A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems

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A *Bacillus subtilis* gene, required for genetic competence, was identified immediately upstream from the previously characterized gene *comA*. The *comA* gene product has been found to exhibit amino acid sequence similarity to the so-called effector class of signal-transduction proteins. DNA sequencing of the new determinant, named *comP*, revealed that the carboxy-terminal domain of the predicted ComP protein is similar in amino acid sequence to that of several sensor members of the bacterial two-component signal-transduction systems. The predicted amino-terminal domain contains several hydrophobic segments, postulated to be membrane-spanning. In vitro-derived *comP* disruptions are epistatic on the expression of all late competence genes tested, including *comG*, *comC*, *comD*, and *comE*, but not on expression of the early gene *comB*. Although *comA* has its own promoter, some transcription of *comA*, especially later in growth, occurs via readthrough from *comP* sequences. A roughly twofold epistatic effect of a *comP* disruption was noted on the downstream *comA* determinant, possibly due to interruption of readthrough transcription from *comP* to *comA*. Overexpression of *comA* fully restored competence to a *comP* mutant, providing evidence that ComA acts after ComP, and consistent with a role for the latter protein in activation of the former, possibly by phosphorylation. ComP probably is involved in transmitting information concerning the nutritional status of the medium, particularly the presence of nitrogen- and carbon-containing nutrients. ComP was also shown to play a role in sporulation, at least partly interchangeable with that of SpoIIJ, another putative sensor protein.

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Genetic competence in *Bacillus subtilis* requires the expression of several gene products that are responsible for the binding, processing, and uptake of transforming DNA (for review, see Dubnau 1989). The manufacture of these proteins, which are encoded by so-called late competence genes, is controlled by an elaborate network of regulatory elements in response to environmental signals about which little is known. The competence regulon is expressed after the transition to stationary phase, and only in glucose minimal salts medium. It is therefore an example of a so-called late-growth-regulated system and is subject to nutritional control. At maximal competence, only 10–20% of the cells are competent, and these can be separated from the noncompetent cells and shown to be physiologically unique (Hadden and Nester 1968; Haseltine Cahn and Fox 1968). Thus, a nonterminal differentiation process results in the elabo-

ration of two cell types. To investigate the regulation of this system, we have characterized a series of mutations that are epistatic on the expression of late competence genes and that appear to play a regulatory role.

One such regulatory element (*comA*) was defined by a Tn917*lac* insertion (Albano et al. 1987; Hahn et al. 1987; Guillen et al. 1989; Weinrauch et al. 1989) and was shown to specify a protein essential for the expression of late competence genes. The DNA sequence of *comA* revealed that its product closely resembled members of the effector (or response regulator) family of bacterial signal-transduction proteins (for review, see Stock et al. 1989). Effector proteins usually serve to activate the expression of a set of target genes in response to environmental signaling. In several cases, the activities of effector proteins are known to be regulated by phosphorylation and dephosphorylation. The amino-terminal moieties of effector proteins contain conserved amino acid sequences that include the aspartate residue(s) that

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appear to serve as phosphorylation sites. A second component of these signaling systems, the sensor, is often membrane-associated. Sensors can be autophosphorylated at a conserved histidine residue and can transfer this phosphate to their cognate effector. The various sensor proteins possess a conserved carboxy-terminal transmitter domain, which includes the conserved histidine residue.

The participation of the putative effector ComA in competence development suggested that a sensor protein might also be involved. *comB* is a second regulatory gene required for the development of competence (Guillen et al. 1989; Weinrauch et al. 1989). Inactivation of *comB* produces a phenotype identical to that of *comA* mutants, and the two genes are closely linked on the *B. subtilis* chromosome. However, the predicted amino acid sequence of ComB revealed no similarity to known sensor proteins. We have now identified an additional regulatory gene for competence, *comP*, which appears to specify a membrane-localized sensor protein and is involved in regulating sporulation as well as competence. Evidence is presented indicating that *comP* and *comA* work together in transmitting environmental signals to the competence regulon and that these signals include information concerning the presence of nitrogen and carbon sources.

Results

Disruption of the open reading frame upstream of comA

DNA sequence determination upstream from *comA* revealed part of an open reading frame (ORF) with a potential stem-loop terminator overlapping the *comA* promoter. To determine whether these DNA sequences were required for competence, we replaced the wild-type upstream ORF in the chromosome by mutant versions using double-crossover events. Plasmid DNA (pBD441 and pBD442) containing a Cm^R cassette in two different orientations inserted in the cloned copy of the upstream ORF was linearized and used to transform a *com*⁺ strain. The Cm^R cassette was inserted in the right-most *Bcl*I site, indicated below in Figure 2. The resulting Cm^R transformants exhibited levels of transformation ~200- to 400-fold lower than that of the isogenic wild-type strain, demonstrating that the upstream ORF encoded a product required for genetic competence. This gene was named *comP*. The strains carrying disruptions in *comP* in two different orientations were designated BD1658 and BD1659.

Dependence of late com genes on comP

It was shown previously that the products of the sporulation genes, *spoOA* and *spoOH*, as well as the early competence genes, *comA*, *comB*, and *sin*, were required for the expression of several late expressing *com* genes (Albano et al. 1987; Guillen et al. 1989). We therefore wished to determine whether *comP* was also required for the expression of late *com* genes. DNA from strains carrying the Em^R late *com-lacZ* fusions, *comC530*,

comD413, *comE518*, *comG12*, and two early fusions, *comB138* and *comA124*, was used to transform strain BD1658, which contains the Cm^R disruption of *comP* in the chromosome. This was possible because the *comP* mutants exhibited some residual transformability. Cm^R Em^R transformants were grown through the one-step competence regimen, and β -galactosidase activity was measured as a function of growth stage. Figure 1 shows that the expression of *comB138* was unaffected by the *comP* disruption, whereas the expression of *comA124* was decreased about twofold after T_0 . This twofold decrease was also observed in the strain carrying the Cm^R cassette in *comP* in the reverse orientation. In contrast, a complete blockage in expression was observed for all the late *com* genes. These results showed that *comP* was required for expression of late competence genes and that the disruption also caused a relatively small decrease in *comA* expression. It is interesting that this effect on *comA* expression is only apparent for the later time points. It appears that expression of *comA* has two components; an early one that is not *comP*-dependent, and a later *comP*-dependent component.

Nucleotide sequence of comP

Figure 2 shows a genetic and physical map of the *comB-degQ-comP-comA* region of the *B. subtilis* chromosome (Guillen et al. 1989; Nakano and Zuber 1989; Weinrauch et al. 1989). The 1.4-kb *Hind*III fragment that contained the *comA* gene was sequenced previously (Dubnau 1989; Weinrauch et al. 1989) and found to contain part of *comP*. We isolated a 3-kb *Eco*V-*Asu*II fragment from pED3, which included the entire *comP* gene, and determined its nucleotide sequence on both strands. The sequence revealed one major ORF (Fig. 3), which extended from position 335 to a TAA stop codon at position 2642, encoding a protein of 749 amino acids with a deduced molecular weight of 86,763. The TTG start codon was preceded by the potential Shine-Dalgarno (Shine and Dalgarno 1974) sequence AGGTGGA.

The predicted amino acid sequence of the ComP protein was compared to the translated GenBank data base. Similarity was found to members of the sensor class of bacterial signal-transduction proteins. Figure 4 shows a comparison of ComP to the most closely related of these sensor proteins. All of the residues noted by Stock et al. (1989) to be completely conserved in the other proteins of this class are also conserved in ComP. Also noteworthy is the striking series of hydrophobic segments in the amino-terminal half of the predicted amino acid sequence (Fig. 5).

Transcription mapping

To localize the 3' end of the *comP* transcript(s), a 1.8-kb *Xma*III-*Sst*I probe was 3'-end-labeled at the *Xma*III site (Fig. 2). The probe was hybridized to RNA isolated from an isogenic *com*⁺ strain (BD630) at T_{-2} , T_{-1} , and T_1 , and from a *com*⁺ strain carrying *comP* and *comA* on a multicopy plasmid, at T_{-1} . The results are shown in Figure

Figure 1. Epistatic effects of *comP* mutation on expression of *com* genes. *comP* was inactivated by insertion of a *Cm*^R cassette, as described in Methods. The effects of this insertion on expression of *lacZ* transcriptional fusion to the indicated *com* genes are shown. The fusions used were *comA124*, *comB138*, *comC530*, *comD413*, *comE518*, and *comG12* (Albano et al. 1987). ($\diamond$) *com*⁺; ($\blacksquare$) *comP* disruption (orientation 1); ($\blacksquare$) *comP* disruption (orientation 2). The time points refer to hours before or after the departure from exponential growth (defined as *T*₀).

6A. The *comP* mRNA appeared to have a major 3' terminus at a site ~450 residues downstream from the labeled *Xma*III site. This would place the major *comP* terminator approximately at the position of a dyad symmetry element that overlaps the *comA* promoter (Fig. 3). Because the *Xma*III–*Sst*I probe encompassed the *comA* gene, together with the putative *comA* terminator

(Weinrauch et al. 1989), we would expect any readthrough past the major *comP* termination site to generate a transcript of 1.6 kb (Fig. 2). In fact, such a potential readthrough transcript was observed in the *T*₋₁ and *T*₁ samples, but not at *T*₋₂. These results suggested that readthrough transcription of the *comA* transcript, possibly from the *comP* promoter, may occur beginning

Figure 2. Physical and genetic map of the *comP* region of the *B. subtilis* chromosome. The locations of the genetic determinants are from Guillen et al. (1989) and Nakano and Zuber (1989). The position of promoters and transcriptional terminators are indicated. A *Cm*^R chloramphenicol acetyltransferase (CAT) cassette was inserted in a *Bcl*II site in both orientations, as indicated. (BH) *Bam*HI; (H) *Hind*III; (EV) *Eco*RV; (B) *Bcl*II; (X) *Xma*III; (A) *Asu*II; (S) *Sst*I.

Competence regulatory gene in *B. subtilis*

1 100
TTAATTAAAGCGGAGTTACTCAATACATTTCGGTATTATTGGAAATATATAAACAGAAATGTATTCTGCTATAGAACAAATTAAATTTAGATAAAGAAAA

200
AAAAGAGCTAATAAAGGAATGCTTATTAAGTTATACAAAGGGGATACAGATGCAAGACCTAATTAAGTACTTTTAAATTATCCTGAGGCTTTAAAAAA

300
ATTGAAAAATAAAGAAGCCTGCCTTATAGGTTTTGATGTGCAAGAACTGAAACAATAATTAAAGCTTATAATGATTATTATCTGGCTGATCCAATAACCC

SD

Met Lys Asn Leu Ile Lys Lys Phe Thr Ile Ala Val Ile Val Leu Ser Ile
GTCAATGGGGTGATTAATAGGTGGATTAATA TTG AAG AAC TTA ATA AAA AAA TTC ACA ATA GCT GTA ATT GTT TTG AGT ATC

400
Leu Tyr Ile Ser Tyr Thr Thr Tyr Ile Ser Met Asn Gly Ile Ile Ile Gly Thr Lys Ile His Lys Asn Asp Lys
CTT TAT ATA AGC TAT ACA ACT TAT ATC AGT ATG AAT GGA ATT ATT ATT GGG ACT AAG ATT CAT AAA AAT GAT AAA

500
Ser Gln Phe Met Ile Glu Glu Ile Ser Glu Ser Ser Tyr Gly Gln Phe Val Gly Leu Arg Gln Ala Asp Ile Ile
AGT CAA TTT ATG ATA GAA GAA ATA TCG GAA TCT TCA TAT GGA CAA TTT GTT GGT CTG AGA CAG GCA GAC ATC ATA

600
Leu Lys Ile Asn Lys Glu Lys Pro Ser Asp Lys His Leu Lys Trp Gly Tyr Leu Ser His Ile Asn Ser Leu Asp
TTA AAA ATT AAT AAA GAA AAA CCT TCA GAC AAA CAT TTG AAA TGG GGA TAT TTG AGT CAT ATT AAC AGT CTG GAT

700
Ile Leu Arg Ser Gly Lys Lys Ile His Leu Lys Asp Phe Asp Leu Val Thr Leu Asn Arg Pro Tyr Ser Phe Phe
ATT TTG AGA AGT GGA AAA AAG ATT CAT CTC AAA GAT TTT GAT TTA GTT ACT CTA AAT AGA CCT TAT AGT TTT TTT

800
Leu Phe Val Leu Pro Leu Phe Phe Tyr Phe Leu Ser Ile Ile Cys Ile Phe Tyr Ile Leu Lys Val Asn Lys Lys
CTG TTC GTA CTT CCT TTG TTT TTT TAT TTT TTA AGC ATA ATA TGT ATT TTT TAT ATA CTT AAA GTA AAT AAA AAA

900
Arg Arg Ser Phe Ala Ala Tyr Ile Leu Ile Leu Leu Leu Leu Asp Ile Ser Ile Ala Tyr Ile Ser Ala Gly Gly
AGG AGA TCA TTT GCT GCC TAT ATT CTT ATT CTA TTG TTA TTA GAC ATT TCG ATA GCA TAT ATA AGT GCA GGC GGA

1000
Pro Phe Arg Gly His Ile Ile Asn Arg Tyr Ile Asn Leu Phe Thr Phe Ile Ser Ser Pro Ile Leu Tyr Leu Gln
CCA TTT AGA GGA CAT ATA ATC AAT CGG TAT ATT AAC TTG TTT ACT TTT ATA TCT TCT CCT ATT CTT TAT CTT CAA

1100
Phe Ile Gln Arg Tyr Leu Gly Glu Ile Gly Lys Thr Phe Leu Asn Arg Ile Ser Phe Leu Tyr Ile Ile Pro Ile
TTT ATA CAA AGA TAC CTT GGA GAA ATA GGT AAA ACT TTT TTG AAT AGA ATT TCT TTT CTT TAT ATC ATA CCA ATA

1200
Ser Phe Ala Thr Leu Thr Leu Phe Ser Phe Ser Ala Ile Tyr Leu His Leu Asn Lys Tyr Lys Tyr Ala Glu His
TCT TTT GCA ACT TTG ACT CTA TTT TCT TTT TCA GCG ATA TAC TTA CAT CTT AAT AAA TAT AAA TAT GCT GAG CAT

Ile Ile Phe Thr Gly Asn Tyr Ile Phe Pro Ala Leu Ala Ser Ala Ser Leu Leu Val Leu Ile Pro Phe Gly Leu
ATA ATA TTT ACA GGT AAT TAT ATT TTC CCG GCA TTA GCT TCG GCG TCA TTA CTA GTA CTA ATT CCG TTC GGG TTA

Figure 3. (Continued on following page.)

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      1300
Val Tyr Gln Phe Val Ala Asn Lys Met Phe Asp Ile Glu Phe Ile Leu Gly Arg Met Arg Tyr Tyr Ala Leu Leu
GTA TAC CAA TTT GTA GCC AAT AAG ATG TTT GAT ATA GAG TTT ATC TTA GGA AGA ATG AGA TAC TAT GCT CTA CTT

      1400
Ala Met Ile Pro Thr Leu Leu Ile Val Gly Ala Leu Val Leu Phe Asp Val Met Asp Ile Gln Met Asn Pro Val
GCC ATG ATA CCA ACT CTT CTA ATA GTT GGT GCA TTA GTT CTG TTT GAT GTA ATG GAC ATC CAG ATG AAC CCT GTG

      1500
Arg Gln Thr Val Phe Phe Phe Val Val Met Phe Ala Val Phe Tyr Phe Lys Glu Val Met Asp Phe Lys Phe Arg
CGT CAA ACT GTA TTT TTT TTC GTT GTC ATG TTC GCT GTC TTT TAT TTT AAA GAG GTC ATG GAT TTT AAA TTT CGG

Leu Lys Arg Phe Ser Glu Lys Phe Asn Tyr Gln Asp Ser Ile Phe Lys Tyr Thr Gln Leu Met Arg Gly Val Thr
TTA AAA CGT TTC TCC GAA AAA TTC AAC TAT CAG GAC AGT ATT TTT AAA TAT ACT CAG CTA ATG AGG GGT GTA ACT
HindIII
-- -- --

      1600
Ser Leu Gln Gln Val Phe Lys Glu Leu Lys Asn Thr Ile Leu Asp Val Leu Leu Val Ser Lys Ala Tyr Thr Phe
TCT CTT CAA CAA GTT TTT AAA GAA CTG AAA AAT ACT ATA CTG GAT GTT TTG CTT GTA AGC AAA GCT TAT ACC TTT
BclI
- - - - -

      1700
Glu Val Thr Pro Asp His Lys Val Ile Phe Leu Asp Lys His Glu Val Gly Pro Asp Trp Asn Phe Tyr Gln Glu
GAG GTT ACT CCT GAT CAC AAA GTG ATA TTT TTA GAT AAG CAT GAA GTT GGA CCG GAC TGG AAT TTT TAT CAA GAG

      1800
Glu Phe Glu Asn Val Thr Ser Glu Ile Gly Lys Ile Ile Glu Val Asn Gln Gly Phe Leu Met Lys Val Gly Glu
GAA TTT GAA AAC GTA ACT TCA GAA ATT GGG AAA ATT ATA GAA GTC AAT CAA GGC TTT CTT ATG AAA GTT GGT GAA

Arg Gly Gly Ser Ser Tyr Val Leu Leu Cys Leu Ser Asn Ile Asn Thr Pro Arg Leu Thr Arg Asp Glu Ile Ser
CGA GGC GGT AGT TCT TAT GTT CTG CTT TGT TTA TCT AAT ATT AAC ACT CCC CGG CTA ACT CGT GAT GAA ATA TCG

      1900
Trp Leu Lys Thr Leu Ser Phe Tyr Thr Ser Val Ser Met Glu Asn Val Leu His Ile Glu Glu Leu Met Glu His
TGG CTG AAA ACA CTG TCT TTT TAT ACA AGT GTG TCC ATG GAA AAT GTC CTG CAT ATT GAG GAG CTC ATG GAA CAT

      2000
Leu Lys Asp Leu Lys Gln Glu Gly Thr Asn Pro Ile Trp Leu Lys Lys Leu Met Phe Ala Ile Glu Glu Lys Gln
TTG AAG GAC TTA AAA CAA GAG GGA ACC AAC CCC ATC TGG CTG AAA AAG CTA ATG TTT GCA ATC GAA GAA AAA CAG

      2100
Arg Ser Gly Leu Ala Arg Asp Leu His Asp Ser Val Leu Gln Asp Leu Ile Ser Leu Lys Arg Gln Cys Glu Leu
CGT TCA GGA CTC GCC CGC GAT CTC CAC GAT TCG GTT CTT CAG GAT TTG ATT TCC TTA AAA CGC CAG TGT GAG CTG
HindIII
--- ---

Phe Leu Gly Asp Phe Lys Lys Asp Asp Asn Pro Cys Arg Glu Glu Val Gln Tyr Lys Leu Val Gln Met Asn Glu
TTT TTG GGT GAT TTC AAG AAG GAT GAT AAT CCG TGC CGT GAA GAG GTG CAG TAC AAG CTT GTA CAG ATG AAT GAG

      2200
Gln Met Ser Asp Val Ile Ser Met Thr Arg Gly Thr Cys His Glu Leu Arg Pro Pro Val Leu Tyr Asp Leu Gly
CAG ATG TCT GAT GTG ATT TCG ATG ACG AGG GGG ACG TGT CAT GAG CTG CGG CCG CCA GTT CTG TAT GAT CTT GGA

      2300
Leu Val Lys Ala Leu Ser Lys Leu Val Ala His Glu Gln Glu Arg Val Pro Phe His Ile Arg Leu Asn Thr Gly
TTG GTG AAG GCG CTG TCG AAG CTG GTG GCG CAC GAG CAG GAG CGG GTT CCG TTT CAT ATC CGT TTA AAT ACC GGG

      2400
Arg Phe Thr Ala Ser Leu Asp Leu Asp Ser Gln Leu Asn Leu Tyr Arg Ile Ile Gln Glu Phe Leu Ser Asn Ala
AGA TTT ACG GCT TCC CTT GAT CTG GAT TCG CAG CTG AAT TTG TAC CGG ATC ATT CAA GAG TTT CTG TCT AAT GCG

Val Lys His Ser Gln Ala Thr Asp Val Leu Ile Met Leu Ile Ser Ile Gln Asn Lys Ile Val Leu His Tyr Glu
GTC AAG CAC TCT CAG GCG ACG GAT GTG CTG ATT ATG CTC ATC AGT ATT CAA AAC AAA ATC GTT CTT CAT TAT GAG
BclI

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Figure 3. (Continued.)

Figure 3. DNA sequence of *comP*. The translated DNA sequence of *comP* is presented, together with its presumed Shine–Dalgarno (SD) (Shine and Dalgarno 1974) and transcriptional termination sequence (ter). The proposed start site for *comA* transcription is also indicated.

at some time after T_{-2} . The data in Figure 1 are consistent with this idea: Disruption of *comP* by insertion of a Cm^R cassette prevented the rise in *comA* expression usually noted after about T_{-2} .

Previous attempts to localize the *comA* start site by primer extension had failed, presumably due to the low abundance of *comA* mRNA (Weinrauch et al. 1989). We therefore repeated these experiments, using T_1 RNA from a strain carrying *comA* in multicopy (Fig. 6B). This attempt was successful, localizing the 5' termini (Fig. 3) of the *comA* transcript within 2 bases of the approximate positions identified by S1 nuclease mapping in our previous publication (Weinrauch et al. 1989).

Complementation of the *comP* defect by *comA* in multicopy

A 3-kb *EcoRV*–*AsuII* fragment carrying *comP* and a 1.4-kb *HindIII* fragment carrying *comA* were each cloned into pBD445 to create pBD457 and pBD458, respectively. The vector plasmid that was derived from pIM13 (Monod et al. 1986; Projan et al. 1987) had a copy number of ~150–200 and conferred resistance to kanamycin. We reported previously that a similar multicopy plasmid carrying *comA* overproduced the ComA protein (Weinrauch et al. 1989). pBD457 carrying multicopy *comP* was introduced by transformation into BD1658 and BD1777 carrying the chromosomal *comP* and *comP comA* defects, respectively. pBD458 plasmid DNA, carrying multicopy *comA* was also transformed into BD1777. Table 1 shows that the strains carrying the single and double defects exhibited 200- and 300-fold deficiencies in transformation. The presence of *comP* in multicopy restored transformation to wild-type levels in the strain carrying the *comP* defect but not in the strain carrying the *comA comP* defect, although a relatively small augmentation of transformability was observed in the latter case. In addition, the *comA* gene in multicopy fully complemented the *comP comA* double mutation.

These results suggested that ComA acts on the competence pathway at a point after ComP. This is certainly consistent with a role for ComP as a sensor kinase, acting on the *comA* product. Other effector genes in

multicopy have been reported to complement sensor defects (Weston and Kadner 1987; Slauch et al. 1988). The ability of the multicopy *comA* strain to become competent in the absence of the *comP* product provides an opportunity to explore further the role of the latter protein. If the *comP* product is a sensor that transmits information to ComA, then the regulation of competence in the complemented strains may differ in some way from that in the wild type. This difference might offer a clue as to the nature of the signals normally detected by ComP. We have reported that the expression of late competence genes is repressed by the addition of glutamine and by the substitution of glycerol for glucose in competence medium (Albano et al. 1987). The data in Figure 7A confirm these results by using *comG12*, a transcriptional Tn917lacZ fusion to *comG*. Figure 7B shows the effects of the multicopy *comA* plasmid. Two changes are apparent: First, the repressing effect of glutamine was reversed; second, the effect of glycerol substitution for glucose was largely reversed by the multicopy *comA* plasmid. These observations will be discussed further below.

Sporulation phenotypes of *comP* and *spoIIJ* mutants

We observed that the *comP* disruption mutant grown on solid medium tryptose blood agar base (TBAB) appeared to be oligosporogenic, judging by colony morphology. In contrast, the *comA* mutant appeared to be Spo^+ . Quantitative spore tests, using heat resistance as a measure of sporulation, has confirmed these impressions, using a set of isogenic strains; the *comP* mutant sporulated 10–20% as well as the wild type in nutrient sporulation medium (NSM), although it seemed to approach the wild-type level of sporulation upon more prolonged incubation (Fig. 8). This behavior was similar to that of the oligosporogenic *spoIIJ* mutant, which was depressed ~25-fold in sporulation frequency in our genetic background (Fig. 8; Perego et al. 1989; Antoniewski et al. 1990). *spoIIJ* encodes a regulatory protein for sporulation that exhibits amino acid sequence similarity to the family of bacterial signal-transduction sensors (P. Stra-

Figure 4. Similarity of ComP to other sensor proteins. Amino acid residues identical to those in ComP are shaded. The positions of residues reported to be universally conserved in this family of proteins are indicated with asterisks (Stock et al. 1989). The asparagine residue at position 358 in FixL was depicted by Stock et al. (1989), as aligned with the conserved asparagine residues 8 positions downstream in the other proteins. The alignment is as plausible because it improves the fit near the conserved histidine residue 4 positions downstream from the conserved asparagine. Similarly, the G residue at position 477 in UhpB was depicted by Stock et al. (1989), as aligned with the conserved G residues 15 positions downstream in the other proteins. The alignments were obtained with FASTP (Lipman and Pearson 1985), followed by several minor manual adjustments.

gier, unpubl.; Perergo et al. 1989; Antoniewski et al. 1990). Interestingly, double mutants carrying both a Tn917 insertion inactivating *spoIIJ* and a *comP* disruption sporulated only ~0.1% as well as the wild-type strain (Fig. 8). Thus, it appears that *comP* is a regulatory gene for sporulation, as well as for competence. A strain carrying a Tn917 disruption of *spoIIJ* is fully competent, and this mutation has no discernible epistatic effect on expression of the late competence genes (data not shown). The *comP spoIIJ* double mutant is no less competent than the *comP* mutant itself (data not shown). Thus, there is no evidence that *spoIIJ* plays a role in regulating competence.

Discussion

We have identified and partially characterized a new regulatory gene for both competence and sporulation that

resembles sensor members of bacterial signal-transduction systems in its carboxy-terminal amino acid sequence. The similarity to this class of proteins is most striking in the cases of *degS* (Henner et al. 1988; Kunst et al. 1988), *narX* (Nohno et al. 1989), and *uhpB* (Friedrich and Kadner 1987). Applying the RDF2 program (Pearson and Lipman 1988) to test the significance of these comparisons yielded the highly significant values of 14.7, 12.6, and 12.5 sd, respectively, for the comparison of the ComP sequence with those of DegS, NarX, and UhpB. In addition, the several amino acid residues identified by Stock et al. (1989), as conserved in all known sensor proteins, appear to be present in ComP. *degS* is a *B. subtilis* regulatory gene required for the full expression of a group of degradative enzymes. *degU*, the cognate effector gene for *degS*, is required for the development of competence (Henner et al. 1988; Tanaka and Kawata 1988; Msadek et al. 1990; J. Hahn and D.

Figure 5. Hydrophobicity profile of ComP. The algorithm of Kyte and Doolittle (1982) was used to generate this profile, with a moving window of 19 residues. The dashed line shows the cutoff of mean hydrophobicity 1.6, suggested by these investigators as a minimum criterion for a membrane-spanning segment. The eight segments predicted on this basis to be membrane-spanning are numbered. The arrows represent the positions of turn sequences predicted by the method of Garnier et al. (1978).

Dubnau, unpubl.). *narX* and *uhpB* are *Escherichia coli* regulatory genes for nitrate utilization and for sugar phosphate transport, respectively.

The hydrophobicity profile (Fig. 5) implies that ComP is organized in two domains: A large relatively hydrophobic amino-terminal moiety with several potential membrane-spanning segments, and a hydrophilic carboxy-terminal domain. These each comprise about half the protein. Similarity with other sensors is confined to the carboxy-terminal portion of ComP. DegS does not appear to possess a hydrophobic amino terminus and is a

smaller protein than ComP. UhpB and NarX each contain hydrophobic amino termini. In the case of NarX, two potential membrane-spanning segments have been identified (Nohno et al. 1989). This appears to be the case as well for the sensor proteins VirA, CpxA, and EnvZ, in which such inferences from amino acid sequences concerning membrane topology have been supported by biochemical and genetic data (Forst et al. 1987; Weber and Silverman 1988; Melchers et al. 1989). In these proteins, the residues between the two membrane-spanning segments are believed to be external to the cell membrane. The UhpB protein has also been found to be membrane-localized (Weston and Kadner 1987), but UhpB (Friedrich and Kadner 1987), like ComP, is somewhat atypical in that more than two potential membrane-spanning segments appear to be present. The membrane topologies of these related proteins are likely to be more complex than those envisaged in the case of sensors with only two hydrophobic segments.

In Figure 9, we propose a working model for the topology of ComP. We have based this model on several arguments. First, the hydrophobicity profile (Fig. 5) has identified eight potential transmembrane segments of appropriate length (~20 residues) to span a typical bilayer. This conclusion was reached using the criterion of Kyte and Doolittle (1982) that the mean hydrophobicity must exceed 1.6 over about a 20-residue stretch. Segments IV and VIII exhibited somewhat marginal hydrophobicities by this criterion, as shown. The eight segments were also identified as membrane-spanning by the statistically based method of Klein et al. (1985) and by the method of Engelman et al. (1986). Second, the procedure of Garnier et al. (1978) predicted locations for turn sequences exclusively outside the eight potential transmembrane segments (Fig. 5). Third, it has been pointed out that individual transmembrane segments tend to be organized with an excess of positive charges on the cytoplasmic side of the cell membrane (von Heijne 1986,1989). As shown in Figure 8, this asymmetry is respected by our model in all cases except for

Figure 6. Transcription mapping of *comP* and *comA*. (A) 3' S1 nuclease mapping of *comP* and *comA*. RNA from the indicated stages was hybridized to 1.8-kb 3'-labeled probe, as described in Results. The positions of the 1.6-kb and 450-bp protected fragments are indicated. (L) Size markers; (-S1 and +S1) probe alone without and with S1 nuclease treatment. The right-most lane contained RNA isolated at T_{-1} from a strain carrying *comA* and *comP* in multicopy. The position of the 1-kb size marker was determined from a longer exposure of the same gel. (B) Primer extension analysis of *comA* transcription. The primer extension products are indicated by arrows. A dideoxy sequencing ladder, obtained with the same primer used to obtain the extension products, is also shown. The four lanes of the sequencing gel were relabeled so that the complementary bases could be read, making it possible to directly infer the sequence of the coding strand. (*) The positions of the two inferred 5' transcriptional termini.

Table 1. Complementation by *comA* and *comP* in multicopy

Chromosomal genotype	Plasmid genotype	Leu ⁺ transformation ^a
<i>com</i> ⁺	—	1
<i>comP</i>	—	4×10^{-3}
<i>comP comA</i>	—	3×10^{-3}
<i>comP</i>	<i>comP</i> ⁺	1.6
<i>comP comA</i>	<i>comP</i> ⁺	1.5×10^{-2}
<i>comP comA</i>	<i>comA</i> ⁺	2

^aExpressed as Leu⁺ transformants per colony-forming unit, normalized to the wild-type (*com*⁺) strain.

segment I. Although the suggested topology is certainly not correct in all details, we favor a model in which several hydrophobic segments serve to anchor ComP in the membrane, with its conserved carboxy-terminal domain located in the cytoplasm.

Various findings suggested strongly that the products of *comP* and *comA* (and possibly *comB*) operate as members of a single regulatory pathway. First, inactivation of any one of these genes, or of several different pairs of them, resulted in a several hundredfold decrease in transformability (Guillen et al. 1989, and Y. Weinrauch and D. Dubnau, unpubl.). Because the residual competence is roughly the same in these various mutants, it appears likely that the three genes do not operate in independent pathways. Second, the ability of overexpressed ComA protein not only to complement *comA* but also *comP* defects strongly implies that the *comA* protein functions at a later point than ComP and

Figure 7. Effects of ComA overexpression on expression of a transcriptional *comG-lacZ* fusion in various media. The effects of glutamine and glycerol substitution for glucose on *comG12-lacZ* expression are shown in the absence (A) and presence (B) of overproduced ComA protein. (○) Competence medium; (□) competence medium plus glutamine; (■) competence medium with glycerol and no glucose added.

in the same pathway. Finally, the similarity of these two proteins to known effector (response regulator) and sensor (signal-transducing) proteins is consistent with the idea that ComP operates prior to ComA. We therefore suggest the following working hypothesis, based on similar suggestions for other two-component signal-transduction systems (for review, see Stock et al. 1989). ComP is an integral membrane protein that senses an extracellular signal, using its external or membrane-spanning moieties. Information is then relayed to the ComP cytosolic carboxy-terminal domain, via a conformational change that alters the activity of ComP-associated kinase. This, in turn, alters the phosphorylation state of ComA, activating this protein. We have argued elsewhere (Weinrauch et al. 1989) that ComA is a transcriptional activator, capable of increasing the transcription rate of one or more competence determinants. The ComA targets are unknown.

Our evidence implies that *comA* expression, itself, may be subject to regulation, at least partly at the level of transcriptional readthrough (Figs. 1 and 6). We have attempted to map the 5' end of the *comP* transcript by S1 nuclease and primer extension experiments (data not shown). Although these experiments were ambiguous with respect to the start site of *comP* transcription, they did suggest that the latter decreased after T_0 . In spite of these potential complexities of unknown significance, transcription of both *comP* and of *comA* occurs during vegetative growth and, in the case of *comA* at least, in

Figure 8. Sporulation of isogenic wild-type (■), *comP* (Δ), *spoIIJ* (●), and double mutant *comP spoIIJ* (□) strains in NSM. The time scale refers to hours after the departure from exponential growth (T_0). The dashed lines present total colony-forming units; the solid lines present heat-resistant colony-forming units.

Figure 9. Model for *com* membrane topology. The proposed transmembrane segments are numbered I–VIII. The net charge in each extra-membrane segment is shown, as are the residue numbers delimiting the membrane-spanning segments.

all media (Weinrauch et al. 1989). This is consistent with a role for these as regulatory genes involved in signal transduction.

The data in Figure 7 provide a hint as to the nature of the extracellular signals detected by ComP. When the *comP* defect was complemented by overproduction of ComA, expression of a late competence gene was no longer inhibited by an excess of glutamine or by substitution of glycerol for glucose. This suggested that ComP may detect the level of nitrogen and carbon sources in the medium. It does not necessarily suggest that the nutritional status of the medium is directly sensed by the ComP protein. For instance, a molecule may be produced in response to changes in nutrient availability and may, in turn, bind to ComP, providing an essential signal to initiate competence. Although the overproduction of ComA bypasses regulation by glutamine and glycerol substitution, it does not completely bypass the nutritional control of competence. For instance, the strain carrying *comA* in multicopy does not express β -galactosidase from the *comG12* fusion in complex media (data not shown).

The involvement of *comP* in sporulation is of particular interest. The much larger effect of the *comP spoIIJ* double mutation on sporulation frequency than that of either mutation by itself suggests that the two sensors may ordinarily be able to substitute for one another. Perhaps both ComP and SpoIIJ are capable of partici-

pating in a sporulation-specific signal-transduction pathway, possibly involving the effector proteins SpoOA or SpoOF or both. The regulatory interrelationships among these (and other) factors promise to be quite complex. For instance, the products of *spoOE*, *spoOB*, and *spoOF* are involved in activating *spoOA* for sporulation (Hoch et al. 1985) but apparently not for competence (Albano et al. 1987). Although ComA is required for competence, probably as a target for activation by ComP, it is not required for sporulation, at least under the conditions tested. Finally, it is worth noting that an additional pair of signal-transduction proteins, *degS* and *degU*, is involved in regulating competence as well as sporulation (Henner et al. 1988; Tanaka and Kawata 1988; Msadek et al. 1990; J. Hahn and D. Dubnau, unpubl.). How are we to understand these complex requirements? The possible responses of *B. subtilis* to the onset of stationary phase are manifold: Sporulation, competence, motility, degradative enzyme production, surfactin synthesis, etc. Precisely which global system(s) will be derepressed in a given instance may depend critically on conditions. The presence of a certain constellation of signals may satisfy the requirements for initiation of competence, another for sporulation, and a third for both degradative enzyme production and sporulation. The several signal-transduction pathways may serve to funnel this information to suitable processing systems, resulting in the activation of appropriate responses.

Methods

Strains and plasmids

All *B. subtilis* strains were isogenic with BD630 (*leu-8 metB5 hisH2*). *E. coli* JM109 was used as a host for recombinant M13 phage. The plasmid pBD439 has been described previously (Weinrauch et al. 1989). It carries the entire *comA* gene, as well as 400 bp of upstream sequences containing the 3' end of the *comP* gene. A chloramphenicol resistance (Cm^R) cassette was isolated on a 1.3-kb *SauII*A fragment from pED1 (Guillen et al. 1989) and inserted in two different orientations into one of the two *Bcl*I sites of pBD439 to inactivate the *comP* ORF. The resultant plasmids pBD441 and pBD442 were then tested by restriction digest analysis to confirm the location of the Cm^R cassette in the proper *Bcl*I site. A third plasmid, pBD444, resulted from the removal of the entire 1-kb *Bcl*I fragment containing the *comA* gene, part of the *comP* ORF, and the 5' end of a reading frame downstream from *comA* and known not to be required for competence (Weinrauch et al. 1989). This 1-kb fragment was replaced by the Cm^R cassette described above. Plasmid pBD445 was constructed using a *Bam*HI–*Taq*I fragment containing the kanamycin resistance (Km^R) determinant from pUB110 ligated to the *Mbo*I–*Cla*I fragment from pIM13 (Monod et al. 1986). It therefore confers only Km^R and replicates using the high-copy-number pIM13 replicon. A 4.5-kb *Eco*RV fragment, isolated from pED3 (Weinrauch et al. 1989) and containing *comP* and *comA*, was inserted into *Hind*III–*Mbo*I-cleaved pBD347 after filling in with the Klenow fragment of DNA polymerase I to give pBD459. pBD347 is a derivative of pIM13 in which the erythromycin resistance (Em^R) determinant has been replaced by a Cm^R gene from pC194 (Monod et al. 1986). The strain, PY390, carrying a Tn917 insertion in the *spoIIJ* gene, was obtained from P. Youngman. Tn917 confers resistance to erythromycin.

Media

Solid media for *B. subtilis* were TBAB (Difco Laboratories) and minimal medium (Anagnostopoulos and Spizizen 1961) with appropriate growth supplements. Chloramphenicol, kanamycin, or erythromycin (5 $\mu\text{g}/\text{ml}$) were added as required. Liquid media used for *B. subtilis* were VY [25 grams of veal infusion (Difco), 5 grams of yeast extract (Difco), 1000 ml of water] for growth of phage and recipient cultures in transduction experiments, and competence medium (Contente and Dubnau 1979b) for growth to competence. When added as supplements, amino acids were used at concentrations of 50 $\mu\text{g}/\text{ml}$. Liquid media used for *E. coli* experiments were L broth or 2 $\times$ yeast tryptone (2 $\times$ YT) (Miller 1972). Solid medium consisted of LB agar, supplemented with ampicillin (100 $\mu\text{g}/\text{ml}$) or X-gal (40 $\mu\text{g}/\text{ml}$), as required. For measurement of sporulation, strains were grown in NSM (Schaeffer et al. 1965).

Transformation and isolation of plasmid DNA

B. subtilis strains were screened for plasmid by the rapid plasmid isolation procedure described by Contente and Dubnau (1979a). Large-scale preparation of *B. subtilis* plasmid DNA was by SDS–NaCl treatment of plasmid-bearing strains, as described by Guerry et al. (1973), followed by CsCl-ethidium bromide purification. Growth of cultures to competence and transformation was performed as described previously, by the one-step procedure (Albano et al. 1987).

Determination of sporulation frequency

Strains were grown for the indicated times in liquid NSM with

shaking at 37°C. The inocula for these cultures were standardized so that equal numbers of exponentially growing cells were inoculated into each culture. The numbers of heat-resistant and total colony-forming units were measured by plating on TBAB agar, with and without incubation of the serial dilutions at 80°C for 30 min before plating.

S1 nuclease protection and primer extension mapping

RNA was prepared from the wild-type *com*⁺ strain (BD630), which was grown in competence medium. Cells were harvested 1 and 2 hr before T_0 (the time of transition from the exponential to the stationary growth phase), and at 1 hr after T_0 . RNA was also prepared at T_{-1} from strain BD1785, which contains the *comA* and *comP* genes on a multicopy plasmid (pBD459). RNA was extracted, as described by Ulmanen et al. (1985). S1 nuclease mapping was carried out, as described previously (Berk and Sharp 1977; Favaloro et al. 1980; Guillen et al. 1989). For primer extension analysis, the primer 5'-CCGTTTCC-AAAATTGTCTTG-3' was used. This primer was complementary to a region of *comA* mRNA 74 bases from the presumed start site of transcription (Weinrauch et al. 1989). The primer was 5' end-labeled with polynucleotide kinase, as described (Weinrauch et al. 1989). A total of 5 ng of labeled primer was mixed with 50 μg of total RNA from BD1785, which carries *comA* and *comP* on a multicopy plasmid. The RNA was dissolved in 16 μl of buffer containing 50 mM Tris (pH 8.0), 30 mM KCl, 8 mM MgCl_2 , 1 mM dithiothreitol, and 1 μl of RNasin (Promega Biotech). The mixtures were heated at 80°C for 10 min and cooled slowly to 42°C. A total of 4 μl of a mixture of four deoxynucleotide triphosphates (each at 10 mM) and 1 μl of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL) were added to the annealed samples, which were then incubated at 42°C for 30 min. Analysis of the extended products was carried out by electrophoresis in 6% polyacrylamide/urea gels.

DNA sequencing

Sequencing of DNA was by the dideoxynucleotide chain-termination method (Sanger et al. 1977) with [α -³⁵S]dATP (sp. act. 500 Ci/mmol; Dupont, NEN Research Products, Boston, MA). Sequencing reactions were performed with modified T7 DNA polymerase kits (Sequenase; U.S. Biochemical Corp.). A 3-kb *Eco*RV–*Asu*II fragment isolated from pED3 was cloned into M13mp18 in both orientations. Two *Hind*III fragments of 1.4 and 0.5 kb from within the 3-kb *Eco*RV–*Asu*II region were also subcloned into M13mp18. The nucleotide sequence was determined completely on both strands and across restriction sites used for cloning, by using the M13 universal primer and 17-mer primers made from known sequences. The sequence of *comP* was submitted to The Genetic Sequence Data Bank (GenBank) with the accession number M29851.

β -Galactosidase determination

Samples were taken at the indicated times during growth in competence medium, and β -galactosidase activity was determined as described previously (Gryczan et al. 1984). β -Galactosidase activities are expressed as units per milligram of protein.

Computer analysis

Protein sequences were compared to a translated version of the GenBank data base by using the TFasta and FASTP programs (Lipman and Pearson 1985; Pearson and Lipman 1988). The

RDF2 randomization program was used to test the significance of sequence comparisons, with 100 uniform shuffles (Pearson and Lipman 1988). The hydrophobicity profile of the ComP protein was determined by using the scales of Kyte and Doolittle (1982) and Engelman et al. (1986), with a moving window of 19 residues. The profile determined with the Kyte–Doolittle scale was further analyzed by the procedure of Klein et al. (1985) by using a window of 17 residues and the linear inequality given in their paper. The amino acid sequence of the predicted ComP protein was analyzed for turns by using the alpha program in the MBIR package of sequence analysis programs (Department of Cell Biology, Baylor College of Medicine, Houston, TX). This program is based on the method of Garnier et al. (1978).

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