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A New *Bacillus subtilis* Gene, *med*, Encodes a Positive Regulator of *comK*

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***Bacillus subtilis degR*, a positive regulator of the production of degradative enzymes, is negatively regulated by the competence transcription factor ComK which is overproduced in *mecA* null mutants. We used transposon Tn*10* to search for a mutation that reduced the repression level of *degR* caused by a *mecA* mutation. A new gene exerting positive regulation on *comK* was obtained and designated *med* (suppressor of *mecA* effect on *degR*). Sequence determination, Northern analysis, and primer extension analyses revealed that the *med* gene contained an open reading frame (ORF) composed of 317 codons and was transcribed into an approximately 1,250-nucleotide mRNA together with its short downstream gene. The expression of *comK* is positively regulated by factors such as ComK itself, ComS (SrfA)-MecA, DegU, SinR, and AbrB. Quantitative analyses using *comK′-′lacZ*, *srfA-lacZ*, *degU′-′lacZ*, and *sinR′-′lacZ* fusions showed that disruption of *med* caused a significant decrease in *comK* expression in both *mecA*$^+$ and *mecA* strains, while expression of *srfA*, *sinR*, and *degU* was not affected by the mutation. An epistatic analysis revealed that overproduction of ComK resulted in alteration of *med* expression, suggesting a regulatory loop between *comK* and *med*. Several possible mechanisms for positive regulation of *comK* by Med are discussed.**

Bacillus subtilis is a gram-positive bacterium living in soil, where cells likely encounter severe fluctuations in their environment. Since a deficiency of nutrients, desiccation, and many other stresses are unavoidable in nature, a number of adaptive response systems have evolved to respond to such stresses; in *B. subtilis* these responses include sporulation, chemotaxis, competence development, and degradative enzyme synthesis. Very often, pathways leading to individual adaptive responses cross each other at molecules that transmit environmental or internal signals to downstream pathways (9).

Competence development is an exquisite adaptive process in which a subpopulation of cells differentiate into those that have the ability to take up external DNA. This process is initiated in response to information about cell density, growth phase, and medium components (6). Cells normally become competent in early stationary phase in minimal media supplemented with glucose and several essential amino acids (6), and extracellular signaling molecules are involved in the initiation of development of competence (22, 42, 43). These molecules, ComX and competence-stimulating factor, are secreted in response to high cell density and then stimulate initiation of competence development via ComA in ComP-dependent and -independent manners, respectively. ComP and ComA constitute a two-component system, the former being a membrane-embedded histidine protein kinase and the latter a response regulator (22, 42, 43, 54). ComP phosphorylates its cognate regulator ComA in response to an external signal, ComX (22, 54). Competence-stimulating factor may also be involved in regulation of phosphorylation of ComA (42, 43). The phosphorylated ComA protein activates transcription of the *srfA* operon (30, 38), which encodes the proteins necessary for the synthesis of a secondary metabolite, surfactin (2). Although the enzymes for surfactin synthesis are not required for competence development (52), activation of the *srfA* operon by phosphorylated ComA results in the expression of *comS*, a gene embedded in the *srfA* operon (4, 5, 15). The small ComS peptide activates the competence transcription factor ComK by antagonizing the effects of MecA and ClpC (MecB), which inactivate ComK through protein-protein interactions (5, 10, 18, 19, 25, 46). Another positive regulator of competence development, SinR, may be required for optimal *comS* expression (10, 21). In addition, the Spo0A-AbrB system acts on *comK* regulation independently of the route involving MecA and ClpC, and CodY is also required for nutritional repression of *comK* (11, 12, 41, 48). The importance of all of this regulation of *comK* is that ComK is the direct positive regulator of transcription of the late competence genes that encode the apparatus for processing and incorporating exogenous DNA into the cell (12, 48, 50, 51). ComK also activates genes such as *sacB*, *nucA*, and *recA* which are not related to competence development (14, 25, 49).

Degradative enzyme synthesis in *B. subtilis* is also a component of the adaptive response, as a two-component system, DegS-DegU, plays an important role in the production of extracellular proteases (20, 26). DegS, a sensor kinase, is thought to respond to environmental or internal stimuli, autophosphorylate, and then transfer the phosphate to DegU (3, 27). Phosphorylated DegU stimulates the production of extracellular proteases and levansucrase, while unphosphorylated DegU is required for transcription of *comK* and thus participates in competence development (3, 12, 48). The production of the exoproteases is also subject to additional positive and negative regulation by several factors (20, 26). One such factor is *degR*, which in multicopy causes overproduction of extracellular proteases and in single copy contributes to positive regulation of *aprE* (subtilisin) expression (28, 29, 33, 55). DegR is a 60-amino-acid polypeptide with homology to the N-terminal region of DegS (36), and it has been shown that multiple copies of *degR* stabilize the phosphorylated form of DegU (28). Transcription of *degR* is dependent on σ^D, a sigma factor involved in chemotaxis, suggesting interaction between the

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TABLE 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
CU741	*trpC2 leuC7*	53
ODM40	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)]	35
QB4650	*trpC2 mecA*::Km^r	F. Kunst
CU741ma	*trpC2 leuC7 mecA*::Sp^r	32
ODM40mak	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] *mecA*::Km^r	QB4650→ODM40
ODM401	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] *mecA*::Km^r pIC333	pIC333, ODM40mak→ODM40
MM41	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] *mecA*::Km^r *med*::Tn*10* (Sp^r)	This work
ODM41	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] *med*::Tn*10* (Sp^r)	MM41→ODM40
OMM42	*trpC2 leuC7 med*::Tn*10* (Sp^r)	MM41→CU741
OMM43	*trpC2 leuC7 med*::pPHLMED	pPHLMED→CU741
ODM42	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] *med*::pPHLMED	pPHLMED→ODM40
ODM43	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] ORFE::Tc^r	pORFE→ODM40
ODM44	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] ORFE::Tc^r *mecA*::Sp^r	CU741ma→ODM43
ODM45	*trpC2 leuC7 amyE*::[*degR3'-'lacZ* (Cm^r)] ORFC::pORFC	pORFC→ODM40
OMM46	*trpC2 leuC7 med3-lacZ* (Em^r) *med mecA*::Sp^r	pMED301, CU741ma→CU741
LAB358	*pheA1 sfp* SPβ *c2del2*::Tn*917*::pXL5 (Cm^r)	31
OSM100	*trpC2 leuC7 sfp* SPβ *c2del2*::Tn*917*::pXL5 (Cm^r)	LAB358→CU741
OSM101	*trpC2 leuC7 sfp* SPβ *c2del2*::Tn*917*::pXL5 (Cm^r) *med*::pPHLMED	OMM43→OSM100
TT714	*trpC2 leuC7 degU'-'lacZ* (Cm^r)	27
OUM101	*trpC2 leuC7 degU'-'lacZ* (Cm^r) *med*::pPHLMED	OMM43→TT714
IS458	*metB5 hisA1 leuA8 sinR'-'lacZ* (Cm^r)	7
ORM100	*trpC2 leuC sinR'-'lacZ* (Cm^r)	IS458→CU741
ORM101	*trpC2 leuC sinR'-'lacZ* (Cm^r) *med*::pPHLMED	OMM43→ORM100
OCM100	*trpC2 leuC7 comK'-'lacZ* (Km^r)	34
OCM101	*trpC2 leuC7 comK'-'lacZ* (Km^r) *med*::pPHLMED	pPHLMED→OCM100
OCM102	*trpC2 leuC7 comK'-'lacZ* (Km^r) *mecA*::Sp^r	34
OCM103	*trpC2 leuC7 comK'-'lacZ* (Km^r) *med*::pPHLMED *mecA*::Sp^r	CU741ma→OCM101
CU741c	*trpC2 leuC7 comK*::Km^r	32
OMM100	*trpC2 leuC7 med5-lacZ* (Em^r)	pMED501→CU741
OMM101	*trpC2 leuC7 med5-lacZ* (Em^r) *comK*::Km^r	CU741c→OMM100
OMM102	*trpC2 leuC7 med5-lacZ* (Em^r) *mecA*::Sp^r	CU741ma→OMM100
OMM103	*trpC2 leuC7 med5-lacZ* (Em^r) *mecA*::Sp^r *comK*::Km^r	OMM101, OMM102→OMM100
BD1512	*hisA1 leu9 metB5 comG'-'lacZ* (Cm^r)	1
OGM100	*trpC2 leuC7 comG'-'lacZ* (Cm^r)	BD1512→CU741
OGM101	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *med*::pPHLMED	pPHLMED→OGM100
OGM102	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *mecA*::Sp^r	CU741ma→OGM100
OGM103	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *mecA*::Sp^r *med*::pPHLMED	CU741ma→OGM101
OGM104	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *comK*::Km^r	CU741c→OGM100
OGM105	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *mecA*::Sp^r *comK*::Km^r	CU741ma, CU741c→OGM100
OGM106	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *med*::pPHLMED *comK*::Km^r	CU741c→OGM101
OGM107	*trpC2 leuC7 comG'-'lacZ* (Cm^r) *med*::pPHLMED *mecA*::Sp^r *comK*::Km^r	CU741ma, CU741c→OGM101
Plasmids		
pIC333	Em^r mini-Tn*10* (Sp^r)	44
pPHL	pUC18 carrying Pm^r	This work
pPHLMED	pPHL carrying a part of *med*	This work
pHV501	Em^r	47
pMED301	pHV501 carrying a part of *med*	This work
pMED501	pHV501 carrying a C-terminal part of *med*	This work
pORFE	pDH88 carrying an ORF E region inserted by Tc^r	This work
pORFC	pPHL carrying a part of ORF C	This work

pathways controlling induction of chemotaxis genes and regulation of exoprotease production (35).

Recently we have found that the transcription of *degR* is negatively controlled by ComK overproduced as a result of disruption of the *mecA* gene (32). This observation provided an opportunity to study the regulation of *degR* in conjunction with *comK*; therefore, we sought factors participating in repression of *degR* by ComK. We identified a gene, designated *med* (suppressor of *mecA* effect on *degR*), which encodes a positive regulator of *comK*. Here we describe the isolation and characterization of the *med* gene.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Toyobo Co. (Tokyo, Japan). The long and accurate PCR amplification kit, DNA blunting kit, DNA ligation kit, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were bought from Takara Shuzo Co. (Shiga, Japan). The synthetic oligonucleotides were commercially prepared by Sawaday Technology Co. (Tokyo, Japan). For PCR, we used a model PJ2000 apparatus (Perkin-Elmer Cetus). Phleomycin and spectinomycin were purchased from Sigma Co. Kanamycin was from Meiji Seika Co. (Tokyo, Japan), and chloramphenicol, tetracycline, and erythromycin were from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Strains and plasmids. Bacterial strains and plasmids are listed in Table 1. Strains ODM401, OMM103, OGM105, and OGM107 were constructed by co-

TABLE 2. Oligonucleotides used for this study

Name	Sequence	Location
Med-Bg-1	5′-GTGGATGTGGACAAACTCCG-3′	2971–2990
Med-Bg-2	5′-AAATGTCATGTCGACCGGTG-3′	2120–2101
ORFE-X	5′-CATTCTAGACGGGCCATGGAAAGAAGAAACAG-3′	2626–2649
ORFE-B	5′-TATGGATCCGTTAAACCTTCTCAGGAATGTAA-3′	4569–4547
Med-1	5′-GAAGCTTCATAAGAGGGGCGTC-3′	3166–3182
Med-2	5′-GAGATCTTGGCCCCTTTGATAA-3′	3437–3421
Med-3	5′-biotin-CAGGAAAGAGCATGCCGACCTTCTC-3′	3030–3006
ORFC-H	5′-GTCAAGCTTCGGCCTTTCATTATG-3′	2313–2329
ORFC-P	5′-ATCCTGCAGCCGTCTTCCTCTGTTT-3′	2661–2644
Med-501-H	5′-GTAAAGCTTTATCAGAAAATGAA-3′	3504–3526
Med-501-Bg	5′-TGCAGATCTTACTCGTTTTTTGGCAGCT-3′	3878–3859

transformation of the host strains with DNAs shown in Table 1, since the cells carrying the temperature-sensitive replicon (pIC333) or the *mecA* and *comK* mutants are not suitable hosts for transformation (13, 44, 50). For the study of *degR* expression, we used strain ODM40 and its derivatives that carry a *degR* control region shorter than that in the original strain ODM50 (35), since it directs a higher expression level of *degR′-′lacZ*, enabling us to identify white and blue colony phenotypes easily.

Plasmid pPHLMED was constructed in the following two steps. A *Pvu*II-*Sma*I fragment of pIC22 (45) containing the phleomycin resistance (Pm^r) gene was inserted into the *Sca*I site of pUC19 (56), generating insertion vector pPHL. A 326-bp fragment internal to open reading frame (ORF) D was amplified by PCR with primers Med-1 and Med-2 (Table 2), digested with *Hin*dIII and *Bgl*II, blunt ended with T4 DNA polymerase, and then cloned into the *Sma*I site of pPHL, resulting in pPHLMED. Plasmid pORFC was made by insertion between the *Hin*dIII and *Pst*I sites in pPHL of a 349-bp PCR fragment amplified by using ORFC-H and ORFC-P (Table 2) as primers. pMED301 was generated by cloning into *Hin*dIII- and *Bam*HI-treated pHV501 (47) a PCR-amplified fragment obtained by using Med-1 and Med-2 (Table 2) as primers and treated with *Hin*dIII and *Bgl*II. pMED501 was made as follows. A 375-bp fragment containing the stop codon of *med* was PCR amplified by using Med-501-H and Med-501-Bg (Table 2) as primers. The resultant DNA fragment was digested with *Hin*dIII and *Bgl*II and cloned between *Hin*dIII and *Bam*HI sites of pHV501. Plasmids pPHLMED, pMED301, pMED501, and pORFC were introduced into the chromosome of the CU741 derivatives by Campbell-type recombination. For construction of pORFE, a 1,944-bp fragment spanning all of ORF D (*med*) and ORF E was PCR amplified by using ORFE-X and ORFE-B (Table 2) as primers, digested with *Xba*I and *Bam*HI, and cloned into the *Xba*I and *Bgl*II sites of pDH88 (16). The tetracycline resistance (Tc^r) gene cassette carrying the *Sac*I sites on both ends was prepared from pBEST304 (17) and inserted into the *Sac*I site of the resultant plasmid. This plasmid, pORFE, was linearized by digestion with *Bam*HI and used for disruption of ORF E by a double-crossover event.

Media and antibiotics. The media used were Luria-Bertani broth (LB), LB agar, antibiotic medium 3 (Difco Laboratories), modified competence (MC) medium (20), and Schaeffer's sporulation medium (40). 2 × SG medium was described previously (8). Concentrations of the antibiotics added to the media were 100 μg/ml for spectinomycin unless otherwise specified, 15 μg/ml for tetracycline, 10 μg/ml for kanamycin, 5 μg/ml for chloramphenicol and phleomycin and 0.5 μg/ml for erythromycin.

Electroporation. Electroporation of plasmids into *Escherichia coli* cells was carried out with a Gene Pulser (Bio-Rad). Preparation of competent cells and electroporation were done according to the manufacturer's recommendation.

Transposon insertion mutagenesis. The mini-Tn*10* delivery vector pIC333 (44) was introduced into strain ODM40 by cotransformation with DNA containing *mecA*::Km^r (kanamycin resistance). Ten test tubes containing 1 ml of LB medium were inoculated with 10 different erythromycin-resistant (Em^r) colonies and incubated overnight at 28°C. Cells were diluted 100-fold in LB medium, grown for 4 h at 28°C, and then shifted to 37°C. After incubation for further 4 h, the cells were plated on LB agar plates containing spectinomycin (50 μg/ml), kanamycin, chloramphenicol, and X-Gal (100 μg/ml). DNA regions flanking the transposon insertion sites were retrieved as follows: total DNA was isolated from a blue colony, and the resultant DNA was digested with either *Eco*RI, *Hin*dIII, or *Sac*I, ligated, and transformed into *E. coli* JM103 by electroporation. The plasmids carried in the transformants were designated pMED-Tn-E, pMED-Tn-H, and pMED-Tn-S, respectively.

Sequence determination. The chromosomal DNA sequences cloned in pMED-Tn-E, pMED-Tn-H, and pMED-Tn-S were first determined. The DNA regions beyond those in the plasmids were obtained by the following procedures and sequenced. The chromosomal DNA of CU741 was completely digested with *Bgl*II and self-ligated and then subjected to PCR with oligonucleotides Med-Bg-1 and Med-Bg-2 (Table 2) as primers. An approximately 5,500-bp DNA fragment thus obtained was used as the template for sequencing reactions. All sequences were determined on both strands by using a model 377 DNA sequencer (Perkin-Elmer) and a dye terminator cycle sequencing kit.

Northern analysis. RNA was isolated as previously described (35). DNA labeling by PCR was done by using a PCR DIG (digoxigenin) Probe Synthesis kit (Boehringer Mannheim) and oligonucleotides Med-1 and Med-2 (Table 2) as primers. Electrophoresis of RNA was performed in the presence of 5% formamide, and Northern blotting to a nylon membrane (Boehringer Mannheim) was carried out as instructed by the manufacturer. Hybridized bands were detected with a DIG Luminescent Detection kit (Boehringer Mannheim), and the sizes of RNAs were determined by comparison with RNA size markers purchased from Promega.

Primer extension analysis. The procedure for primer extension analysis of *degR* and the primer used were described previously (35).

Determination of sporulation frequency. Cells were grown in 2 × SG medium at 37°C for 24 h. Serial dilutions of the cells were plated on LB agar plates before and after heating at 80°C for 10 min.

β-Galactosidase assay. Cells were grown in Schaeffer's sporulation medium as described in the figure legends and processed as described previously (36). β-Galactosidase activity is expressed in Miller units (23).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession no. D86376.

RESULTS

Restoration of *degR* expression repressed in a *mecA* mutant by insertion of Tn*10* transposon. We have shown previously that a null mutation of *mecA* markedly reduces β-galactosidase activity driven by *degR′-′lacZ* and that this reduction is due to repression of *degR* by the competence transcription factor ComK overproduced by the *mecA* mutation (32). The expression of *comK* is subject to regulation by diverse regulatory factors including ComS, Spo0A, DegU, SinR, AbrB, and ComK itself (10, 12, 21, 48). We therefore expected that we could isolate in this experimental system a novel mutant(s) in which the expression of *comK* was reduced. To isolate such mutants, we performed mutagenesis on strain ODM401 (*degR′-′lacZ mecA*::Km^r) by insertion of the mini-Tn*10* transposon which is delivered from the pIC333 vector (Sp^r [spectinomycin resistant] Em^r). Since replication of pIC333 is temperature sensitive at 37°C, it is possible that we obtain cells carrying the Tn*10* transposon in the chromosome by a temperature shift up from 28 to 37°C, followed by selection for Sp^r Em^s colonies (see Materials and Methods).

ODM401 cells form white colonies on LB agar containing X-Gal, since the expression of the *degR′-′lacZ* fusion is prevented by the *mecA* mutation. Scores of pale blue colonies were obtained by the transposon mutagenesis method, and one of them, designated MM41, was subjected to further study. After the MM41 cells were checked for the reproducibility of blue colony formation on an X-Gal plate, the total DNA was isolated, and the DNA region flanking the Tn*10* insertion site was retrieved in three Sp^r plasmids, pMED-Tn-E, pMED-

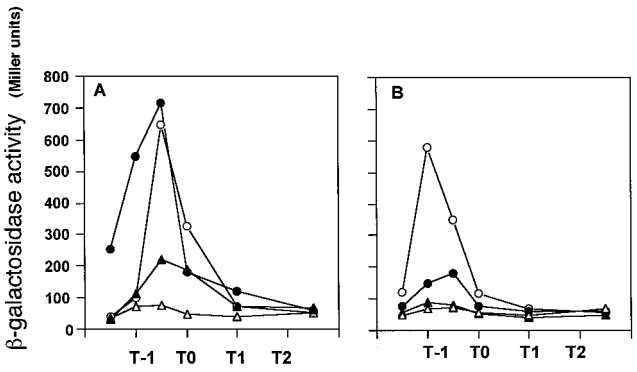

FIG. 1. Partial restoration by *med* mutation of *degR'-'lacZ* expression repressed by *mecA* deficiency. Cells were grown in Schaeffer's sporulation medium. β-Galactosidase activities were determined as described in Materials and Methods and are shown in Miller units. Numbers on the *x* axis represent the growth time in hours relative to the end of the vegetative growth (T0). (A) ○, ODM40 (*mec*$^+$); △, ODM40mak (*mecA*); ▲, MM41 (*mecA med*); ●, ODM41 (*mec*$^+$*med*). (B) ○, ODM40 (*mec*$^+$); △, ODM40mak (*mecA*); ●, ODM42 (*mecA med*:: pPHLMED); ▲, ODM44 (*mecA* ORF E::Tc^r).

Tn-H, and pMED-Tn-S (see Materials and Methods). Sequence determination revealed that the insertion had generated a 9-bp duplication of the sequence 5′-GGCCAAGTA-3′. That the Tn*10* insertion event had caused no detectable DNA rearrangement in the flanking regions of the insertion site was confirmed by Southern blot analyses using a probe around the transposon insertion site (data not shown). To examine whether the observed phenotype is linked to the Tn*10* insertion, we transformed ODM40 with DNA from MM41 by selecting for Sp^r (Tn*10* insertion) and Km^r (*mecA* mutation). All of the colonies obtained were found to be blue (data not shown), showing that the Tn*10* insertion is responsible for the phenotype. We designated the locus of the Tn*10* insertion *med* (suppressor of the *mecA* effect on *degR*).

To determine the extent to which the *med* mutation allows the expression of *degR* in the *mecA* background, we measured β-galactosidase activities derived from *degR'-'lacZ* in strains carrying the *med* mutation. While the expression of *degR'-'lacZ* was much lower in strain ODM40mak (*mecA med*$^+$) than in strain ODM40 (*mecA*$^+$ *med*$^+$), it was restored to 35% of the control level in strain MM41 (*mecA med*) (Fig. 1A). The levels and profiles of β-galactosidase synthesis were similar in strains ODM40 (*med*$^+$ *mecA*$^+$) and ODM41 (*med mecA*$^+$), indicating that the *med* mutation alone does not affect the expression of *degR'-'lacZ*. These results show that the *med* mutation antagonizes the negative effect of the *mecA* mutation on *degR* expression.

Nucleotide sequence of the *med* region. We sequenced the 2.4-kb chromosomal DNA region flanking the mini-Tn*10* insertion site cloned in the three plasmids pMED-Tn-E, pMED-Tn-H, and pMED-Tn-S, as well as the DNA regions further upstream and downstream of the 2.4-kb region (see Materials and Methods). The results of the sequence determination are shown in Fig. 2. A computer analysis revealed that the sequenced region contained a C-terminal region of *argF* and six complete ORFs preceded by putative Shine-Dalgarno (SD) sequences (Fig. 2). The *argF* gene is the last member of the *argCJBDF* operon, which is located at 102° on the *B. subtilis* genetic map (24). Three inverted repeat structures followed by T stretches were found in the sequenced region, two between *argF* and ORF A and one between ORFs E and F. The transposon was found to be inserted at codon 172 of 317-codon ORF D. To test whether disruption of ORF D by a sequence other than Tn*10* causes the same phenotype, we inserted pPHLMED (see Materials and Methods) at codon 172 of ORF D and found that insertion of the plasmid had a similar effect on the expression of *degR'-'lacZ* in the *mecA* background (Fig. 1B).

The proteins predicted from all six ORFs had no significant amino acid sequence similarity to known proteins in the PIR or SwissProt data bank.

Northern and primer extension analyses. There are spacings of 84, 91, and 14 bp between ORFs B and C, ORFs C and D, and ORFs D and E, respectively, but there is no apparent rho-independent terminator in the spacings (Fig. 2). To analyze transcription around these regions, we first performed a Northern analysis using total RNA isolated from the CU741 cells and a PCR-amplified DNA fragment within ORF D as a probe (see Materials and Methods). As shown in Fig. 3, an RNA band with a size of approximately 1,250 bases was detected. Since the observed size coincides with the sum of the sizes of ORFs D and E (1,156 bases) and a rho-independent terminator-like structure is present directly downstream from the stop codon of ORF E, it is likely that the RNA band corresponds to the transcript containing the ORF D and E regions. A more slowly migrating faint band (approximately 2,800 bases long) was barely detectable (dotted arrow in Fig. 3). This may be a transcript initiated from a possible upstream promoter of ORF B. Contribution of this mRNA to the expression of *med* would be minor. The diffuse band of less than 1,250 bases could represent degradation products.

If the foregoing prediction is true, a transcription start site should be present in the region between ORFs C and D. To test this, we performed a primer extension analysis. Total RNAs were isolated from the CU741 cells grown for various lengths of time in sporulation medium and used as templates. As shown in Fig. 4, two transcription start points were detected for RNA isolated from the cells in the logarithmic growth phase, while only the upstream start point was detected for RNA from the stationary-phase cells. The upstream and downstream start sites were preceded by putative promoter sequences 5′-AAGACA-16 bp-GACAAA-3′ and 5′-TTTTCA-16 bp-ATTAAG-3′, respectively. The putative −35 and −10 sequences contained four and three matches, respectively, out of six nucleotides compared to those of the consensus sequence of the σ^A-type promoter (Fig. 4).

The results described above are consistent with the notion that the expression of ORFs D and E depends mainly on transcription started between ORFs C and D. This predicts that insertion of an *E. coli* plasmid in ORF C does not affect the transcription level of ORF D. Plasmid pORFC was inserted by Campbell-type recombination in ORF C, and RNAs were isolated from the resultant strain ODM45 and its parent ODM40. Figure 4 shows that plasmid insertion in ORF C did not affect the intensity of the bands (lanes 7 and 8). From these results, we conclude that ORFs D and E constitute an operon.

Suppression of *mecA* effect on *degR* is not due to polar effect of Tn*10* insertion. Since ORF E is located downstream of ORF D and the two ORFs constitute an operon (see above), it is possible that the observed restoration of *degR* expression is due to a polar effect on ORF E but not the Tn*10* insertion in ORF D. To address this issue, we examined the expression of *degR'-'lacZ* in strain ODM46 in which ORF D is disrupted at codon 172 by insertion of pMED301 (see Materials and Methods) and the expression of ORF E is ensured by an IPTG-inducible promoter, P_{spac} (Fig. 5B). As shown in Fig. 5A, a *degR* transcript was detected in total RNA isolated from the *mec*$^+$ control strain CU741 (lane 1) but not in RNA isolated from the *mecA* strain CU741ma (lane 2). These results corroborate the

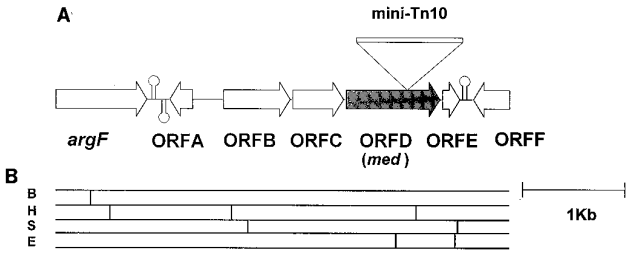

FIG. 2. Schematic representation of the ORFs (A), restriction map (B), and entire 4,608-bp sequence (C). Open arrows depict ORFs deduced from the nucleotide sequence, and the shaded arrow indicates the *med* gene. The Tn*10* target site is shown by the boxed sequence in panel C. Arrows under the nucleotide sequence show inverted repeats. Amino acid sequences below and above the nucleotide sequence indicate those of the ORFs shown in panel A. Underlined sequences show the putative SD sequences. Asterisks indicate stop codons. Abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I.

C

```
AGATCTGCAGCTGGGGCGCGGTGAGACTGTCGCTGATACGGCAAAAGTGCTGTCAGGCTATGTGGATGCCATCATGATCCGGACCTTTGAACATGAAAAGGTGGAGGAACTTGCCAAAGA  120
 D  L  Q  L  G  R  G  E  T  V  A  D  T  A  K  V  L  S  G  Y  V  D  A  I  M  I  R  T  F  E  H  E  K  V  E  E  L  A  K  E
AGCTGACATTCCGGTGATCAATGGACTTACCGATAAATACCACCCATGCCAGGCGCTGGCGGATCTCTTGACGATTAAGGAGATAAAAGGGAAGCTTAAAGGCGTAAAAGTCGCGTACAT  240
 A  D  I  P  V  I  N  G  L  T  D  K  Y  H  P  C  Q  A  L  A  D  L  L  T  I  K  E  I  K  G  K  L  K  G  V  K  V  A  Y  I
CGGTGACGGAAATAACGTGGCGCACTCCTTGATGATCGGCTGCGCGAAAATGGGCTGTGATATCTCGATCGCTTCACCAAAGGGATATGAAGTGTTAGATGAAGCAGCTGAAGCGGCAAA  360
 G  D  G  N  N  V  A  H  S  L  M  I  G  C  A  K  M  G  C  D  I  S  I  A  S  P  K  G  Y  E  V  L  D  E  A  A  E  A  A  K
GACATATGCACTTCAATCCGGCTCTTCTGTCACGCTGACAGATGATCCGATTGAAGCTGTAAAAGACGCGGACGTGATTTATTCTGATGTGTTTACAAGCATGGGGCAGGAAGCGGAAGA  480
 T  Y  A  L  Q  S  G  S  S  V  T  L  T  D  D  P  I  E  A  V  K  D  A  D  V  I  Y  S  D  V  F  T  S  M  G  Q  E  A  E  E
GCAAGAACGTCTTGCTGTCTTTGCGCCTTATCAGGTGAATGCGGCGCTGGTCAGCCATGCCAAGCCTGACTATACATTTTACATTGTCTCCCTGCGCATCGCGAGGAAGAAGTGACTGC  600
 Q  E  R  L  A  V  F  A  P  Y  Q  V  N  A  A  L  V  S  H  A  K  P  D  Y  T  F  L  H  C  L  P  A  H  R  E  E  E  V  T  A
GGAGATTATTGACGGGCCGAACTCTGCAGTGTTCCAGCAGGCGGAAAACCGTCTTCATGTGCAAAAAGCACTGCTGAAGGCCATTTTATATAAAGGGGAATCATCAAAAAACTGCTGAGC  720
 E  I  I  D  G  P  N  S  A  V  F  Q  Q  A  E  N  R  L  H  V  Q  K  A  L  L  K  A  I  L  Y  K  G  E  S  S  K  N  C  *
CAAACTCAGCAGTTTTTTGGATGGCAACATATTCACAGCCGCTTAGCGCATACTACAGCAAAAAGGAAAAGGAGGAAAAACAGTGGGACAGCAGCACCATTCCGGCCCGGACAGAAATCG  840
CCTAATAACGGTGTCTACGTAGAAATCGGCGAGACAGGGAGCATGGTTAAAAACCCTCAAAAGGTCCATCTGTCAGCAGGGAAATGTTCCCCGAAACATCAAACCACAACCGGCTGTGG  960
                                                                                                 *  H  Q  S  A  N  K  N  M  P  M  I  E  A  L  V  M  I
ACCTACAAAAGAAAGCCGTGACCACTAAAAAACCCAAAACAATAAATGTTTTGGGTTTTTGGCTTTTTAATGCTGGCTTGCATTTTTGTTCATAGGCATGATTTCTGCTAAAACCATGAT  1080
 L  V  Y  L  V  V  A  I  V  S  S  T  K  F  N  Y  T  V  S  N  M  S  A  V  L  Y  C  A  M  H  S  L  L  F  T  W  I  F  A  I
TAAGACATACAGTACAACAGCAATGACAGATGATGTTTTGAAATTATACGTAACGCTGTTCATGCTTGCAACCAAGTAGCATGCCATATGTGATAAAAGGAACGTCCAAATAAAGGCAAT  1200
 I  Y  R  M ← ORFA
AATATATCGCACGTTTCCACCTCGTCCTTCTCATTCTATTCTAAAAGTTATCATACCATACAAAAAGCGAAGGGGGAATCCAATTTTAGCAGAAAGTGAGGTGACAAAAGAGTCTGATTC  1320
GTCACACTTTAATGGTAAAATATAAAACGTCAATCAGTATAATAGCCAAAAATGTAAAAGGCTAGGACTTTACCTTATTCGTTTCTTTTCTATTTACATAAGATAGCGTGAGATGAAGTG  1440
TGAAAGGAGGCGGGCAAGCGATGGGAATAACGGAAAGATACTTTCAACTGGGAACGGAGCATAATGTCATTCATCTGCCCTATCGCCCGAACGGCTTCGGCATCTTTATCTTGGGGGACA  1560
            ORFB →M  G  I  T  E  R  Y  F  Q  L  G  T  E  H  N  V  I  H  L  P  Y  R  P  N  G  F  G  I  F  I  L  G  G  D  R
GAACCCACTTTGTCGGACATGATTCAAGCTTTTGGCAGCAGCATTATGGCAGAAATCAGCTTCTGAGCATGTTGATGAATGAAGGATACACTCTTTTTAACAGCAATCTGTTCGGAAGGC  1680
 T  H  F  V  G  H  D  S  S  F  W  Q  Q  H  Y  G  R  N  Q  L  L  S  M  L  M  N  E  G  Y  T  L  F  N  S  N  L  F  G  R  H
ATTGGGGGGCGGAAAAAGCAGTCACCTATGCCAAACAGCTGATACATTCTGTGCTGAAGCAGGAAATCCTCAATCCGAAGATTCATATTTTAGCAGAAGGCATGGGTGCTCTCGTGGCCG  1800
 W  G  A  E  K  A  V  T  Y  A  K  Q  L  I  H  S  V  L  K  Q  E  I  L  N  P  K  I  H  I  L  A  E  G  M  G  A  L  V  A  D
ATGAACTGCTTCGTTTTTCCCCGGATCATATCCGCTCAGCCGCAATGTTGAATCCTTGTCTCGATCTTCAGGCACATTATGAATCGGAAAAAGAAAATAAATTTTTCTATAAGCAATTTT  1920
 E  L  R  F  S  P  D  H  I  R  S  A  A  M  L  N  P  C  L  D  L  Q  A  H  Y  E  S  E  K  E  N  K  F  F  Y  K  Q  F  L
TGAAAGAAGTATCGAAAAGCTACGGAATTTCTGAAAAAGAAGCGGAAGCGATTCGTTATAAAACAATTCAAAGCTACCCGAGCGGCGTACCGGTTCATATATGGCAGCGTATGACCGGAG  2040
 K  E  V  S  K  S  Y  G  I  S  E  K  E  A  E  A  I  R  Y  K  T  I  Q  S  Y  P  S  G  V  P  V  H  I  W  Q  R  M  T  G  A
CTCCATATCCCTACAGCGTACATGCGGAAGTTTTCAAGGAAAAACAGCAAAAGCAAGGCTCACCGGTCGACATGACATTTCATTTATTTGAGCATCCAAGCAGAATTTATGCATCAATTT  2160
 P  Y  P  Y  S  V  H  A  E  V  F  K  E  K  Q  Q  K  Q  G  S  P  V  D  M  T  F  H  L  F  E  H  P  S  R  I  Y  A  S  I  C
GCAAATTCTTTCATAGTCATGAAAAAGAATTATAGAGAATATAATGACAACAGCCTTCTCTTTTGCGGAGGCTGTTTTGTTTTGGGAAATGAAAAACCGGAAGGGCGGCAGATGCAATGG  2280
 K  F  F  H  S  H  E  K  E  L  *                                                                                ORFC →M  E
AATCAACGCTGATTGTAGGTGCGGACGAATTTTTCGGCCTTTCATTATGTGAGCGAATGATGGATGAAGGCATACATGTTGACGTTGTTCTGGCGGAAACAGAAGATAAAATGAGACAAA  2400
 S  T  L  I  V  G  A  D  E  F  F  G  L  S  L  C  E  R  M  M  D  E  G  I  H  V  D  V  V  L  A  E  T  E  D  K  M  R  Q  M
TGTACTTAGAAGAAAGACTCATGTGGCTTGGGAGAAATGAACTCTTTCGCCAGCTTGAACACATTGGAGACCAAAATTATGATACAATTTGTATCCAGTTCGGAAGTTTTTTGCCTTTAG  2520
 Y  L  E  E  R  L  M  W  L  G  R  N  E  L  F  R  Q  L  E  H  I  G  D  Q  N  Y  D  T  I  C  I  Q  F  G  S  F  L  P  L  D
ACCAATATGATTCTCCTTATATTTTAGTATATGAAGAAGACCGAAAAGAATGGGATAAACGTGAAAAAACAGGCTCGGAAAAAACCGTGATCCTGCCGAAAATGTACGGGCCATGGAAAG  2640
 Q  Y  D  S  P  Y  I  L  V  Y  E  E  D  R  K  E  W  D  K  R  E  K  T  G  S  E  K  T  V  I  L  P  K  M  Y  G  P  W  K  E
AAGAAACAGAGGAAGACGGCTATTACACAAACGACGTGGCTGACGAGTTGCTGAGATTCCTGTTAGAACCAAGCCGTCATAGCAAAGATCAGCTTTTTGAATTGCAAGTCACAGAGAAAA  2760
 E  T  E  E  D  G  Y  Y  T  N  D  V  A  D  E  L  L  R  F  L  L  E  P  S  R  H  S  K  D  Q  L  F  E  L  Q  V  T  E  K  T
CCTCAAAAGAAGAAGCAAAAACAAAAATTATCGAGTGGAAAAGACAATTTTCATCAATTTTCGACAAATATTAAGAAAAACCTTCCATGTTTCGAGTTTTCTAGATACAATAAACGTATA  2880
 S  K  E  E  A  K  T  K  I  I  E  W  K  R  Q  F  S  S  I  F  D  K  Y  *
AAAATAATACATGTTATAGCTTGTCAAAAGGAGTTGAACATGACTTGATCACAAGGCTTGTCATGATCTTTTCTGTCCTCCTTTTATTGAGTGGATGTGGACAAACTCCGTTCAAAGGAA  3000
                                    ORFD →M  I  T  R  L  V  M  I  F  S  V  L  L  L  L  S  G  C  G  Q  T  P  F  K  G  K
AAATTGAGAAGGTCGGCATGCTCTTTCCTGATACGATTAATGACCTCGTATGGGGCACAAAAGGGTATAAAGGATTACTGAATATACAATCCAAATACAATGTGGACGTCTATTATAAAG  3120
 I  E  K  V  G  M  L  F  P  D  T  I  N  D  L  V  W  G  T  K  G  Y  K  G  L  L  N  I  Q  S  K  Y  N  V  D  V  Y  Y  K  E
AAGGCGTTAAAACAGAAGAAGATATCATAAATGCGATTGAGGATTTTCATAAGAGGGGCGTCAATCTGCTCTACGGCCATGGGAGTGAATATGCTGAGGTGTTTAACTTGGTCGGCGAAG  3240
 G  V  K  T  E  E  D  I  I  N  A  I  E  D  F  H  K  R  G  V  N  L  L  Y  G  H  G  S  E  Y  A  E  V  F  N  L  V  G  E  D
ATTATCCAGATATGGAATTCGTCATTTCCAATGCGAAAGCGAAAGCTGATAATGTAACAAGTGTCCATTTTAGCGGGGAAGCAATGGGCTTTTTTGGAGGAATGACTGCTGCCCATATGT  3360
 Y  P  D  M  E  F  V  I  S  N  A  K  A  K  A  D  N  V  T  S  V  H  F  S  G  E  A  M  G  F  F  G  G  M  T  A  A  H  M  S
CGAAAACGAATCAGGTCGGTGTAATTGCTTCCTTTACGTGGCAGCCTGAGGTTGACGGTTTTATCAAAGGGGCCAAGTATGAAAATCCGGATATAGAGGTAAATACGAAATATACGGATC  3480
 K  T  N  Q  V  G  V  I  A  S  F  T  W  Q  P  E  V  D  G  F  I  K  G  A  K  Y  E  N  P  D  I  E  V  N  T  K  Y  T  D  H
ATTGGGATGATGATACAACAGCAGTAAAGCTTTATCAGAAAATGAAGAACGAAGGCGCGGATGTTGTGTATCCCGCCGGAGACGGGTATAATGTTCCTGTTATTCAGCAAATCAAAAAAG  3600
 W  D  D  D  T  T  A  V  K  L  Y  Q  K  M  K  N  E  G  A  D  V  V  Y  P  A  G  D  G  Y  N  V  P  V  I  Q  Q  I  K  K  D
ACGGTCTTTATGCCATAGGCTACGTCACAGATCAATCGGATCTGGGCGAGAATACCGTATTAACCAGCACGGTGCAAAATGTGGACAAGGCCTATGAAATCATTGCTGAACAATTTAATA  3720
 G  L  Y  A  I  G  Y  V  T  D  Q  S  D  L  G  E  N  T  V  L  T  S  T  V  Q  N  V  D  K  A  Y  E  I  I  A  E  Q  F  N  K
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CATACAACAAAACAGGCGAGCTGCCAAAAAACGAGTAATGGAGTGACGTCACATGCAGCAAGAAAAATCACTTGAATTCTTGCAAATTGCCATGAAATATCTCCCTGAAGCGAAAGAACA  3960
 Y  N  K  T  G  E  L  P  K  N  E  *                ORFE →M  Q  Q  E  K  S  L  E  F  L  Q  I  A  M  K  Y  L  P  E  A  K  E  Q
GCTTGAGAAATCAGGCATTGAGCTCTCAATGGAGGCCATCCAGCCGTTTATGAATCTATTTACAACGGTAATGGCGGAAGCTTATGAGCTTGGCAAGTCTGACGCTAAAGCAGA  4080
 L  E  K  S  G  I  E  L  S  M  E  A  I  Q  P  F  M  N  L  F  T  T  V  M  A  E  A  Y  E  L  G  K  S  D  A  K  S  E  T  E
                                                *  M  I  K  K  K  I  A  S  L  M  P  A  L  E  K  L  A  P  K  I  Q  D  L  S  N  M  I  S
ATAAGAAGAAGCCACTTTTTTGAAAGTGGCTTTTCACATGATTTTCTTTTTAATAGCGGAGAGCATGGGCGCAAGCTCCTTTAAAGCGGGCTTGATTTGATCAAGAGAGTTCATAATTGA  4200
 *
 G  I  Q  D  M  I  H  M  Y  N  I  T  E  E  D  N  T  Q  S  V  Q  S  V  E  E  Q  Q  E  E  E  K  E  E  T  P  K  A  A  D  G
ACCGATTTGGTCCATAATATGCATGTAATTAATCGTCTCTTCGTCGTTTGTCTGAGAAACCTGAGAAACTTCCTCTTGCTGTTCCTCCTCCTTCTCTTCTGTTGGTTTTGCTGCATCGCC  4320
 F  M  M  R  S  F  V  D  L  Q  M ← ORFF
GAACATCATTCTTGAAAACACATCCAACTGCATACGCCTCCTTTTCCATATACCATACTCTATGAGTAAGATGAACTGATAGTTTAGACGAATATATTGCCATGTGAAAAAAAATAGGATA  4440
GAATTAGTACCTGATACTAATAATTGATCACAACCTGATTGATCTTCTAAATTTAAGATATAAAGGAGTCTTCCCTAATGAAAGCTGGAATACTTGGTGTTGGACGTTACATTCCTGAGA  4560
AGGTTTAACAAATCATGATCTTGAAAAAATGGTTGAAACTTCTGACGA                                                                          4608
```

observations shown in Fig. 1A. When total RNA from OMM46 grown in IPTG-containing medium was used, the *degR* transcript was detected (lane 3). These results strongly suggest that ORF D but not ORF E is involved in the control of *degR* expression. The notion was further confirmed by using strain ODM44 (Table 1), in which ORF E has been disrupted by insertion of the Tc^r gene between codons 30 and 31 of 63-codon ORF E. As shown in Fig. 1B, the ORF E disruption did

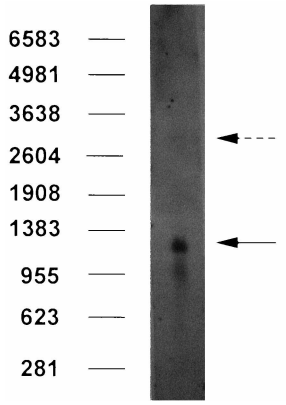

FIG. 3. Northern analysis of total RNA isolated from the CU741 cells, using an internal region of ORF D as a probe. Cells were grown in Schaeffer's sporulation medium and harvested in the mid-log phase. The procedure for detection of RNA is described in Materials and Methods. Numbers on the left show positions of the RNA size markers (base pairs). The solid and dotted arrows indicate the ORF D-containing transcript and a barely detectable transcript, respectively.

not increase the expression level of *degR'-'lacZ* in the *mecA* background. These results indicate that the ORF D deficiency is solely responsible for the restoration of *degR* expression in the *mecA* background, and therefore ORF D corresponds to the *med* locus.

Effect of disruption of *med* on expression of competence-regulating factors. We have shown previously that the inhibitory effect of *mecA* on *degR* expression is relieved by a *comK* null mutation, indicating that ComK overproduced in a *mecA* background represses *degR* expression (32). The observation that the *med* mutation rescued the inhibitory effect of *mecA* deficiency suggested that Med would also function in the competence-developing pathway. Thus, we first examined the effect of *med* disruption on expression of the major positive regulators *srfA* (*comS*), *degU*, and *sinR* in both sporulation and competence media. The results showed that *med* deficiency did not affect the expression of these genes significantly in sporulation medium (Table 3). There was a 30% reduction in the expression levels of *degU'-'lacZ* and *sinR'-'lacZ* in the cells grown in competence medium, the significance of which is uncertain, since the transformation efficiency was not affected significantly in the *med* background (data not shown).

We next examined the effect of the *med* mutation on sporulation, since Spo0A and AbrB constitute a regulatory branch different from those involving the above-mentioned three factors (11, 12, 48). As shown in Table 4, the mutation did not affect sporulation efficiency, showing that Med does not affect the Spo0A function (see also Discussion).

Finally we examined the effect of *med* deficiency on the expression of *comK* and its downstream gene *comG*. The β-galactosidase activity derived from *comK'-'lacZ* was very low in the wild-type strain grown in sporulation medium, and *med* disruption did not change this low-level activity (Fig. 6A). It has been reported that the expression of *comK* is greatly increased by the *mecA* null mutation (12, 48). As shown in Fig. 6A, we obtained a similar result, and this increasing effect was abolished by the *med* disruption mutation. Since the expression level of *comK'-'lacZ* in sporulation medium was low except in the case of the *mecA* strain, we examined the same set of strains for *comK'-'lacZ* expression in MC medium. The level of *comK'-'lacZ* expression in the *med* background was about 30% of the control level (Fig. 6B). Disruption of *mecA* resulted in an enhanced expression of *comK'-'lacZ* as expected, and this enhancement was greatly reduced by the presence of the *med* mutation (Fig. 6B). These results together with those described above show that *comK* is positively regulated by Med, most markedly in the *mec* background, and that the positive regulation is not exerted through expression of *srfA* (*comS*), *degU*, *sinR*, and *spo0A*. In these experiments, the timing of *comK'-'lacZ* expression in the *mecA* background was somewhat different from that observed previously (12, 48), probably because of the different media used. In addition, the expression of *comK'-'lacZ* in the *med* background increased slightly during the exponential growth phase (Fig. 6B). Although the result is reproducible, the reason is unknown at present.

Expression of *comG* would be expected to be reduced in a *med*-deficient mutant, since *comG* is positively regulated by ComK (50, 51). Expression of the *comG'-'lacZ* fusion in the wild-type cell was very low in sporulation medium, but it in-

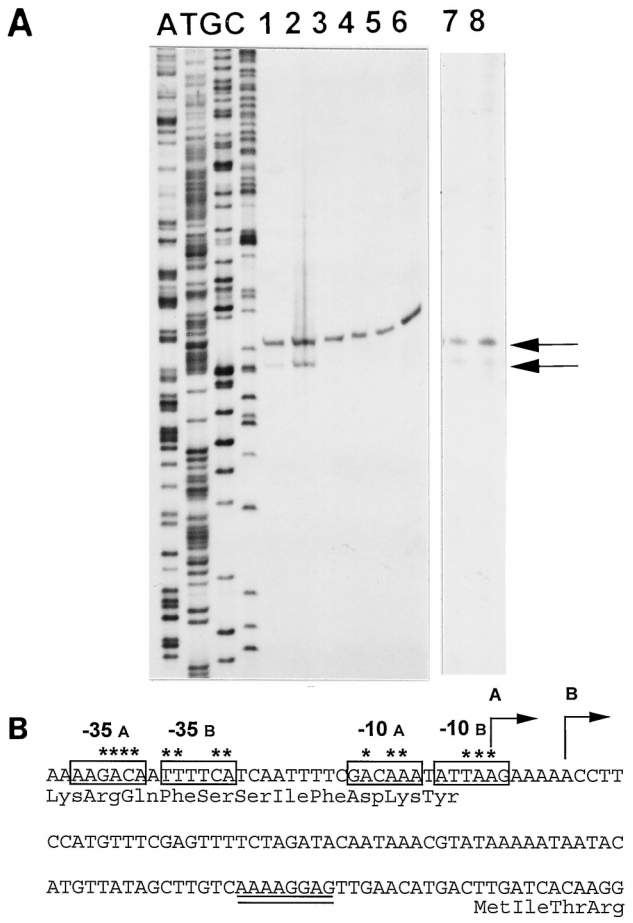

FIG. 4. Transcriptional start points of ORF D and the effect of ORF C disruption on the transcription of ORF D as determined by primer extension analysis. The procedure for primer extension is described in Materials and Methods. The primer used for the extension reaction was oligonucleotide Med-3 (Table 2). Cells were grown in Schaeffer's sporulation medium, and RNAs were isolated from CU741 at hourly intervals starting at an OD_{600} of 0.5 (lanes 1 through 6) and from ODM40 and ODM45 at an OD_{600} of 0.8 (lanes 7 and 8). (A) Arrows indicate the transcriptional start sites of ORF D, and lanes A, T, G, and C represent the sequence ladders of the coding strand obtained with the primer used for the primer extension reaction. (B) The nucleotide sequence before ORF D, showing the transcriptional start points (arrows), the −10 and −35 sequences of the putative promoters (boxes), and the SD sequence (double underline). Asterisks indicate the nucleotides matching the consensus sequence of the σ^A recognized promoter.

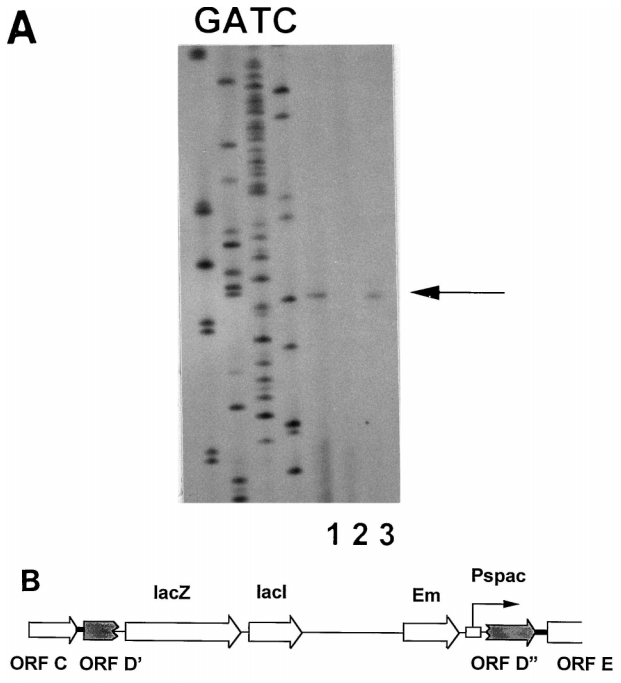

FIG. 5. Effect of ORF D disruption on transcription of *degR* in a *mecA*-deficient mutant. (A) Expression of *degR* determined by primer extension analysis. Cells were grown in Schaeffer's sporulation medium and harvested in the mid-log phase. Hybridization was carried out with RNA isolated from the strains CU741 (*mec*$^+$) (lane 1), CU741ma (*mecA*) (lane 2), and OMM46 (*mecA med*::pMED301) (lane 3) grown in the presence of 0.5 mM IPTG. Sequence ladders are those of the coding strand obtained with the primer used for the primer extension analysis. The arrow shows the *degR* transcript. (B) Schematic representation of the *med* region in OMM46. The large and small arrows show coding regions and the transcriptional start site of the P_{spac} promoter (small open box), respectively. The shaded broken arrows designated ORF D′ and ORF D″ show the N- and C-terminally truncated regions of ORF D, respectively. The thick and thin lines represent the *B. subtilis* chromosome and the DNA region derived from the vector, pHV501, respectively.

creased in the *mecA*-deficient background (Fig. 6C), as would be expected from the high-level expression of *comK* in this mutant. That the increased *comG′-'lacZ* expression was caused by *mecA* deficiency was verified by the observation that β-galactosidase activity was negligible in the *mecA comK* strain (Fig. 6C). In contrast to the mode of *comK′-'lacZ* expression, however, the presence of the *med* mutation in the *mecA* mutant did not result in a reduction of *comG′-'lacZ* expression but caused an approximately twofold increase (Fig. 6C). These results may indicate that high-level expression of *comG* is

TABLE 3. Expression of *degU′-'lacZ*, *srfA-lacZ*, and *sinR′-'lacZ* in strains carrying the *med* mutation

Strain	Relevant genotype	β-Galactosidase activity[a]	
		SM	MC
TT714	*degU′-'lacZ*	85	253
OUM101	*degU′-'lacZ med*	97	180
OSM100	*srfA-lacZ*	723	884
OSM101	*srfA-lacZ med*	790	903
ORM100	*sinR′-'lacZ*	28	45
ORM101	*sinR′-'lacZ med*	32	30

[a] Cells were grown in Schaeffer's sporulation medium (SM) or MC medium. Maximum values (Miller units) of the expression of each *lacZ* fusion are given.

TABLE 4. Sporulation frequency of the *med* mutant

Strain	Relevant genotype	No. of viable cells/ml (10^8)	No. of spores/ml (10^8)	Sporulation frequency (%)
CU741	Wild type	7.8	1.2	15
OMM100	*med*	5.8	1.1	18

attained in the *mecA med* cell in which the expression of *comK* is limited. This interpretation was verified by the observation that the level of *comG′-'lacZ* expression was undetectable in the *mecA med comK* triple mutant (Fig. 6C). In MC medium, similar results were obtained except that *comG′-'lacZ* expression was detected in the wild-type strain and *med* deficiency resulted in a slight decrease in *comG′-'lacZ* expression (Fig. 6D).

Effect of deficiency of competence-regulating genes on *med-lacZ* expression. Since *med* was shown to be involved in *comK* regulation, conversely we examined the effect of the *comK* regulators MecA, ComK, SrfA, AbrB, DegU, and SinR on the expression of the *med* gene. The strains used for this study were derivatives of OMM100, which was constructed by insertion of pMED501 (see Materials and Methods), which carries a *med5-lacZ* transcriptional fusion immediately downstream of the stop codon of the *med* gene. The β-galactosidase activity driven by the *med5-lacZ* fusion in sporulation medium reached

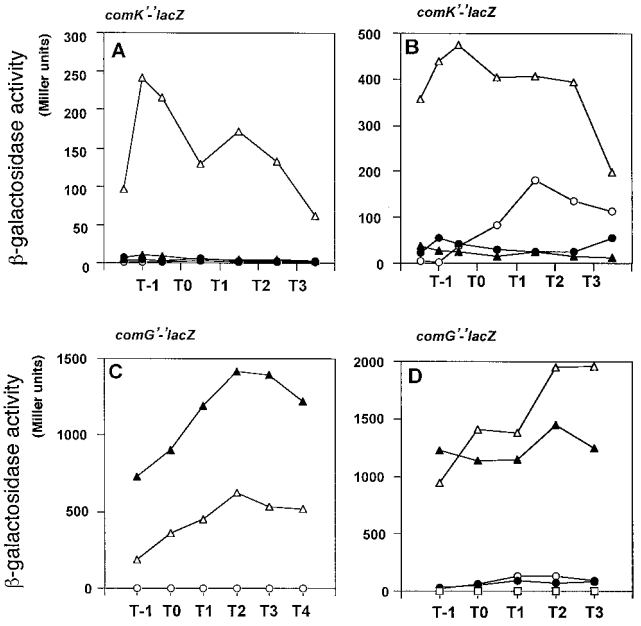

FIG. 6. Effect of *med* mutation on the expression of various regulatory genes. Growth conditions and measurement of β-galactosidase activities were as specified in the legend to Fig. 1 except that sporulation medium was used for experiments A and C and MC medium was used for experiments B and D. β-Galactosidase activities are shown in Miller units. Numbers on the *x* axis represent the growth time in hours relative to the end of the vegetative growth (T0). (A and B) ○, OCM100 (*comK′-'lacZ*); ●, OCM101 (*comK′-'lacZ med*); △, OCM102 (*comK′-'lacZ mecA*); ▲, OCM103 (*comK′-'lacZ mecA med*). (C) ○, OGM100 (*comG′-'lacZ*), OGM101 (*comG′-'lacZ med*), and OGM107 (*comG′-'lacZ mecA med comK*); △, OGM102 (*comG′-'lacZ mecA*); ▲, OGM103 (*comG′-'lacZ mecA med*). (D) ○, OGM100 (*comG′-'lacZ*); ●, OGM101 (*comG′-'lacZ med*); △, OGM102 (*comG′-'lacZ mecA*); ▲, OGM103 (*comG′-'lacZ mecA med*); □, OGM104 (*comG′-'lacZ comK*), OGM105 (*comG′-'lacZ mecA comK*), OGM106 (*comG′-'lacZ med comK*), and OGM107 (*comG′-'lacZ mecA med comK*).

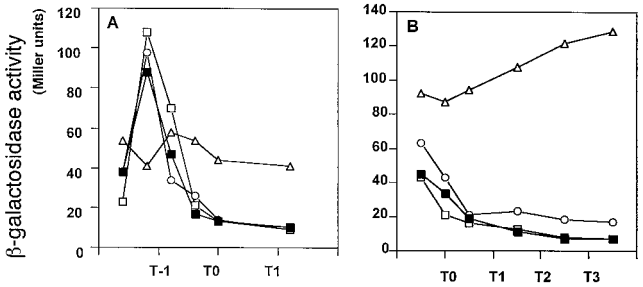

FIG. 7. Effects of mutations of competence regulatory factors on the expression of *med5-lacZ*. Growth conditions and measurement of β-galactosidase activities were as specified in the legend to Fig. 1. β-Galactosidase activities are shown in Miller units. Numbers on the *x* axis represent the growth time in hours relative to the end of the vegetative growth (T0). Sporulation medium was used for experiment A, and MC medium was used for experiment B. ○, OMM100 (*med5-lacZ*); □, OMM101 (*med5-lacZ comK*); △, OMM102 (*med5-lacZ mecA*); ■, OMM103 (*med5-lacZ mecA comK*).

the maximum toward the end of the logarithmic growth phase and rapidly dropped at the beginning of the stationary phase (Fig. 7A). The decrease, however, might be due to the turnover of β-galactosidase and may not reflect a decrease in *med* expression, since the effect of Med on *comK* expression was seen from 1 to 2.5 h after the end of vegetative growth (Fig. 6), and thus Med should be present in the cell during this period. Disruption of *comK* had no effect on the expression of *med5-lacZ* (Fig. 7A). This result is not unexpected, since *comK* expression is negligible in this medium (Fig. 6A). Similarly, *srfA*, *abrB*, and *degU* deficiency did not affect the expression of the fusion (data not shown). The *sinR* mutation slightly reduced the expression of *med5-lacZ* (data not shown), the significance of which is uncertain. On the other hand, disruption of *mecA* resulted in an altered expression of *med5-lacZ* (Fig. 7A), suggesting that *med* is regulated in some way by ComK overproduced by the *mecA* mutation. To test this possibility, the expression of *med5-lacZ* in a strain carrying both the *mecA* and *comK* mutations was examined, and the result showed that the expression profile of *med5-lacZ* became similar to that in the wild-type cell (Fig. 7A). In MC medium, the level of β-galactosidase derived from the *med5-lacZ* fusion in the *comK* background was about 70% of that in the *comK*$^+$ cells (Fig. 7B). Disruption of *mecA* resulted in constitutive expression of *med5-lacZ*, but this expression mode was abolished by introduction of *comK* deficiency (Fig. 7B). These results show that ComK is involved in the regulation of *med*. Since Med positively regulates *comK* in MC medium as shown above, it appears that there is a positive regulatory loop between *med* and *comK* at least in competence medium.

DISCUSSION

It has been postulated that competence-developing signals converge at the competence transcription factor ComK through several pathways including Spo0A-AbrB, ComP/A-SrfA (ComS)-Mec, DegU, SinR, and AbrB (9–12, 21, 34, 48). In addition *comK* is also regulated by its own gene product (12, 48). By analyses of epistatic effects of *mec* mutations on various genes involved in competence development, ComK has been shown to work downstream of the *mec* gene products (10, 12, 48). We report in this paper isolation and characterization of a new gene, *med*, whose disruption partially relieved *degR* expression prevented by overproduced ComK in a *mecA*-deficient mutant. We concluded that Med is a positive regulator of *comK*, since *med* disruption led to a dramatic reduction in *comK'-'lacZ* expression in the *mecA* background (Fig. 6A and B) and to a significant reduction in a *mecA*$^+$ strain (Fig. 6B). The expression of *comG'-'lacZ*, however, was not prevented in the *mecA med* double mutant (Fig. 6C and D), an observation in contrast to the finding that the fluctuation of the ComK level sharply influences transformation frequency and *comG* expression (48). One interpretation for the discrepancy is that although *comK* expression is greatly reduced in the *mecA med* mutant, ComK activity is high enough to support *comG* expression, but this level of ComK activity does not support positive regulation of *comK* expression itself in the autoregulatory circuit of *comK* (12, 48). The data shown in Fig. 1 are intriguing in this respect: the reduced level of *degR'-'lacZ* expression caused by *mecA* was restored by Tn*10* insertion in *med*, but the extent of restoration was about 35% under the condition in which the expression of *comK'-'lacZ* was undetectable (Fig. 6A). Furthermore, it was found that the expression of *comG'-'lacZ* was undetectable in a *mecA med comK* triple mutant (Fig. 6C and D), which supports the idea that a substantial amount of ComK activity remains in the *mecA med* cells and that this residual ComK activity is responsible for *comG* expression. It has been demonstrated that transposon insertion in *degU* greatly reduced *comK* expression in a *mecA*-deficient mutant, but expression of the *comG'-'lacZ* fusion was not affected in a *degU mecA* mutant (34, 39). This phenomenon could also be explained on this basis. Hahn et al. suggested recently that both *comK* and *comG* are positively regulated by ComK in different modes; they postulated that DegU is a coactivator and required for the expression of *comK* as a transient positive regulator but not for *comG* expression (10). This type of regulation could also be applicable to *comK* regulation by Med. The finding that the ratio of the expression level of *comK* was greater than that of *comG* in the *mecA*$^+$ *med*$^+$ and *mecA*$^+$ *med* strains (Fig. 6B and D) but the transformation efficiency was indistinguishable between the two strains suggests a mechanism by which *comG* is expressed with a reduced amount of ComK.

Which pathway among those affecting the ComK level could be the target of Med? Med may exert its effect on *comK* independently of the ComS-MecA system in the competence regulatory network, since the gene was identified by Tn*10* insertion in a *mecA* background. Disruption of *med* did not have a significant effect on the expression of *sinR'-'lacZ* and *degU'-'lacZ* fusions (Table 3). Sporulation was not affected by the *med* mutation (Table 4), indicating that Spo0A is not the target of Med. It is unlikely that the effect of Med on *comK* is exerted through positive regulation of *abrB*, since the requirement of *abrB* for *comK* expression is bypassed by the *mecA* mutation (11), while *med* was identified in the *mecA* background. These results imply a new regulatory branch for Med. However, it remains possible that Med affects the activity of SinR or DegU and thereby exerts a positive effect on *comK* expression. It may also be possible that Med regulates activity or stability of ComK, since the twofold enhancement of *comG* expression (Fig. 6C) could be due to an alteration of ComK. The data described in this study, however, do not rule out the possibility that Med is involved in *comK* transcription directly. Future work will address whether Med enhances *comK* expression itself at the transcription level.

The deduced amino acid sequence of the protein encoded by *med* did not have any significant similarity to those of the known proteins in the PIR and SwissProt data banks, precluding extrapolation of the Med function. It is noteworthy, however, that Med has a putative transmembrane domain and a potential lipopeptide modification site at its N-terminal region, as revealed by a computer analysis (data not shown). Whether

these features contribute to the function of Med remains to be examined.

The transcription of *degR* is dependent on the alternative sigma factor σ^D (35). We have shown that neither *mecA* nor *mecB* null mutations affect the expression of *sigD*, the gene for σ^D, and the reduced expression of *degR* in *mec* null mutants is recovered in *mecA comK* or *mecB comK* double mutants, indicating that ComK is responsible for the repression of *degR* (32). The observations that both the *degU* and *med* null mutations reduce the expression of *comK* and suppress the inhibition of *degR* caused by *mec* deficiency are in line with the foregoing results. Recently Rashid et al. reported that the expression of *sigD* is prevented by *mec* null mutations and that this inhibition is not restored by *comK* deficiency (37). This is in contrast to our findings, and we could not confirm their results.

It is difficult to deduce the physiological role of Med in vivo from the experimental results obtained in this study. The only effect of *med* deficiency was the reduced expression of *comK*. The signal transduction that results in activation of ComK involves nutritional signals in addition to cell density signals (6, 9). It may be possible that Med participates in one of these processes, although *med* deficiency did not affect transformation or sporulation in the laboratory condition.

The present study adds another *comK* regulator, Med, to the multiple regulatory factors of *comK* (10). This exquisitely regulated system may give to the cell more chances to integrate various signals into ComK, which is not only the regulator of late competence genes but also the regulator of adaptive response genes such as *degR*, *sacB*, *nucA*, and *recA* (14, 25, 32, 49). We presume that the regulatory system including Med would give an advantage of survival in nature to the living cell.

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