The initiation of sporulation in Bacillus subtilis is regulated by the phosphorelay, a complex signal transduction system consisting of kinases and response regulators. The key component of a phosphorelay is the phosphotransferase, which recognizes two response regulators and transfers a phosphoryl group between them. In this reaction, the phosphoryl of one response regulator is transferred to a histidine on the phosphotransferase before phosphorylating an aspartate of the second response regulator. The phosphorylated histidine on the Spo0B phosphotransferase was found to be His30. Site-directed mutation of His30 to alanine destroyed its phosphotransferase activity in vitro and strains constructed with this mutation were unable to sporulate. None of the other 10 histidines of Spo0B was implicated in the phosphotransferase reaction. A structurally vulnerable site, histidine 23, was also identified through the mutational study. The His30 of Spo0B resides in a domain with little sequence homology to functionally equivalent domains in the phosphorelays of other bacterial and yeast systems, suggesting that the two types of phosphotransfer domains evolved convergently.

Two-component signal transduction systems generally consist of a sensor kinase and a response regulator. The sensor kinase interprets specific signals resulting in an autophosphorylation reaction where a phosphate from ATP is transferred to a histidine residue on the kinase (1). The phosphoryl group is then donated to an aspartate residue on the response regulator to which the kinase is mated. The phosphorylation of the response regulator serves to modify another domain in the protein to be catalytically active or in the case of transcription factors, to bind to DNA and stimulate transcription from specific promoters. In micro-organisms, there are dozens of such two-component systems that are used to regulate many different processes of the cell (2, 3). There exist more complex signal transduction systems based on this simple paradigm. The phosphorelay (Fig. 1) was named to describe the flow of phosphate between signaling molecules involved in the initiation of the developmental process of sporulation in Bacillus subtilis (4). In the phosphorelay, at least two sensor kinases, KinA and KinB, are used to feed phosphate into the system (5, 6). The primary response regulator target of these kinases is Spo0F, consisting solely of a response regulator domain of 124 amino acids that serves as an intermediate in the phosphotransfer scheme. The goal of the phosphorelay is to phosphorylate the Spo0A transcription factor to activate developmental transcription (7, 8). This is accomplished by transferring the phosphoryl group from Spo0F–P to Spo0A by means of the Spo0B response regulator phosphotransferase, an enzyme first discovered in the phosphorelay and unique to it.

The Spo0B response regulator phosphotransferase is a critical junction between the information processing portion of the phosphorelay and transcription activation. When the phosphorelay was discovered, it was speculated that the multitude of signals that a cell is required to process before choosing between vegetative growth and development requires a signal transduction system capable of recognizing many signals and the more components of the pathway, the more targets for regulation (4). Subsequent studies have revealed the prophetic nature of this conclusion. It is now clear that both Spo0F and Spo0A are subject to dephosphorylation reactions from protein aspartate phosphatases that process signals antithetical to sporulation (9, 10). Thus, information conducive to development promotes the phosphorylation of the phosphorelay via the kinases while information counter to sporulation stimulates the loss of phosphate from the phosphorelay by phosphatases (9, 11, 12). The phosphorelay is actually the core of a signal integration mechanism capable of recognizing and dealing with a large variety of signals.

The Spo0B response regulator phosphotransferase has not been found, as yet, to be the target of regulation by any signals. The spo0B gene is co-transcribed with a gene for the essential GTPase, Obg, which is representative of a large family of related proteins found in other micro-organisms and higher organisms (13). However, no interactions between Spo0B and Obg have been observed.

In the course of the reaction catalyzed by Spo0B, the enzyme becomes phosphorylated and the phosphorylated residue has the properties of a phosphoramidate, assumed to be histidine-phosphate (4). In this report, we establish the identity of the phosphorylated residue and, based on the motif in which it resides, speculate about the origin and role of such proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation of Phosphorylated Spo0B Protein (Spo0B–P)**—Spo0B was phosphorylated in 30 μl of a reaction mixture containing 50 mM HEPES, pH 8.5, 50 mM KCl, 20 mM MgCl2, 0.1 mM EDTA, 5% glycerol, 0.2 mM γ-[32P]ATP (LEN Life Science Products). KinA was added to a final concentration of 1 μM and the reaction mixture was incubated at room temperature for 2 min, followed by addition of Spo0F and Spo0B.
at 10 μM each. After another incubation for 10 min, the reaction was stopped by adding 0.2 volume of 5× protein loading buffer and separated on a 15% SDS-PAGE according to the method of Laemmli (14). The gel was exposed to x-ray film at room temperature for 3 min, and the band corresponding to Spo0B–P was localized and excised. The gel pieces were ground in an Eppendorf tube, and protein was extracted in 800 μl of freshly prepared 50 mM ammonium bicarbonate, pH 8.0, for 2 h at 4 °C with constant shaking. The gel debris was removed by centrifugation at 10,000 rpm for 10 min at 4 °C, and the supernatant was lyophilized; the resulting pellet was dissolved in water, and stored at −80 °C before use. Alternatively, Spo0B–P was eluted from the gel by electrophoresis in 1× Tris-glycine running buffer, and dialyzed against a buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 1 mM EDTA.

**Phosphoamino Acid Analysis**—Radiolabeled Spo0B–P was hydrolyzed to a mixture of individual amino acids with 3 N HCl at 115 °C for 5 h, as described previously with minor modifications (15). The phosphohistidine standard was prepared by a reaction of potassium phosphoramide (20 mg) and histidine (2 mg) in water (200 μl) for 2 h at room temperature, as described (16). The hydrolysate was spotted onto a thin-layer chromatography plate along with the standards of unlabelled phosphoarginine (Sigma) and phosphohistidine. The plate was visualized by autoradiography and the standards were visualized by spraying the plate with ninhydrin in ethanol.

**Analysis of Endoproteinase Lys-C-digested Phospho-Spo0B**—Spo0B protein was phosphorylated in a mixture containing KinA, Spo0F, and ATP as above described and the reaction mixture was resolved on a 10% Tris-Tricine PAGE (17). The gel was stained with 0.025% Coomassie Brilliant Blue 250 in 40% methanol, 7% acetic acid for 30 min, then destained in the same solution until the bands could be visualized. Bands corresponding to Spo0B–P were excised, placed in an Eppendorf tube, and washed with 300 μl of 50 mM CH3CN, 200 mM NaHCO3 for 15 min at room temperature, followed by two additional washes with 150 μl of the same buffer. The shrunk gel was then cut into 1 × 2-mm pieces and dried down using a SpeedVac (Savant). 20 μl (2 μg) of sequencing-grade endoproteinase Lys-C (Promega) in 25 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA was added as 5-μl aliquots. Each aliquot was allowed to soak in before adding the next aliquot. A 50-μl aliquot of 200 mM NaHCO3 solution was finally added to just cover the gel. The digestion was allowed to proceed overnight at 37 °C. The supernatant was transferred to another tube and the gel pieces were extracted twice with 200 μl of 60% CH3CN, 200 mM NaHCO3, for 20 min with shaking at room temperature. The combined solution was dried down by SpeedVac, the pellet dissolved in sample buffer, and analyzed on a 4–10% 18% Tris-Tricine SDS-PAGE. After staining and destaining to reveal the molecular weight markers, the gel was exposed to x-ray film.

**Site-directed Mutagenesis**—Mutagenesis was done by the PCR reaction using protocols with either (i) double sets of primers and Taq polymerase (Boehringer Mannheim) or (ii) a megaprimer method using Pfu polymerase (Boehringer Mannheim) (18, 19). (i) The PCR conditions were: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, for 35 cycles, with a final extension at 72 °C for 15 min. The plasmid OB-16 (20) was used as template, and primers used were the following: two external primers, OB5Nde and OB5Bam; OB5Nde and OB5Bam 5′-GCC GGA TCC ACA AAC AAT ATG; the forward and reverse primers for H30A mutation, 5′-GCC ATT CCC GGG CGT ATT GGA TG and 5′-CAT CCA ATC AGG CCG GGA ATG GC; the forward and reverse primers for H64A mutation, 5′-GAC GCA AAG GCC GA TCA AAG C and 5′-GCT TGG ATG CCT GGT C; and the forward and reverse primers for H88A mutation, 5′-GGA AAA CGG CTT ATA TGA CG and 5′-GCT CAT ATA AGC GGT TGT CC. PCR products were subcloned into pET28a (+) vector (Novagen) in the NdeI and BamHI sites, resulting in OB/PET28, OB30A/PET28, OB6H4A/PET28, and OB6H8A/PET28. (ii) PCR reaction with a 5′ primer, 5′-CAT AAA GAA TTA TCG CCT CTT GAA AAT C and 5′-ATG GAG CTT AGT CAG CCG GTT GAA AAT C, in the second run of PCR reaction. H32Q and/or H72Q mutation(s) were generated by a degenerate mutagenic primer having an A/T mixture at the third codon of histidine, 5′-CAT GCC GGA AAA TTG GGC CAA GCA GAT TTT GAA AAT C and 5′-GCA GAT CTT GCT GCT GAA AAT C, and a 3′-mutagenic primer was used to generate a megaprimer containing the entire PCR products. This PCR product was purified with 5′-CAT AAA GAA TTA TCG CCT CTT GAA AAT C and 5′-ATG GAG CTT AGT CAG CCG GTT GAA AAT C, which introduced a stop codon at residue 155 and a BamHI site, 5′-ATG GAG CTT AGT CAG CCG GTT GAA AAT C, in the second run of PCR reaction. H32Q and/or H72Q mutation(s) were sequenced using Sequenase II sequencing kit (Amersham Pharmacia Biotech) to confirm the authenticity of the spo0B gene and expected mutations.

**Protein Expression and Purification**—spo0B/H23Q, spo0B/H27Q, and spo0B/H32Q/H72Q genes were PCR-amplified using Pfu polymerase with the chromosomal DNA of each corresponding B. subtilis strain as template, and OB5Nde and OB5Bam as 5′ and 3′ primers, respectively. These PCR fragments were subcloned into pET28a (+) vector to generate the N-terminal (His)6-tagged proteins. Each of the PCR products was transformed into the Escherichia coli strain BL21(DE3) (Novagen). The transformant cells were grown in 1 liter of BL21 supplemented with 5 μg/ml kanamycin and 5 μg/ml chloramphenicol. After removing the cell debris by centrifuging at 17,000 rpm in the SS-34 rotor of a Sorvall centrifuge and sonicated in 50 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM β-mercaptoethanol, 5 μg/ml phenylmethylsulfonyl fluoride. After removing the cell debris by centrifuging at 17,000 rpm in the SS-34 rotor of a Sorvall centrifuge for 50 min, the supernatant was applied onto a 2-ml nickel-nitrilotriacetic acid column equilibrated with the lysis buffer. Following extensive washing with the same buffer until A280 was below 0.05, the His-tagged proteins were eluted with 100 ml of the lysis buffer containing a gradient of 0–100 mM imidazole in the lysis buffer. Fractions were analyzed by SDS-PAGE, and protein-containing fractions were pooled and dialyzed against a buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 1 mM EDTA. Protein concentration was determined by the Bradford dye-binding assay (Bio-Rad) using bovine serum albumin as standard.

**Analysis of in Vivo Function of the Mutant Proteins**—The integrative plasmids encoding the wild type Spo0B N-terminal fragment were constructed by EcoRI/BamHI digestion of the plasmid pJIH4222–5 (22), and this fragment, which contains both the promoter region and N-terminal encoding portion of spo0B, was subcloned into the integrative vector, pJM103, resulting in Spo0B/pJM103. The fragments with individual H30A, H64A, and H88A mutations were obtained from the EcoRV-MsiI digestion of the corresponding pET28a (+) constructs and replace the corresponding fragment in Spo0B/pJM103. The orientations were verified by restriction enzyme digestion, and the mutations were confirmed by direct DNA sequencing.

All integrative constructs were transformed into the B. subtilis strain JH642 (trpC2 phe-1) according to the published procedure (23), and Cmr transformants were selected on Schaeffer sporulation plates (24) supplemented with 5 μg/ml chloramphenicol. Sporulation efficiency was
The Spo0B phosphoamino acid was detected by autoradiography. The threonine standards were visualized by staining with 2% ninhydrin, and 17% (w/w) ammonia (2:2:1; v/v). The phosphoarginine and phosphohistidine standards, together with phosphoarginine and phosphohistidine standards, were visualized by staining with 2% ninhydrin, and the Spo0B phosphoamino acid was detected by autoradiography. The film was aligned with the TLC plate on the origin, and the positions of standards are marked as dotted circles.

RESULTS

Spo0B Is Phosphorylated on a Histidine Residue—It had been shown previously that Spo0B is phosphorylated when it catalyzes the transfer of a phosphoryl group from Spo0F-P to Spo0A. The phospho-Spo0B (Spo0B-P) exhibited acid lability and alkali stability that were indicative of phosphoramidates, such as phosphohistidine, phospholysine, and/or phosphoarginine (4). To determine which of these amino acid(s) was actually phosphorylated, Spo0B was phosphorylated using the KinA Spo0F-P Spo0Bphorelay reactions and Spo0B-P was purified by separation on a SDS-polyacrylamide gel. The isolated Spo0B-P was alkaline-hydrolyzed into a mixture of individual amino acids and analyzed by one-dimensional thin layer chromatography (TLC), with the standards phosphohistidine and phosphoarginine (Fig. 2). The spot of 32P-labeled phosphoamino acid produced by Spo0B-P hydrolysis co-migrated with the phosphohistidine standard. As a positive control, NtrB-P, a histidine kinase in the nitrogen regulation system of E. coli, which is known to be phosphorylated on a histidine residue (25, 26), was analyzed in parallel with Spo0B-P. The chromatographic migration patterns were identical for both proteins. A secondary radiolabeled spot, which migrated slightly slower than the histidine standard, was observed for both Spo0B and NtrB (Fig. 2). This species is not phospholysine, which has a mobility less than phosphoarginine (15). As has been reported for the histidine kinase, VirA, of Agrobacterium tumefaciens (27), another form of phosphohistidine where the phosphoryl group resides on a different nitrogen of the imidazole ring (28) might account for this. Spo0B, therefore, appears to be phosphorylated solely on a histidine residue.

Protease Digestion Analysis Localized the Phosphorylation Site to the First 88 Residues of Spo0B—There are 11 histidine residues in Spo0B (Fig. 3A). In order to identify which histidine was phosphorylated, an analytic protease digestion was performed. Endoproteinase Lys-C was chosen based on the distinct histidine-containing fragment sizes expected from a complete digestion (Fig. 3A). The last five histidine residues (His119, His132, His141, His152, and His163) reside on a minimal 8.8-kDa peptide, whereas the first three histidine residues (His23, His27, and His30) lie on a 3.4-kDa fragment. Spo0B-32P protein was digested as described, and the results of autoradiography are presented in Fig. 3B. Only one radiolabeled peptide was observed in the digestion mixture. The apparent size of this peptide is smaller than the 6-kDa molecular size marker, indicating it is not the 8.8-kDa fragment. These data, therefore, indicate that none of the last five histidines is the phosphorylation site, but do not definitively identify the phosphorylation site without additional data. The role of the remaining six histidine residues was subsequently examined by site-directed mutagenesis. Because the Spo0B-phosphorylated intermediate is likely to be critical in the phosphorelay, a complete elimination of sporulation would be expected if the phosphorylation site was altered.

 SITE-DIRECTED MUTAGENESIS AND IN VIVO ANALYSIS—Histidine residues 23, 27, 30, 64, 75, and 88 were mutated individually as listed in Table I. Functional transcriptional units with C-terminal truncated spo0B genes carrying each individual mutation were constructed on an integration vector, pJM103. The resulting plasmids were introduced and integrated into the chromosomal spo0B locus by Campbell-type recombination in order to investigate the effect on sporulation of individual histidine mutations. After integration of the plasmids into the chromosome, the recombinants contained two copies of the spo0B gene; one copy encoded a C-terminal truncated and nonfunctional protein, as shown previously (22), while the other copy was a fully functional intact spo0B gene. Depending on where the recombination event takes place, the intact gene can be wild type, or contain the His mutation in the recombinants, as shown in Fig. 4. If the Spo0B mutant cannot support the initiation of sporulation, we expect to find both Spo and Spo− phenotypes among the transformant colonies on Schaeffer sporulation plates. As shown in Table I, only mutations at two of these histidine residues, 23 and 30, produced diminished sporulation phenotypes. Both Spo− and Spo+ colonies were observed in transformation with plasmid donors having either H23Q, H23Q/H27Q or H30A mutations, while the others gave only Spo− colonies. Spore counts in liquid medium were performed with these mutants in order to obtain a more quantitative comparison. H30A completely eliminated spore formation (frequency less than 10−8); H23Q yielded ~104-fold decreased sporulation frequency compared with that of wild type; however, H23Q/H27Q double mutations somehow rescued the defect of the H23Q single mutation to only a ~10-fold decrease relative to that of wild type. The H23A mutant, however, behaves like wild type Spo0B. One explanation for the peculiar histidine 23 mutation is that H23Q disrupts interactions with one of the Spo0B relay partners, Spo0F or Spo0A, and by introducing an additional H27Q mutation, the interactions are re-established. Alternatively, the H23Q mutation may produce a less stable protein, while the second H27Q mutation complements the instability introduced by H23Q. In vitro phosphotransfer experiments were done to further characterize these mutants. Nevertheless, if Spo0B phosphorylation is essential for its phosphotransferase activity, this result indicates that the putative site of phosphorylation is either H23 or H30.

In Vitro Studies of Spo0B Proteins Confirm That His30 Is the Site of Phosphorylation—A total of seven Spo0B proteins were expressed and purified as N-(His)6-tagged proteins, including wild type, H23Q, H27Q, H23Q/H27Q, H30A, H64A, and H88A. Like the wild type protein, all mutants behave as dimers as indicated by native gel analysis. In addition, phosphorylation of the N-terminal His6-tagged wild type Spo0B was similar to the
wild type protein, indicating that the N-terminal His tag does not significantly affect Spo0B catalytic activity (data not shown). Each protein was analyzed for its ability to be phosphorylated by Spo0F. The autoradiography of the separated proteins of these reactions is presented in Fig. 5. It is clear that only H30A is unable to be phosphorylated by Spo0F. Despite the defect seen in vivo, the H23Q mutant can accept the phosphoryl group from Spo0F perfectly well. It is curious that the H23Q mutant protein migrated at a slightly different position even in the presence of SDS. The result shown in Fig. 5 demonstrates that the single alanine substitution at residue 30 can prevent Spo0B phosphorylation.

It remained possible that phosphorylation of Spo0B arises from a side reaction and is not necessary for the phosphotransferase function of Spo0B. Such a situation was found in the phosphorylation of the GTPase, Obg, where the protein becomes phosphorylated during the GTPase reaction but histidine phosphorylation is not part of the catalytic mechanism (29). In order to test this possibility, these Spo0B mutants were assayed for their phosphotransferase activity from Spo0F to Spo0A. The H30A mutant neither receives the phosphate from the Spo0F–P nor transfers the phosphoryl group to Spo0A (Fig. 6). The unrelated histidine mutants (H23Q, H64A, H88A, and H23Q/H27Q), however, resembled the wild type Spo0B: they were phosphorylated by Spo0F–P and were able to phosphorylate Spo0A (Fig. 6). Thus, the phosphorylation of Spo0B on the His30 appears to be indispensable for its phosphotransferase activity.

The abnormality of the H23Q mutant was clearly illustrated by native gel electrophoresis as well. As shown in Fig. 7, both phosphorylated and unphosphorylated forms of Spo0F and Spo0B can be resolved on a 13% native PAGE. The position of Spo0F–P comes from comparing lanes 1 and 2. In lanes 7–10, no phosphorylation occurred because KinA was not present in the reaction mixture. It is clear that H23Q (Fig. 7, lane 9) migrated at a different rate relative to those of the other Spo0B proteins. In addition, the H27Q mutation somehow corrected the abnormality of H23Q and therefore, the H23Q/H27Q mutant migrated to the same position as the wild type protein (Fig. 7, lane 8). When KinA was added into the reaction, all Spo0B present in the reaction mixture was phosphorylated under this reaction condition, and the three Spo0B–P proteins examined here migrated faster than did the unphosphorylated Spo0B. This is opposite to the behavior of Spo0F, which runs faster than Spo0F–P. Additionally, no change in H30A migration was observed in the presence of KinA, Spo0F, and ATP, indicating no phosphorylation occurred (Fig. 7, lanes 3 and 7). This is consistent with the result shown in Fig. 5. Furthermore, the position of unphosphorylatable H30A was identical to that of the wild type protein, implying that its global folding was not affected by its mutation (Fig. 7, lane 7 versus lane 10) and the inability to phosphorylate was not a result of protein misfolding.

**DISCUSSION**

Although the phosphorelay signal transduction system was first discovered in the information processing pathway to the initiation of sporulation in *B. subtilis*, it is now clear that such pathways are found in other bacteria (30, 31) and yeast (32).
The key element in a phosphorelay is the Spo0B equivalent that catalyzes the transfer of a phosphoryl group from one response regulator to another. In the *B. subtilis* phosphorelay, this enzymatic activity is found on an individual protein as are the kinase and Spo0F response regulator. Phosphorelays also exist in multi-domain enzymes such as BvgS or ArcB, where all the enzymatic activities, except the ultimate response regulator transcription factor, reside in a single protein (30, 31). It is highly likely that all of the “unusual” histidine kinases that incorporate a response regulator domain function by means of a phosphorelay. Regardless of the domain structure of the proteins involved, the catalytic mechanism follows the original His-Asp-His-Asp paradigm described for the *B. subtilis* phosphorelay (4).

There are several key properties of the His-Asp-His-Asp phosphorelay that are not universally appreciated. The phosphates on aspartate residues are in the form of a mixed anhydride and susceptible to hydrolysis spontaneously, by autophosphatase activity and by external phosphatases including the kinase that originally phosphorylated it. Phosphorelay signal transduction systems allow effectors other than those that regulate the kinase to influence the phosphorylation level of the system. The Spo0B phosphotransferase reaction is readily reversible, and the equilibrium of the reaction at similar response regulator concentrations is close to an equal distribution of phosphate on both the substrate and the product. Thus, the level of Spo0F–P and Spo0A–P in the cell is likely to be roughly the same excluding other unknown factors that modify the phosphate distribution. The consequence of reversibility is that any influence on the stability of the phosphorylated state of one of the response regulators will immediately affect the phosphorylation level of the other. This was dramatically demonstrated by the Rap phosphatases that specifically dephosphorylate Spo0F–P and reverse the course of gene expression regulated by Spo0A–P (4, 9). The Rap phosphatases and the Spo0E phosphatase that dephosphorylates Spo0A–P, form part of the proof that the phosphorelay evolved as a mechanism by which many potential regulators could influence in both positive and negative ways the outcome of the signal transduction pathway (4). Such a system provides a means to integrate all these signals into a single output, Spo0A–P (10). This signal integration capacity is likely to be extremely important for the developmental process of sporulation, which is a cellular event and subject to environmental, metabolic, and cell cycle regulatory influences.

Response regulators are ideally suited to serve as the targets for regulation in this system. The active phosphorylated state results from a phosphoryl-aspartate, which, because of its position in the molecule, may maximize conformational changes allowing a variety of regulatory interactions with other molecules (33). Acyl-phosphates are subject to hydrolysis activities that limit the lifetime of the phosphorylated state. Protein-protein interactions may activate such inherent reactions (34), and such interactions have been postulated to limit, for example, the transcriptional lifetime of the Spo0A–P protein (12). In contrast, the Spo0B phosphotransferase has not as yet been

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2 J. A. Hoch, unpublished results.
found to be subject to outside regulatory influences. Recently, however, a phosphatase specific for the phosphohistidine of the ArcB phosphotransferase domain has been identified (35), indicating the possibility that some putative regulatory input(s) through Spo0B remains to be found.

In this report, histidine 30 was identified to be essential for Spo0B's function. Its in vivo activity and phosphorylation activity were destroyed by a single alanine substitution at this residue, a result also demonstrated for the His64 mutation of Histidine 30 is located on the C terminus of Spo0B and is essential for the function of the phosphotransferase domain (30). Additionally, a positive charged residue is present at position 27 may establish new interactions with Gln23, thus replacing any unfavorable constraints produced by Gln23.

The origin, specificity, and relationship of the Spo0B phosphotransferase to two-component kinases are of some interest. The residues surrounding the active histidine of the yeast Spo0B equivalent, YPD1, suggest a relationship to the histidines of E. coli CheA kinase and to the phosphotransferase domains of the composite “unusual” kinases BarA, ArcB, and BvgS (32). The latter two are known to be part of phosphorylation systems. Spo0B, on the other hand, has little homology to these proteins in the region around the active site histidine residue by directly comparing their primary sequence. The active site histidine of the CheA kinase (36) and the ArcB phosphotransferase domain (30) are located in a contiguous region of one subunit known to form a four-helix bundle. The structure of Spo0B is now known, and the active histidine is similarly placed within a four-helix bundle made up of two helices from each subunit of the active dimer (20). These helices are amphipathic in nature, and only those residues spaced on the same helical side of the active histidine have potentially important functional consequences. These residues are located at i ± 1, i ± 3, and i ± 4 positions with respect to the active histidine (i) and are listed in Table II. An invariant glycine is found at i + 4 position in all domains expect Spo0B, which has a glycine at i − 4 position instead. This glycine residue has been proposed to carve out an empty space for the active histidine (30). Additionally, a positive charged residue is present at i + 1 position in most of the cases, which may neutralize the negatively charged phosphoryl group (30); yet Spo0B has an arginine at i − 1 position. It seems that Spo0B has an inverted orientation with respect to other phosphotransferase domains. These differences may arise from the different origins of the four-helix bundle in each case. Convergent evolution may ac-

![Fig. 6. Autoradiography showing the phosphotransferase activities of the wild type and mutant Spo0B proteins. Reaction conditions are similar to those in Fig. 4, except that the reaction was initiated by adding Spo0B (10 μM) and Spo0A (10 μM) simultaneously to a mixture with Spo0F at 10 μM concentration.](image)

![Fig. 7. Native gel analysis of Spo0B proteins. Individual Spo0B protein (10 μM) was mixed with Spo0F (10 μM) and ATP (400 μM) (lanes 7–10). Phosphorylation was initiated by adding KinA into a second aliquot of the corresponding mixtures in lanes 1–6, yielding a final concentration of 0.2 μM; then, the reaction was continued for 30 min (lanes 3–6). Lane 1 contains only Spo0F and KinA without ATP, whereas ATP was added in lane 2. Samples were quenched by 0.3 volume of 4× native gel sample buffer, frozen on dry ice, and analyzed as described by Zapf et al. (37). Protein migration patterns were revealed by Coomassie staining.](image)

| Protein      | -4 | -3 | -1 | 0  | +1 | +3 | +4 |
|--------------|----|----|----|----|----|----|----|
| Spo0B        | G  | H  | R  | H  | D  | M  | N  |
| ArcB<sup>a</sup> | V  | E  | G  | H  | K  | K  | G  |
| BvgS<sup>b</sup> | S  | D  | A  | H  | R  | A  | G  |
| EvgS<sup>c</sup> | H  | Q  | I  | H  | R  | H  | G  |
| BarA<sup>d</sup> | V  | D  | I  | H  | K  | H  | G  |
| InvA<sup>e</sup> | I  | E  | I  | H  | R  | H  | G  |
| RcaC<sup>f</sup> | Q  | H  | A  | H  | K  | A  | G  |
| YPD1<sup>g</sup> | D  | N  | S  | H  | F  | K  | G  |
| CheA<sup>h</sup> | F  | R  | A  | H  | S  | K  | G  |

<sup>a</sup> Minus indicates the residue located on the N-terminal side of the helix; plus indicates the residue on the C terminus.

<sup>b</sup> E. coli.

<sup>c</sup> B. pertussis.

<sup>d</sup> P. syringae.

<sup>e</sup> Fremyella diplosiphon.

<sup>f</sup> Saccharomyces cerevisiae.

The residues in proximity of the active histidine of the histidine phosphotransfer domain in multistep phosphorelay signal transduction systems.

<sup>g</sup> K. I. Varughese, and J. A. Hoch, unpublished results.
count for the similar function of Spo0B and these kinase domains. It will be of interest to determine if the evolutionary origin of Spo0B portends other functions for this protein.

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