). This suggested that RsmP formed helical/filamentous structures in vivo extending from one pole to the other at a cellular location consistent with a possible and essential cytoskeletal function, and therefore in accordance with our results showing that RsmP was indeed involved in determining rod-shaped morphology.

RsmP Forms Filamentous Structures in Vitro—RsmP was able to form filamentous structures in vivo, as observed with GFP localization. We therefore tried to confirm RsmP function as a cytoskeletal protein and whether RsmP could form filaments in vitro. First, we used a polyhistidine-tagged version of RsmP (His-RsmP) purified from E. coli by affinity chromatography, but most of the protein was present in inclusion bodies, a typical characteristic of IFs (45, 47). This was solved by overnight incubation of the overexpressing E. coli strain at 17 °C, and the soluble RsmP proteins were purified and used in an “in vitro” polymerization assay using the cross-linking agent DTBP widely used to study the polymerization of eukaryotic IF proteins (48–50). His-RsmP was incubated in the presence of DTBP, and the reaction was stopped after 15 or 30 min by adding 1 M Tris-HCl, pH 8. The reaction product was loaded onto an SDS-PAGE and detected by Western blot using commercial anti-histidine antibodies. As shown in Fig. 6A, His-RsmP formed dimers, trimers, and tetramers in the presence of DTBP. Furthermore, the polymerization reaction was reversed by adding 100–150 mM dithiothreitol (DTT) at 37 °C for 30 min, confirming the specificity of the polymerization reaction (Fig. 6A).

DTBP, and the reaction was stopped after 15 or 30 min by adding 1 M Tris-HCl, pH 8. The reaction product was loaded onto an SDS-PAGE and detected by Western blot using commercial anti-histidine antibodies. As shown in Fig. 6A, His-RsmP formed dimers, trimers, and tetramers in the presence of DTBP. Furthermore, the polymerization reaction was reversed by adding 100–150 mM dithiothreitol (DTT) at 37 °C for 30 min, confirming the specificity of the polymerization reaction (Fig. 6A).

To further confirm the ability of RsmP to form filamentous structures in vitro, we used a complementary strategy. In fact, most IF proteins have the ability to form filaments in vitro without divalent cations, nucleotides, or other exogenous factors (47). Because of the high insolubility of IFs, in vitro assembly first requires the solubilization of the IF protein in a strong denaturating agent, and filaments are obtained after dialyzing the protein against physiological or low ionic strength buffers at a neutral pH (51). This was the method used to demonstrate that CreS from C. crescentus and FilP from S. coelicolor formed filamentous structures in vitro (28, 29). Therefore, we purified RsmP under strong denaturing conditions (8 M urea), and once purified, RsmP was spontaneously able to self-assemble into filaments after removal of urea by several dialysis steps. The filaments formed were confirmed by transmission electron microscopy (Fig. 7); they appeared similar to the filaments described for the Ccrp59 (coiled-coil rich proteins) from H. pylori (30). RsmP formed straight and branched filamentous structures measuring on average 2000 nm long and 50 nm diameter, thus definitively confirming its function as a cytoskeletal protein.

RsmP Is Preferentially Phosphorylated by the Ser/Thr Protein Kinase Cg_PknA—Eukaryotic IF proteins can occasionally be regulated post-translationally by glycosylation or phosphorylation (41, 52). Indeed, phosphorylation on their head and tail domains as well as the dynamics of their phosphorylation/dephosphorylation play a major role in regulating the assembly/
Characterization and Phosphorylation of C. glutamicum RsmP

A

RsmP

DTPBP

DTT

- - +

+ - +

+ + +

170

130

95

kDa

- 15 min 30 min 30 min

tetramer

trimer

dimer

monomer

B

WT

WT + DTPBP

Phosphoablative

Phosphomimetic

Phosphoablative

Phosphomimetic

+ + +

+ - +

+ + +

+tetramer

trimer

dimer

monomer

FIGURE 6. A, in vitro polymerization assay of the wild-type His-tagged RsmP from C. glutamicum using the cross-linking agent DTPBP. Protein RsmP was incubated for 15 or 30 min in the presence of a 10 times molar excess of DTPBP, and the reaction was stopped by adding 1 M Tris. Proteins were separated on a 12% SDS gel, electroblotted onto PVDF membranes (Millipore), and detected using anti-His antibodies. The specificity of the polymerization reaction was tested by treatment with 100 mM DTT. Note the formation of dimers, trimers, and tetramers in the presence of DTPBP. B, in vitro polymerization assays of the His-tagged wild-type RsmP protein, the His-tagged RsmP protein S6A/T168A/T211A (phosphoablative protein), and the His-tagged RsmP protein S6D/T168D/T211D (phosphomimetic protein). Note that mutant proteins can also form dimers, trimers, and tetramers in the presence of DTPBP.

disassembly of these filaments and consequently their functions (53). As RsmP shares characteristics of IF elements, we investigated whether RsmP could be post-translationally modified by phosphorylation via the STPKs present in the genome of C. glutamicum (33). The kinases Cg_PknA, Cg_PknB, and Cg_PknL from C. glutamicum were expressed as GST-tagged fusion proteins and purified from E. coli as reported earlier (33). Each of the purified kinases was incubated with RsmP and [γ-32P]ATP and resolved by SDS-PAGE, and their phosphorylation profiles were analyzed by autoradiography. The presence of an intense radioactive signal indicated that RsmP was preferentially phosphorylated by Cg_PknA (Fig. 8A), and to a lesser extent by Cg_PknL, although no signal was observed in the presence of Cg_PknB. These results clearly indicated that RsmP is a specific substrate and interacts with C. glutamicum STPKs in vitro, suggesting that this key protein controlling rod-shaped morphology might be regulated in corynebacteria by multiple extracellular signals.

RsmP Is Phosphorylated on Ser and Thr Residues—To identify which residues of RsmP corresponded to the phosphorylated site(s), a mass spectrometric approach was used. RsmP was incubated with cold ATP in the presence of Cg_PknA (the most active kinase for RsmP, see Fig. 8A) and subjected to mass spectrometric analysis after tryptic and chymotryptic digestion, as described previously (34). Analysis of tryptic and chymotryptic digests allowed the characterization of three phosphorylation sites in RsmP corresponding to Ser-6, Thr-168, and Thr-211, which lie outside the coiled-coil regions (Table 3 and Fig. 3). Definitive identification of the Ser-6, Thr-168, and Thr-211 residues identified by mass spectrometry was achieved by successive site-directed mutagenesis by introducing mutations that prevented their specific phosphorylation. Thus, all three residues were replaced by alanine, yielding the mutant RsmP_S6A/T168A/T211A. This mutant protein (phosphoablative mutant) was expressed in E. coli, purified, and incubated with [γ-32P]ATP and Cg_PknA (Fig. 8B). Phosphorylation of the phosphoablative mutant protein appeared to be completely abolished, confirming the identification of all three sites of phosphorylation. An additional round of mass spectrometric analysis was also performed directly on the Ala mutant protein, which failed to identify any additional phosphate group.

RsmP Phosphorylation Negatively Regulates Filament Formation—Phosphorylation of IFs have been reported to block vimentin and desmin polymerization in vitro as well as to disassemble pre-existing filaments (54, 55). To investigate the consequences of phosphorylation on RsmP filament forming activity, Ser-6, Thr-168, and Thr-211 residues were replaced by aspartic acid residues able to mimic the phosphorylated isoform of the protein, generating the RsmP_S6D/T168D/T211D protein (phosphomimetic mutant) (56–59). The three RsmP alleles (wild-type, phosphoablative, or phosphomimetic) were expressed in E. coli, purified, and used in in vitro cross-linking experiments. As showed in Fig. 6B, the three proteins could form dimers, trimers, and tetramers in the presence of DTT, suggesting that phosphorylation did not affect the polymerization activity of RsmP. However, for the eukaryotic IF vimentin, phosphorylation in the N-terminal head domain induced disassembly of vimentin filaments and resulted in the release of tetrameric subunits (60), thus leading to the conclusion that
phosphorylation does not affect the formation of tetramers but the assembly of tetramers into filaments. Therefore, we hypothesized that RsmP might behave in a similar way. The three alleles were cloned into pEAG6 to obtain the three enhanced GFP fusions, which were introduced into \textit{C. glutamicum} and observed by fluorescence microscopy. As shown in Fig. 9, RsmP, corresponding to the phosphomimetic protein, leads to the loss of protein localization along the cell and concentration at the poles, although the phosphoablative mutant showed a similar localization as in the wild type. These results demonstrate that modification of the phosphorylation sites by phosphomimetic residues affects localization in a manner consistent with control of localization by phosphorylation and thus confirmed the critical role of phosphorylation in regulating RsmP localization within the cell.

**DISCUSSION**

In all actinobacteria studied, cell elongation occurs at the cell poles and is supported by the coiled coil-rich protein DivIVA (19–23). This protein is regulated by phosphorylation in \textit{M. tuberculosis} (22, 57, 61) but not in \textit{C. glutamicum}, suggesting that corynebacteria uses different cell elongation control mechanisms. In this study, we characterized a novel protein, conserved almost exclusively in all \textit{Corynebacterium} species and overexpressed in the DivIVA-depleted strain. This protein, renamed here RsmP, corresponds to a coiled coil-rich protein with a central rod domain of two coiled-coil segments flanked by head and tail domains. This structural organization is similar to those of DivIVA, CreS, and FilP proteins and resembles the organization of eukaryotic intermediate filaments. In addition, RsmP can polymerize to form filamentous structures \textit{in vitro} without the addition of any external cofactors, and \textit{in vivo} the RsmP structures extend from one cell pole to the other like CreS. The structural organization of RsmP, the partial insolubility of this protein, and its ability to polymerize spontaneously are archetypical characteristics of eukaryotic intermediate filaments. Moreover, this protein is essential to \textit{C. glutamicum} cell viability, with overexpression and partial depletion of the gene producing severe morphological alterations, thus indicating that RsmP is involved in maintenance of corynebacterial cell shape.

In eukaryotic cells, intermediate filaments are usually controlled by different post-translational modifications such as phosphorylation. In previous reports, we have characterized the four STPKs of \textit{C. glutamicum} and demonstrated that the PknA and PknB kinases were involved in cell division and PG biosynthesis. Moreover, different STPK substrates have been identified and characterized as proteins involved in cell division and PG biosynthesis thus confirming the critical role of Ser/Thr phosphorylation in regulating such pathways (34, 62). Therefore, we hypothesized that RsmP could be regulated via STPK phosphorylation, and this was confirmed in this study by evidence that corynebacterial PknA and PknL can phosphorylate RsmP. To our knowledge, this is the first study of a bacterial IF-like protein being regulated by phosphorylation as none of the prokaryotic intermediate filament-like proteins (FilP, CreS, and Ccrp) were described previously as being phosphorylated.

\textit{In vitro} phosphorylation assays coupled with mass spectrometric analysis led us to identify three phosphorylated residues in RsmP. None of them is located in the coiled-coil regions, and apparently their replacement does not affect the RsmP polymerization ability, as it is the case with eukaryotic intermediate filaments, where phosphorylation prevents polymerization of tetramers into filaments (31). Determination of the phosphorylation sites provided the essential groundwork for mechanistic/functional studies on RsmP and demonstrated the efficacy of combining genetics and mass spectrometric analyses with precise identification of phosphoacceptors, a prerequisite for a further understanding of the RsmP regulation. Moreover, strains with defined mutations within the phosphorylation sites will be extremely helpful in establishing the role of RsmP phosphorylation-dependent regulation in corynebacterial growth and cell division, and in fact, the localization pattern of RsmP is affected by phosphorylation. Whereas overexpression

**TABLE 3**

Sequence of the phosphorylated peptides identified in \textit{Cg3264} as determined by mass spectrometry

| Phosphorylated tryptic and chymotryptic peptide sequence   | No. of detected phosphate groups, LC-ESI/MS/MS | Phosphorylated residue(s) |
|-----------------------------------------------------------|-----------------------------------------------|--------------------------|
| (163–191) MQRGEVTPKSM_DSNLQFGTQDSSVPTLDAVR              | 1                                            | Ser-6                    |
| (198–225) YADALGAAQLTNPVYSDR                             | 1                                            | Thr-168                  |
| (163–191) MQRGEV TPKSM_DSNLQFGTQDSSVPTLDAVR              | 1                                            | Thr-168                  |
| (−3)−7 GSHMANPLpSK                                         | 1                                            | Ser-6                    |
| (−3)−7 GSHMANPLpSKGWK                                      | 1                                            | Ser-6                    |

**FIGURE 8.** \textit{A}, \textit{in vitro} phosphorylation of \textit{C. glutamicum} RsmP by corynebacterial STPKs. The recombinant STPKs (Cg_PknA, Cg_PknB, and Cg_PknL) were expressed and purified as described previously (33). Recombinant RsmP was treated with the tobacco etch virus protease to remove the N-terminal His tag and then incubated with [γ-32P]ATP and the different kinases. Samples were separated by SDS-PAGE, Coomassie-stained (upper panel), and visualized by autoradiography (lower panel). Upper bands illustrate the autokinase activity of each STPK, and lower bands reflect RsmP phosphorylation. \textit{B}, \textit{in vitro} phosphorylation of RsmP mutants by Cg_PknA. The different RsmP mutants were treated with the tobacco etch virus protease to remove the N-terminal His tag and then used in phosphorylation assays in equal amounts in the presence of [γ-32P]ATP and Cg_PknA. The RsmP_WT, RsmP_S6A, RsmP_T168A, RsmP_T211A, and RsmP_S6A/T168A/T211A mutant proteins were separated by SDS-PAGE and stained with Coomassie Blue (upper panel), and the radioactive bands were revealed by autoradiography (lower panel).
of the wild-type protein and the phosphoablative mutant exhibit a similar localization forming an internal cytoskeleton extending from one cell pole to the other, overexpression of the phosphomimetic mutant results in localization predominantly at the cells poles.

In light of these data, we propose two possible roles for RsmP in C. glutamicum cell elongation. First, RsmP may be able to polymerize from pole to pole to generate an internal scaffold that supports the lateral wall during cell elongation, as it is the case in B. subtilis or E. coli where this function is carried out by actin-like MreB homologues (6, 63). Therefore, MreB function may then be involved in the control of RsmP polymerization and subsequently in cell elongation. C. glutamicum has a well known bacillary-to-coccoid pleomorphism during late exponential growth phase (64). This pleomorphism has been linked to bolA in E. coli (65, 66), but to date no other molecular factors have been identified that would explain the cell-shape shift in response to nutrient deprivation in other bacteria. Therefore, PknA and PknL may phosphorylate RsmP under these conditions to produce a larger number of smaller cells to distribute the stress caused by starvation over a larger number of individuals, increasing the probability of survival of at least a few of them (67).

Alternatively, RsmP may be another element of the cytoskeletal structure recruited at the cell poles by DivIVA, which directs PG synthesis for cell elongation in actinobacteria. In this hypothesis, RsmP would provide an additional internal support, probably by protein-protein interaction through its coiled-coil domains. Therefore, RsmP may be the substrate of the Pkn-mediated signal transduction of the polar PG synthesis complex in C. glutamicum. Indeed, the corynebacteria-specific conservation of RsmP, the striking lack of DivIVA phosphorylation in corynebacteria (well documented in other actinomycetes), the up-regulation of RsmP in DivIVA-depleted strains, and the polar localization of the phosphomimetic mutant support a corynebacteria-specific role of RsmP in polar PG synthesis.

In conclusion, the data reported here provide significant and novel insight into the bacterial cytoskeleton and function of cytoskeletal elements. Especially significant, this study confirmed that C. glutamicum possesses an original and specific system for establishing and maintaining rod-shaped morphology. Furthermore, these findings could be useful to extend our present understanding in cell division and cell-shape determination in Gram-positive bacteria toward the design of new anti-microbial drugs. The essential protein RsmP could be a useful target to combat emergent corynebacterial pathogens, especially the human pathogen C. diptheriae.

Acknowledgments—We thank M. Becchi and A. Cornut (Institut de Biologie et Chimie des Protéines, Lyon, France) for excellent technical assistance in mass spectrometric analysis and David Hopwood for critical reading of the manuscript.
Characterization and Phosphorylation of C. glutamicum RsmP

27. Graumann, P. L. (2009) Cell Motil. Cytoskeleton 66, 909–914
28. Ausmees, N., Kuhn, J. R., and Jacobs-Wagner, C. (2003) Cell 115, 705–713
29. Bagchi, S., Tomenius, H., Belova, L. M., and Ausmees, N. (2008) Mol. Microbiol. 70, 1037–1050
30. Waidner, B., Specht, M., Dempwolff, F., Haerer, K., Schaetzle, S., Speth, V., Kist, M., and Graumann, P. L. (2009) e002669
31. Parry, D. A., Strelkov, S. V., Burkhard, P., Aebi, U., and Herrmann, H. (2007) Exp. Cell Res. 313, 2204–2216
32. Mateos, L. M., Schäfer, A., Kalinowski, J., Martin, J. F., and Pühler, A. (1996) J. Bacteriol. 178, 5768–5775
33. Fiuza, M., Canova, M. J., Zanella-Cléon, I., Becchi, M., Cozzone, A. J., Mateos, L. M., Kremer, L., Gil, J. A., and Molle, V. (2008) J. Biol. Chem. 283, 18099–18112
34. Fiuza, M., Canova, M. J., Zanella-Cléon, I., Becchi, M., Mateos, L. M., Mengin-Lecreulx, D., Molle, V., and Gil, J. A. (2008) J. Biol. Chem. 283, 36553–36563
35. Daniel, R. A., Harr, E. J., and Errington, J. (2000) Mol. Microbiol. 35, 299–311
36. Mengin-Lecreulx, D., Ayala, J., Bouhss, A., van Heijenoort, J., Parquet, C., and Hara, H. (1998) J. Bacteriol. 180, 4406–4412
37. Elderkin, S., Jones, S., Schumacher, J., Studholme, D., and Buck, M. (2002) J. Mol. Biol. 320, 23–37
38. Darwin, A. J. (2005) Mol. Microbiol. 57, 621–628
39. Jones, D. T. (1999) J. Mol. Biol. 292, 195–202
40. Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., and Kim, P. S. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 8259–8263
41. Herrmann, H., and Aebi, U. (2004) Annu. Rev. Biochem. 73, 749–789
42. Bierman, M., Logan, R., O’Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) Gene 116, 43–49
43. Letek, M., Valbuena, N., Ramos, A., Ordóñez, E., Gil, J. A., and Mateos, L. M. (2006) J. Bacteriol. 188, 409–423
44. Lupas, A., Van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164
45. Herrmann, H., Bär, H., Kreplak, L., Strelkov, S. V., and Aebi, U. (2007) Nat. Rev. Mol. Cell Biol. 8, 562–573
46. Ausmees, N. (2006) J. Mol. Microbiol. Biotechnol. 11, 152–158
47. Domingo, A., Sarria, A. J., Evans, R. M., and Klymkowski, M. W. (1992) The Cytoskeleton: A Practical Approach (Carraway, K. L., and Carraway, C. A. C., eds) pp. 223–255, IRL Press at Oxford University Press, Oxford, United Kingdom
48. Downing, D. T. (1996) Proteins 25, 215–224
49. Steinert, P. M., Marekov, L. N., Fraser, R. D., and Parry, D. A. (1993) J. Mol. Biol. 230, 436–452
50. Charulatha, V., and Rajaram, A. (2001) J. Biomed. Mater. Res. 54, 122–128
51. Steinert, P. M., Idler, W. W., and Zimmerman, S. B. (1976) J. Mol. Biol. 108, 547–567
52. Chung, S. M., and Wang, H. S. (1997) J. Neurol. Sci. 152, 198–209
53. Sihag, R. K., Inagaki, M., Yamaguchi, T., Shea, T. B., and Pant, H. C. (2007) Exp. Cell Res. 313, 2098–2109
54. Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., and Sato, C. (1987) Nature 328, 649–652
55. Geisler, N., and Weber, K. (1988) EMBO J. 7, 15–20
56. Cohen-Gonsaud, M., Barthe, P., Canova, M. J., Stagei-Simon, C., Kremer, L., Roumestand, C., and Molle, V. (2009) J. Biol. Chem. 284, 19290–19300
57. Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C., Cantley, L. C., and Husson, R. N. (2005) Genes Dev. 19, 1692–1704
58. Veyron-Churlet, R., Molle, V., Taylor, R. C., Brown, A. K., Besra, G. S., Zanella-Cléon, I., Fütterer, K., and Kremer, L. (2009) J. Biol. Chem. 284, 6414–6424
59. Veyron-Churlet, R., Zanella-Cléon, I., Cohen-Gonsaud, M., Molle, V., and Kremer, L. (2010) J. Biol. Chem. 285, 12714–12725
60. Eriksson, J. E., He, T., Trojo-Skali, A. V., Härmla-Braskén, A. S., Hellman, I., Chou, Y. H., and Goldman, R. D. (2004) J. Cell Sci. 117, 919–932
61. Hamasha, K., Sahana, M. B., Jani, C., Nyayapathy, S., Kang, C. M., and Rehse, S. J. (2010) Biochem. Biophys. Res. Commun. 391, 664–668
62. Schultz, C., Niebisch, A., Schwaiger, A., Viets, U., Metzger, S., Bramkamp, M., and Bott, M. (2009) Mol. Microbiol. 74, 724–741
63. Vats, P., Shih, Y. L., and Rothfield, L. (2009) Mol. Microbiol. 72, 170–182
64. Fuhrmann, C., Soedarmanto, I., and Lämmler, C. (1997) Zentralbl. Veterinarmed. B. 44, 287–294
65. Santos, J. M., Freire, P., Vicente, M., and Arraiâno, C. M. (1999) Mol. Microbiol. 32, 789–798
66. Freire, P., Moreira, R. N., and Arraiâno, C. M. (2009) J. Mol. Biol. 385, 1345–1351
67. James, G. A., Korber, D. R., Caldwell, D. E., and Costerton, J. W. (1995) J. Bacteriol. 177, 907–915
68. Saitou, N., and Nei, M. (1987) Mol. Biol. Evol. 4, 406–425
69. Zuckerfandl, E., and Pauling, L. (1965) Evolving Genes and Proteins, pp. 97–166, Academic Press, New York
70. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) Mol. Biol. Evol. 24, 1596–1599
71. Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H., and Pühler, A. (1990) J. Bacteriol. 172, 1663–1666
72. Santamaria, R. I., Gil, J. A., and Martin, J. F. (1985) J. Bacteriol. 162, 463–467
73. Canova, M. J., Kremer, L., and Molle, V. (2008) Plasmid 60, 149–153