Staphylococcus aureus Operates Protein-tyrosine Phosphorylation through a Specific Mechanism*

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Protein phosphorylation on tyrosine has been originally characterized in animal systems and has been shown to be involved in several fundamental processes including signal transduction, growth control, and malignancy. It has been later demonstrated to occur also in a number of bacteria, and recent data suggest that it may participate in the control of bacterial pathogenicity. In this work, we provide evidence that the Gram-positive human pathogen Staphylococcus aureus harbors a protein-tyrosine kinase activity. This activity is borne by a protein, termed Cap5B2, whose phosphorylating capacity is expressed only in the presence of a stimulatory protein, either Cap5A1 or Cap5A2, that enhances its affinity for the phosphoryl donor ATP. In fact, the last 27/29 amino acids of the C-terminal domain of either polypeptide are sufficient for stimulating Cap5B2 activity. The stimulation of Cap5B2 by Cap5A1 involves essentially three amino acid residues in a helix of Cap5A1 (Asp202, Glu203, and Asp205) and three residues in a helix (helix 7) of Cap5B2 (Glu190, Lys192, and Lys193), thus suggesting helix-helix interaction between these two proteins. This type of helix-helix interaction resembles the interaction required for the activation of MinD ATPase by MinE protein in the process of septum-site determination, MinD sharing sequence similarity with Cap5B2. Such activation mechanism is described here in a Gram-positive bacterial tyrosine kinase, and differs from the activation mechanism previously proposed for Gram-negative bacteria. Therefore, it appears that S. aureus, and possibly other Gram-positive bacteria, utilizes a specific molecular mechanism for triggering protein-tyrosine kinase activity.

Protein phosphorylation on tyrosine has long been considered a covalent modification specific to eukaryotes. In these organisms, it has been shown to play a key role in the regulation of a variety of fundamental biological functions, such as signal transduction, growth control, and malignant transformation (1). In the last decade, however, conclusive evidence has accumulated that tyrosine phosphorylation is not a phylogenetic constraint specific to eukaryotes, as it has been shown to affect the eukaryotic enzymes, such as genistein, quercetin and tosylsulfone chloromethyl ketone. Also, bacterial kinases are unable to phosphorylate synthetic substrates like poly(Glu100-Tyr30) or angiotensin II, which are, by contrast, easily targeted by eukaryotic kinases (7).

Further analysis of the mechanism of protein-tyrosine phosphorylation reveals the existence of differences not only between bacterial and eukaryotic kinases but also within enzymes of different bacterial origin. In particular, a conspicuous contrast arises when comparing Gram-positive and Gram-negative bacterial strains. In Gram-positive bacteria, two distinct proteins encoded by two different genes are required together for phosphorylation (8, 9), whereas in Gram-negative bacteria, only a single protein is sufficient (10–13). In addition, a specific tyrosine residue, located in the C-terminal domain of Gram-negative, but not Gram-positive, bacterial tyrosine kinases has been shown to be crucial for phosphorylation (14). This is namely the case for protein kinase Wzc from Escherichia coli K12. Besides this crucial tyrosine (Tyr569), Wzc contains five other tyrosine residues clustered at its C-terminal end. A detailed examination of the mode of action of Wzc has shown that its phosphorylation occurs through a two-step process. First, the Tyr569 residue autophosphorylates in an intramolecular reaction, which results in an increased kinase activity of Wzc; then, Wzc can, in turn, phosphorylate the five terminal clustered tyrosines of another Wzc molecule in an intermolecular reaction (15).

Because of the absence of a Tyr569 counterpart in the tyrosine kinases of Gram-positive bacteria, as well as the requirement of two distinct proteins instead of one to generate a tyrosine kinase activity, it could be expected that a particular phosphorylation mechanism would occur in Gram-positive bacteria. Therefore, it seemed of interest to investigate in detail the reaction of protein-tyrosine phosphorylation in Gram-positive bacteria. For that purpose, we analyzed in this work the tyrosine kinase activity of Staphylococcus aureus, a major human pathogen responsible for a diversity of community-acquired and hospital-acquired infections.
**TABLE 1**

| Plasmid       | Description                                                                 | Reference or source          |
|---------------|------------------------------------------------------------------------------|------------------------------|
| pQE30         | Expression vector generating His, fusion protein, Amp<sup>+</sup>             | Qiagen                       |
| pET15b        | Expression vector generating GST fusion protein, Amp<sup>+</sup>             | Novagen                      |
| pET15b-A1     | Encoding Cap5A1 from Met<sup>1</sup> to Asn<sup>227</sup>, His<sub>A1</sub>, cloned in NdeI/BamHI sites, Amp<sup>+</sup> | This study                   |
| pET15b-A2     | Encoding Cap5A2 from Met<sup>1</sup> to Phe<sup>220</sup>, His<sub>A2</sub>, cloned in NdeI/BamHI sites, Amp<sup>+</sup> | This study                   |
| pET15b-B1     | Encoding Cap5B1 from Met<sup>1</sup> to Glu<sup>239</sup>, His<sub>B1</sub>, cloned in NdeI/BamHI sites, Amp<sup>+</sup> | This study                   |
| pET15b-B2     | Encoding Cap5B1 from Met<sup>1</sup> to Ser<sup>232</sup>, His<sub>B2</sub>, cloned in Xhol/BamHI sites, Amp<sup>+</sup> | This study                   |
| pQE30-A1CtB1  | Encoding last 29 amino acids of Cap5B1 (from Val<sup>194</sup> to Asn<sup>227</sup>) fused to Cap5B1 (from Met<sup>1</sup> to Glu<sup>239</sup>), His<sub>A1CtB1</sub>, cloned in BamHI/PstI sites, Amp<sup>+</sup> | This study                   |
| pQE30-A1CtB2  | Encoding last 29 amino acids of Cap5A1 (from Val<sup>194</sup> to Asn<sup>227</sup>) fused to Cap5B2 (from Met<sup>1</sup> to Ser<sup>232</sup>), His<sub>A1CtB2</sub>, cloned in BamHI/HindIII sites, Amp<sup>+</sup> | This study                   |
| pQE30-A2CtB1  | Encoding last 27 amino acids of Cap5B2 (from Leu<sup>194</sup> to Phe<sup>220</sup>) fused to Cap5B1 (from Met<sup>1</sup> to Glu<sup>239</sup>), His<sub>A2CtB1</sub>, cloned in BamHI/HindIII sites, Amp<sup>+</sup> | This study                   |
| pQE30-A2CtB2  | Encoding last 27 amino acids of Cap5A2 (from Leu<sup>194</sup> to Phe<sup>220</sup>) fused to Cap5B2 (from Met<sup>1</sup> to Ser<sup>232</sup>), His<sub>A2CtB2</sub>, cloned in BamHI/HindIII sites, Amp<sup>+</sup> | This study                   |
| pQE30-A1CtB2K | Same as pQE30-A1CtB2, but mutated on K55M                                   | This study                   |
| pGEXVM-A1Ct   | Encoding last 29 amino acids of Cap5A1 from Met<sup>1</sup> to Asn<sup>227</sup>, GSTA1Ct, cloned in BamHI/HindIII sites, Amp<sup>+</sup> | This study                   |
| pGEXVM-A2Ct   | Encoding last 27 amino acids of Cap5A2 from Leu<sup>194</sup> to Phe<sup>220</sup>, GSTA2Ct, cloned in BamHI/HindIII sites, Amp<sup>+</sup> | This study                   |
| pQE30-B2-D189C| Same as pQE30-B2, but mutated on D189C                                      | This study                   |
| pQE30-B2-E190C| Same as pQE30-B2, but mutated on E190C                                      | This study                   |
| pQE30-B2-K192C| Same as pQE30-B2, but mutated on K192C                                      | This study                   |
| pQE30-B2-K193C| Same as pQE30-B2, but mutated on K193C                                      | This study                   |
| pQE30-B2-K188A| Same as pQE30-B2, but mutated on K188A                                      | This study                   |
| pQE30-B2-D189A| Same as pQE30-B2, but mutated on D189A                                      | This study                   |
| pQE30-B2-E190A| Same as pQE30-B2, but mutated on E190A                                      | This study                   |
| pQE30-B2-K192A| Same as pQE30-B2, but mutated on K192A                                      | This study                   |
| pQE30-B2-K193A| Same as pQE30-B2, but mutated on K193A                                      | This study                   |
| pQE30-B2-K195A| Same as pQE30-B2, but mutated on K195A                                      | This study                   |
| pGEXVM-A1Ct-E202C| Same as pGEXVM-A1Ct, but mutated on D202C                                   | This study                   |
| pGEXVM-A1Ct-E203C| Same as pGEXVM-A1Ct, but mutated on E203C                                   | This study                   |
| pGEXVM-A1Ct-D205C| Same as pGEXVM-A1Ct, but mutated on D205C                                   | This study                   |
| pGEXVM-A1Ct-D202A| Same as pGEXVM-A1Ct, but mutated on D202A                                   | This study                   |
| pGEXVM-A1Ct-D203A| Same as pGEXVM-A1Ct, but mutated on E203A                                   | This study                   |
| pGEXVM-A1Ct-E204A| Same as pGEXVM-A1Ct, but mutated on E204A                                   | This study                   |
| pGEXVM-A1Ct-D205A| Same as pGEXVM-A1Ct, but mutated on D205A                                   | This study                   |
| pGEXVM-A1Ct-E207A| Same as pGEXVM-A1Ct, but mutated on E207A                                   | This study                   |

**S. aureus** genome analysis reveals two couples of polypeptides harboring a putative tyrosine kinase activity, the Cap5A1/Cap5B1 (also termed A1/B1) and the Cap5A2/Cap5B2 (also termed A2/B2). Although A1 and B1 are encoded by genes that are located in the cap operon controlling capsule biosynthesis, the genes encoding A2 and B2 are located elsewhere on the genome. In terms of biological role, A1 and B1 are thought to function together as a polysaccharide co-polymerase and because of their respective sequence similarity, A2 and B2 are supposed to have a similar activity. However the precise function of these proteins remains unknown. In this work, the tyrosine phosphorylating activity of A1/B1 and A2/B2 has been biochemically characterized and the amino acid sequences needed for this activity have been determined, leading to a molecular model based on specific protein–protein interaction required for the kinase activity. Such protein–protein interaction is reminiscent of a mechanism found in the MinD/MinE interaction required for the process of septum site determination.

**EXPERIMENTAL PROCEDURES**

**PCR Amplification and Cloning Experiments**—The cap5A1, cap5A2, cap5B1, and cap5B2 genes were PCR-amplified by using genomic DNA of **S. aureus** Reynolds serotype 5 strain, and specific primers to which appropriate restriction sites had been added (supplemental Table SI). The PCR products of cap5A1, cap5A2, cap5B1, and cap5B2 were inserted in pET15b, pQE30 was also inserted in pQE30. The list of plasmids used in this study is presented in Table 1. The nucleotide sequence of all synthesized and mutated genes was checked to ensure proper base replacement and error-free amplification. The plasmids were used to transform **E. coli** XL1-Blue or BL21(DE3) (pREP4GroEL) cells to, respectively, propagate plasmids or express the corresponding proteins. Cells were grown in LB medium containing appropriate antibiotics. DNA sequence analysis, BLAST search, and sequence alignment were performed by using MLRC and ESPript methods (17, 18).

**Site-directed Mutagenesis:**—Site-directed mutagenesis was carried out by using either the Transformer Site-Directed Mutagenesis Kit from Clontech, based on the method developed by Deng and Nickoloff (19) or by PCR amplification. The first strategy was applied to generate single mutations in A1Ct. The corresponding primers used are listed in supplemental Table SI. The selection primer that eliminates the EcoRV site of the pGEXVM plasmid was obtained by creating a new SacI site. This procedure was applied directly to pGEXVM-A1Ct vector to generate substitution of either alanine (D202A, E203A, E204A, D205A, and E207A) or cysteine (D202C, E203C, and D205C) in GST-A1Ct (Table 1). The second strategy was used to create alanine (K188A, D189A, E190A, K192A, K193A, and K195A) and cysteine (D189C, E190C, K192C, and K193C) substitutions in B2. By using the different primers listed in supplemental Table SI two successive PCR amplifications, using pQE30-B2 as template (Table 1), were carried out. The amplified DNA fragment, with appropriate sites at both ends, was restricted by BamHI and HindIII and ligated with pQE30 vector previously opened with the same enzymes. The resulting plasmids are listed in Table 1. To generate lysine substitution (K55M) in A1CtB2, two successive PCR amplifications were performed by using pQE30-A1CtB2 as template.
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and primers A1193nt/B2ct/B2K55M. The resulting plasmid was termed pQE30-A1CtB2K.

Construction of Chimerical Gene Expression Plasmids—Chimerical gene constructions were carried out by PCR amplification to fuse the last 87 bases of capsA1 (A1Ct) and the last 81 bases of capsA2 (A2Ct) to the 5'-end of capsB1 or capsB2 gene. First, primer pairs A1193nt/A1ctB1 and A2205nt/A2ctB1 were used for PCR amplification of, respectively, A1Ct and A2Ct to be fused with capsB1 gene (B1). For fusion with capsB2 (B2), primer pairs were, respectively, A1193nt/A1ctB2 and A2205nt/A2ctB2 (supplemental Table SI). Then, B1 was amplified with primer A1B1nt/B1ct or A2B1nt/B1ct, and B2 was amplified with A1B2nt/B2ct or A2B2nt/B2ct to allow fusion with any one of the A1Ct and A2Ct fragments. Finally, appropriate mixtures of PCR amplification products of A1Ct, A2Ct, B1, and B2 were prepared to perform another PCR amplification by using primer pairs A1193nt/B1ct, A1193nt/B2ct, A2205nt/B1ct, or A2205nt/B2ct and to obtain, respectively, fused DNA fragments A1CtB1, A1CtB2, A2CtB1, and A2CtB2. DNA fragments A1CtB1 and A2CtB1 were restricted by BamHI and PstI and ligated into pQE30 vector opened with the same enzymes. A1CtB2 and A2CtB2 were restricted by BamHI and HindIII and ligated into pQE30 opened accordingly. The resulting plasmids were termed pQE30-A1CtB1, pQE30-A1CtB2, pQE30-A2CtB1, and pQE30-A2CtB2 (Table 1).

Construction of GST-A1Ct, GST-A2Ct, and GST-A1CtB2 Expression Plasmids—The 87-bp capsA1-(577–666) and 81-bp capsA2-(546–626) gene fragments encoding the last 29 and 27 C-terminal amino acids of Cap5A1 and Cap5A2, respectively, were synthesized by PCR amplification using PET15b-capsA1 and PET15b-capsA2 as templates and primer pairs A1193nt/A1ct and A2205nt/A2ct, respectively (supplemental Table SI). The DNA fragments synthesized were restricted by BamHI and HindIII and ligated into pGEXVM vector opened with the same enzymes. Plasmid pQE30-A1CtB2 was also used as the template for PCR amplification using primer pair A1193nt/B2ct to ligate the corresponding DNA fragment in pGEXVM vector. The resulting plasmids were termed pGEXVM-A1Ct, pGEXVM-A2Ct, and pGEXVM-A1CtB2 (Table 1).

Overproduction and Purification of Proteins—His-tagged proteins (wild or mutated B1 and B2, chimerical A1Ct-B1, A1Ct-B2, A2Ct-B1, A2Ct-B2, and A1CtB2K55M) and GST-tagged proteins (A1Ct and A2Ct, and derivatives, and A1CtB2) were overproduced and purified as previously described (15). Purified fractions were collected and dialyzed overnight against a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, and 20% glycerol and stored at 4 °C.

Kinase Assays and Photoaffinity Labeling—In vitro phosphorylation of 1–2 μg of different purified proteins was carried out for 10 min at 37 °C in the presence of 10 μM ATP with 200 μCi/ml [γ-32P]ATP as previously described (16). Photoaffinity labeling was performed as reported by Matsuyama et al. (20), except that the buffer contained 50 mM Tris-HCl, pH 7.5, 20% glycerol, 2 mM MgCl2, 1 mM β-mercaptoethanol, 25 mM [α-32P]ATP, 30 pmol protein of interest, and a varying concentration of non-radioactive ATP, from 0 to 50 μM. After electrophoresis, radioactive proteins were visualized by autoradiography.

Fluorescence Measurement—All experiments were performed at 25 ± 0.1 °C using a Photon technology International Quanta Master I spectrophotometer. Fluorescence measurements were carried out as described previously (21) using a buffer containing 50 mM Tris-HCl, pH 7.5, 20% glycerol, 2 mM MgCl2, and 1 mM β-mercaptoethanol. Tryptophan-intrinsic fluorescence was monitored between 310 and 380 nm. Alternatively, an increasing concentration of N-methylanthraniloyl (MANT)2-ATP (Jenabiosciences GmbH) from 0 to 5 μM was added, and fluorescence resonance-energy transfer (FRET) between tryptophan residues and bound MANT-ATP was monitored through the appearance of a fluorescence emission peak between 400 and 530 nm. Correction for both the variation of volume and the inner-filter effect of MANT-ATP was performed under the same conditions by using N-acetyltryptophanamide. Peak integration was carried out at each MANT-ATP concentration with the Felix 1.21 software (Photon technology International). Curve fitting of the data were performed using the Graphit 2.11 software (Erithacus Software).

Cysteine Oxidation and Disulfide Bond Formation—Oxidation of B2 and A1Ct cysteine residues was adapted from Oudot et al. (22). 0.5 μg of 2 The abbreviations used are: MANT, N-methylanthraniloyl; FRET, fluorescence resonance-energy transfer; GST, glutathione S-transferase.
mutated B2 and/or A1Ct was incubated for 1 h at 4°C with 2 mM CuSO₄. Samples were then directly mixed with an electrophoresis loading buffer in the absence of 2-β-mercaptoethanol and heated at 100°C for 5 min prior to perform electrophoresis in the absence of reducing agent. Samples were then analyzed by Western immunoblotting by using the SuperSignal West HisProbe Kit from Pierce. Finally, blots were revealed by enhanced chemiluminescence using the chemiluminescence reagent from Amersham Biosciences.

RESULTS

Activation of Cap5B2 by the C-terminal Domain of Cap5A—The major difference between protein-tyrosine kinases of Gram-negative and Gram-positive bacteria lies in the fact that the former produce kinase activity from a single polypeptide, whereas the latter require two distinct polypeptides, one homologous to the N-terminal periplasmic region and the other homologous to the C-terminal cytoplasmic region of the Gram-negative counterpart (12). It has been suggested that in Gram-positive bacteria the cytoplasmic peptide would interact with the membrane peptide to mimic the situation encountered in Gram-negative bacteria and, thus, would induce kinase activity. In the case of S. aureus serotype 5, the A1 and A2 polypeptides are located in the membrane, whereas B1 and B2 are in the cytoplasmic fraction (Fig. 1). Preliminary work carried out in our laboratory has shown that the B2 tyrosine kinase activity is enhanced in the presence of its cognate transmembrane partner A2 and, to a greater extent, in the presence of A1/A2 (data not shown).

From these observations, it seemed of interest to determine which regions of A1 and A2 were needed to activate B2 and, possibly, B1 polypeptide (data not shown). To decide between these possibilities, a lysine substitution (K55M) was generated in the Walker A motif of HisA1CtB2 thus rendering the mutated protein unable to sustain phosphorylation (5). We also produced A1CtB2 fused to GST to distinguish it, on the basis of molecular mass, from other GST-protein-tyrosine phosphatase constructs (Fig. 1). We supposed that these fragments could interact with B1 and B2, respectively. To check this hypothesis, A1Ct and A2Ct were synthesized fused to a glutathione S-transferase tag (GST-A1Ct and GST-A2Ct), purified to homogeneity, incubated with either purified HisB1 or HisB2 in the presence of [γ-32P]ATP, and analyzed by SDS-PAGE and autoradiography. As can be seen in Fig. 2, GST-A1Ct and GST-A2Ct were able to elicit the autokinase activity of Cap5B2. The extent of activation by A1Ct and A2Ct appeared to be comparable to that observed when the entire A1 or A2 protein was used (Fig. 2). Furthermore, it was observed that A1Ct and A2Ct seemed to activate B2 more efficiently than A2Ct, respectively. Surprisingly, B1 was not activated by A1, A2, A1Ct, or A2Ct. These results indicated that the cytoplasmic C-terminal part of either A1 or A2 is able to interact with B2 and promote its autophosphorylation. In contrast, B1 does not seem to possess such capacity, even though it exhibits over 57% identity to B2.
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ity of B2 would then be because of the induction of a conformational change of B2 leading to a structural organization more appropriate for kinase activity. First, circular dichroism (CD) experiments were performed to detect possible variations in the secondary structure of B2 when fused or not to A1Ct and A2Ct. The CD spectra appeared to be identical for each of the three proteins analyzed (B2, A1Ct-B2, and A2Ct-B2), and the calculated percentage of α-helix was in the same range (around 45%) for each of them (supplemental Fig. S1A). This result indicated that if structural rearrangements were occurring in B2 the secondary structure of the protein seemed, however, to be essentially unmodified upon fusion to A1Ct and A2Ct. Then, to get information on the tertiary structure of B2 and corresponding chimeras, fluorescence measurements were performed by following the variations of their intrinsic tryptophan fluorescence. Taking advantage of the fact that B2 contains only one tryptophan residue (Trp104) in its sequence, the measurements made at an excitation wavelength of 295 nm showed that B2 alone exhibited a fluorescence emission spectrum characteristic of an accessible tryptophan residue with a maximum at around 346 nm. In the case of A1Ct-B2 and A2Ct-B2, a comparable emission spectrum was observed with, however, a blue shift of the emission fluorescence wavelength maximum to 336 nm, showing that structural modifications occurred in the local environment surrounding the tryptophan residue (supplemental Fig. S1B). It was therefore concluded, at this point, that upon binding of A1Ct and A2Ct, the three-dimensional structure of B2 was changed in connection with an increase in the B2 kinase activity.

CapSA Interaction Increases the Affinity of CapSB2 for ATP and MANT-ATP—Two main possibilities could be envisaged to explain the higher kinase activity displayed by the B2 chimeras. Either the affinity of B2 for ATP was increased by its structural rearrangement, or its capacity to transfer the γ-phosphate moiety of ATP to tyrosine residues was stimulated. To decide between these two possibilities, the affinity of B2, A1Ct-B2, and A2Ct-B2 for MANT-ATP, a nucleotide analogue of ATP, was analyzed (supplemental Fig. S2). The intrinsic fluorescence of Trp104 in B2, A1Ct-B2, or A2Ct-B2 was monitored at 340 nm by the addition of an increasing amount of MANT-ATP. In parallel, the FRET between Trp104 and the MANT moiety of MANT-ATP was studied by following the progressive appearance of a new peak of fluorescence centered at ~420 nm. No variation of Trp104-intrinsic fluorescence and no FRET was observed with B2 alone (supplemental Fig. S2A) When MANT fluorescence intensity was measured then plotted as a function of MANT-ATP concentration, no fluorescence transfer was detected (Fig. 3A). Accordingly, when the Trp104-intrinsic fluorescence was measured and plotted as a function of MANT-ATP concentration, the same observation was made (Fig. 3B). These findings suggested that B2 had a poor affinity for MANT-ATP at the concentration used. To exclude the possibility that the MANT moiety of MANT-ATP might prevent binding of the nucleotide, and thus to verify that B2 had also a poor affinity for authentic ATP, MANT-ATP was replaced with [α-32P]ATP to perform photolabeling experiments on B2. Protein B2 was incubated with a mixture of a fixed amount of radioactive [α-32P]ATP and a varying concentration of non-radioactive ATP, from 0 to 10 μM. As shown in Fig. 3C, no radioactive signal was detected for B2, even when the mixture was devoid of non-radioactive ATP. It was concluded from these experiments that B2 alone was not able to bind ATP.

In contrast, when the fluorescence of chimeras A1Ct-B2 and A2Ct-B2 was measured, both the Trp104-intrinsic fluorescence and FRET between Trp104 and MANT-ATP were found to vary (supplemental Fig. S2, B and C). Indeed, Trp104-intrinsic fluorescence decreased while FRET increased, for each chimera, as a function of MANT-ATP concentration. Curve fitting of the results obtained from the energy transfer experiments allowed estimation of $K_{Dapp}$ for MANT-ATP to be 0.05 and 0.19 μM for A1Ct-B2 and A2Ct-B2, respectively (Fig. 3, A and B). When performing ATP photolabeling for A1Ct-B2 and A2Ct-B2 to determine their $K_{Dapp}$ for ATP (Fig. 3C), we observed that A1Ct-B2 and A2Ct-B2 could bind ATP with a $K_{Dapp}$ value of, respectively, 0.61 and 3.6 μM. The differences observed between the $K_{Dapp}$ values for ATP analogs and ATP were previously reported to result in...
from the increased affinity mediated by the fluorescent probes tethered (21, 23). Furthermore, it was noted that the different effect of A1 and A2 on B2 kinase activation reported in Fig. 2 could be connected with the different $K_{D_{app}}$ values measured for A1Ct-B2 and A2Ct-B2. Together, these results led to conclude that the increase in the kinase activity of B2 induced by its interaction with A1 or A2 was due essentially to an increase of its affinity for ATP, A1 being more efficient than A2 in this stimulation.

Characterization of CapB2-A1Ct Interaction—Previous data had shown that bacterial tyrosine kinases are using the Walker A and B motifs to bind ATP instead of the motifs usually found in eukaryotic protein kinases. For this reason, they share more similarity with certain ATPases than with conventional tyrosine kinases, namely eukaryotic kinases (5). A theoretical comparative analysis of sequence alignments revealed strong similarity between B2 and a well described ATPase, the MinD protein from *Pyrococcus furiosus*, involved in septum site determination (Fig. 4). Indeed, this protein possesses the Walker A and B ATP-binding motifs and share over 35% sequence similarity with B2. The main features of the MinD ATPase mechanism are rather well understood. MinD has basically a low ATPase activity that is greatly enhanced upon interaction with another protein, MinE (24). More precisely, the helix 7 of MinD has been shown to interact with the N-terminal helix of MinE, thus changing the orientation of the P-loop of the Walker A motif and promoting ATPase activity (25). We proposed a molecular model for B2, based on the three-dimensional structure of MinD (26). In this model, helix 7 is present in B2 (supplemental Fig. S3), and the secondary structure of A1Ct is expected to contain an $\alpha$-helix (SOPMA method) (27). Therefore, one could anticipate that, as in MinD/MinE interaction, A1Ct would interact with helix 7 of B2 and thus would trigger its tyrosine kinase activity.

To check this hypothesis and identify the amino acids possibly involved in the B2/A1Ct complex formation, we constructed mutants in which some of the charged residues of either helix 7 of B2 or the helix of A1Ct were replaced with alanine. Thus, six single mutations were made by site-directed mutagenesis in B2 helix 7, and five others were made in A1Ct (Fig. 5A). Each corresponding mutant protein was overproduced and purified to homogeneity. The activation by A1Ct of the kinase activity of B2 was used as a test to identify the amino acids required for the B2/A1Ct interaction and the activation of B2 kinase activity. First, a kinase assay was performed with A1Ct and radioactive ATP in the presence of each of the six B2 mutants and analyzed by SDS-PAGE and autoradiography. The amount of radioactivity incorporated in B2 was measured (Fig. 5B). A control assay with wild B2 and A1Ct was run in parallel. It was observed that alanine substitution of Lys188 or Lys195 in the B2 helix 7 did not affect the kinase activity of B2 upon interaction with A1Ct. These two residues therefore did not seem to be of importance in B2 kinase activation by A1Ct. Surprisingly, D189A substitution in helix 7 conferred to A1Ct a higher capacity to activate B2. This observation suggested that, although Asp189 was not crucial for the interaction, it probably could modulate the efficiency of the interaction between A1Ct and helix 7 of B2. On the other hand, Glu190, Lys192, and especially Lys193, appeared to be key amino acids in B2/A1Ct interaction because their substitution drastically reduced B2 phosphorylation.

Next, a series of experiments was performed to measure the amount of radioactivity incorporated in B2 after incubation with one of the five mutated A1Ct fragments. When Glu204 or Asp207 were each replaced with alanine, mutated A1Ct was able to induce B2 autophosphorylation to the same extent as in the control assay (Fig. 5C). In contrast, when the kinase assay was performed in the presence of A1Ct-D202A, E203A, or A1Ct-D205, it was observed that B2 phosphorylation was reduced by 40–80% (Fig. 5C). Therefore, these data provided evidence that although certain residues did not seem to significantly affect the B2/A1Ct interaction, other residues such as Glu190, Lys192, and Lys193 in B2 and Asp202, Glu203, and Asp205 in A1Ct were of particular importance in the mechanism of B2 kinase activation upon A1Ct binding.

After characterizing some of the important residues for B2 kinase activation upon A1Ct interaction, it seemed of interest to confirm direct interaction between helix 7 of B2 and A1Ct. For this purpose, one amino acid at a time was replaced with one cysteine at different positions in helix 7 and A1Ct, and the interaction between B2 and A1Ct was checked out, in each case, after mixing the two corresponding mutated proteins (Fig. 5A). Close and well oriented cysteine residues were thus expected to form disulfide bonds. The mutated proteins were overproduced, purified to homogeneity, incubated in the presence of Cu$^{2+}$ to favor cysteine oxidation. They were then analyzed by SDS-PAGE, blotted onto membrane, and revealed by incubation with HisProbe$^{TM}$-horse-radish peroxidase (Pierce). As shown in Fig. 5D, a 58-kDa band could be
detected upon mixing A1Ct-D202C or A1Ct-D205C with B2-K192C and mixing A1Ct-D205C with B2-K193C. As a control, no such band was observed when non-mutated B2 was incubated with any mutated A1Ct, and, conversely, when non-mutated A1Ct was assayed with any mutated B2. Considering the molecular mass of His6B2 (27.4 kDa) and GST-ACt (30.1 kDa), these data indicated that B2-K193C could interact with A1Ct-D205C and that B2-K192C could interact with both A1Ct-D205C and A1Ct-D202C and form a bridge to produce this 58-kDa signal. To confirm the presence of A1Ct in this 58-kDa band, it was assayed by using an anti-GST antibody conjugated to horseradish peroxidase. In these conditions, the presence of the 58-kDa molecular species was still observed (data not shown). In addition, to check whether or not cysteine substitution could affect the stimulation of B2 tyrosine kinase activity, a kinase assay was performed after cysteine cross-linking. We detected a 58-kDa phosphorylation signal thus indicating that cysteine substitutions are benign for B2 stimulation (data not shown). A helical-wheel projection of B2 helix 7 and A1Ct helix. The positions of amino acids are indicated on the surface of each α-helix. Non-mutated amino acids are in roman characters. Amino acids not involved in the interaction are italicized. Bold amino acids are those found to be crucial for B2 kinase activity. Bold italicized amino acids are considered less important for kinase activation.

DISCUSSION

The mechanism of phosphorylation by tyrosine kinases and the role of this modification in the synthesis of capsular polysaccharides and exopolysaccharides has been previously investigated in different Gram-negative and Gram-positive bacteria including Streptococcus pneu-
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For instance, in the Gram-positive bacterium *S. pneumoniae*, the protein kinase CpsD can undergo autophosphorylation only when interacting with the transmembrane protein CpsC (28). However, in another Gram-positive species, *B. subtilis*, protein YwqD possesses alone a tyrosine autokinase activity, and a regulatory protein, termed YwqC, plays only an enhancer role when interacting with YwqD (8). Differences of the same type have been reported in Gram-negative bacteria. For example, in *E. coli* K12 and *S. meliloti*, the cytoplasmic domain alone of protein Wzc or protein ExoP, respectively, is able to undergo phosphorylation (10, 12). But, in another serotype of *E. coli*, K30, the cytoplasmic domain is not able to autophosphorylate per se (11). The present results provide a further example in *S. aureus* of the requirement of a complementory polypeptide for a tyrosine kinase to be active, as in the case of *S. pneumoniae*. Indeed, protein CapB2 requires interaction with the transmembrane protein Cap5A1 or Cap5A2 to express tyrosine kinase activity. However, this does not apply to Cap5B1, which remains inactive in phosphorylation even upon interaction with Cap5A1 or Cap5A2. This observation is unexpected because Cap5B1 shares over 57% structural identity with Cap5B2. Thus, in the same bacterial species, two similar proteins, Cap5B1 and Cap5B2, appear to function quite differently in terms of phosphorylation reaction. The mode of action of these various kinases may be even more complex than it appears because of the possible involvement of additional factors or the effect of particular environmental conditions on their activity. This hypothesis is supported namely by Bender et al. (29) who suggested that the phosphorylation of tyrosine kinase CpsD in *S. pneumoniae* would be dependent in vivo on a regulatory factor, not yet identified. Furthermore, tyrosine kinases are often part of multiprotein complexes in which they interact with other polypeptides, such as Wzz or Wzy in *E. coli*, that would participate in the control of their activity (14). In addition, we determined that B2 autophosphorylates through an intermolecular process. This feature is in agreement with those observed in the case of Wzz and CpsD proteins in *E. coli* and *S. pneumoniae*, respectively, but differs from the situation encountered in *B. subtilis* in which YwqD protein autophosphorylates through an intramolecular process (8, 15, 29). All together, these observations suggested that different phosphorylation molecular mechanisms can exist depending on the strain analyzed.

We have found that the cytoplasmic C-terminal domain of Cap5A1 (A1Ct) or Cap5A2 (A2Ct) is sufficient to turn on Cap5B2 activity. This activation occurs when the Cap5A fragments are added to Cap5B2 in the incubation medium or fused to the Cap5B2 protein. It is more pronounced in the presence of A1Ct than with A2Ct, even though these two fragments share high structural identity. However, this observation does not exclude that the interaction between B2 and A1Ct or A2Ct, and consequently the stimulation of Cap5B2, could be dependent on the domain of A1 and A2 that is located outside the bacterial cell wall. Indeed, this domain could bind extracellular components and have an effect on the CapA/CapB2 interaction. If not, another possibility would be that protein CapA behaves like an anchor that would only drive CapB to the membrane.

It has been demonstrated that Cap5B2 interaction with CapA5 induces structural rearrangements of Cap5B2, which increase its affinity for ATP. Interestingly, Cap5B2 shares more structural similarity with ATPases than with conventional tyrosine kinases, namely eukaryotic enzymes. In particular, CapS5B resembles protein MinD, an ATPase involved in septum site determination. Recently, tyrosine kinases and MinD have been unified in the same superfamily on the basis of sequence similarity clustering, and it has been suggested that the tyrosine kinase family emerged from the MinD family through extensive divergence (30). It has been shown that the ATPase activity of MinD is stimulated by its interaction with protein MinE. It seems that the Cap5B2 kinase activity is stimulated through a similar model by the cytoplasmic C-terminal domain of Cap5A1. Indeed, the interaction of Cap5A1 with helix 7 of Cap5B2 is required for Cap5B2 kinase activity. Such a helix is also crucial for the activation of MinD by MinE (25). It would be worth checking whether this type of interaction also takes place in other Gram-positive tyrosine kinases, namely CpsC versus CpsD in *S. pneumoniae* or YwqC versus YwqD in *Bacillus subtilis* (8, 28). Although the Cap5A5/Cap5B2 model is similar to the MinE/MinD model, some structural differences can be noted. Especially the lysine residue Lys11, which is present in the Walker A motif of MinD and required for both MinD interaction with MinE and MinD ATPase activation, is not present in the Cap5B2 sequence (31).

Obviously, the mechanism of phosphorylation of Cap5B2 of *S. aureus* differs significantly from that observed for tyrosine kinase Wzc of *E. coli*. Therefore, among the various protein-tyrosine kinases described so far in bacteria, these two enzymes could be used as a basis to define two classes of tyrosine kinases involved in polysaccharide biosynthesis, each specific to the type of strain analyzed, i.e. Gram-positive or Gram-negative. Further work is now required to investigate the phosphorylation mechanism of Cap5B2 in more detail. From a general standpoint, the aim of this type of study is not only to decipher a fundamental mechanism in bacterial physiology but also, considering the importance of tyrosine kinases in bacterial virulence, to attempt identifying new regulatory systems potentially involved in the control of pathogenicity in bacteria.

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