Note
Med, a Cell-surface Localized Protein Regulating a Competence Transcription Factor Gene, comK, in Bacillus subtilis
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Med was found as a positive regulator for comK, a master regulator for late competence genes. It was found by Western analysis that the ComK level was decreased in a med mutant. Experiments using an alkaline phosphatase fusion with Med and Western analysis of Med were done because a putative lipoprotein signal is found at the N-terminus of Med. The results obtained are consistent with the localization of Med at the cell surface. An implication of the cell-surface localization of Med is discussed in terms of comK regulation.

Key words: competence development; protein localization; Med

Genetic competence in Bacillus subtilis is the ability to process and take up exogenous DNA into the cell. Competence development is regulated by both cell density and nutrient limitation signals. ComA, the response regulator of the ComP-ComA two-component system, is activated by cell-density signals through its phosphorylation. Phosphorylated ComA activates transcription of the sfa operon, which encodes enzymes for the synthesis of surfactin and the competence regulator ComS, located within and out-of-frame with srfAB. There are two known extracellular factors, ComX and PhrC, by which ComA is activated. When B. subtilis cells reach stationary phase, the concentration of ComX becomes high enough to interact with the ComP kinase. On the other hand, the PhrC pentapeptide is imported into the cell by oligopeptide permease (Opp), and probably inhibits the activity of RapC phosphatase, the target of which is phosphorylated ComA. Opp is an ABC transporter composed of five subunits. One subunit, OppA, is a lipoprotein and works as a substrate-binding protein for the transporter. Various lipoproteins have been shown to function as substrate-binding proteins for transporters. In eubacteria the process of lipid modification is well conserved. The diacylglycerol transferase encoded by lgt transfers a diacylglycerol moiety to the cysteine residue downstream from a target signal peptide, followed by the removal of the signal peptide from the lipid-modified substrate by signal peptidase II.

ComK is a master regulator of competence development, which is necessary for transcription of the four late com operons encoding the apparatuses for processing and taking up DNA (comC, comE, comF, and comG). When competence development is repressed, ComK is part of a ternary complex with MecA and ClpC, which belongs to a large family of regulatory subunits of ATP-dependent proteases. The catalytic subunit of the protease, ClpP, associates with this complex, and the resultant ClpCP protease is thought to degrade ComK. In the complex ComK is inactive and cannot bind to its target DNA. Once ComS is produced in the cell by virtue of extracellular regulation through ComP-ComA, it binds to MecA and releases ComK from the complex, resulting in the binding of ComK to AT-rich ComK boxes in the regulatory regions of these operons.

Recently we have found a positive regulatory factor for comK transcription, med. The med and downstream comZ genes constitute an operon involved in competence development. In this paper we present evidence that Med is located at the cell surface.

We reported that expression of comK-lacZ was reduced in the med mutant. To confirm the observation at the protein level, polyclonal antibody was raised in rabbits against a synthetic peptide derived from a C-terminal region of ComK (NH$_2$-KMIYDFILRELGE-COOH). Synthesis of the peptide and immunization were done by Sawady Technology Co. (Tokyo, Japan). This antibody was used to measure ComK in the med mutant grown in competence medium. By using this antibody, an immuno-reactive band with a molecular mass of ComK was detected in med$^+$ cells, but not in comK-deficient cells, indicating that the detected band is indeed ComK (Fig. 1). In the med mutant the amount of ComK was greatly reduced.

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Abbreviation: AP, alkaline phosphatase
the total DNA of CU74115) as a template was digested with pMA5.18). The resultant plasmid was cut with CT-3 proline or alanine into pMA5 carrying the med-phoA region of protein localization.15) This feature strongly suggested that Med may be subject to signal peptide processing and lipid modification. To test this possibility, we constructed a fusion between the N-terminal half of Med and the E. coli alkaline phosphatase (AP) lacking its signal peptide, essentially as described by Dartois et al.16) It has been known that AP is active only when translocated across the membrane,17) and thus AP fusion is used for measurement of protein localization.

A plasmid pMed-PhoA1 used for such experiments carried an in-frame fusion between an N-terminal region of med containing the putative signal peptide followed by the lipo-modification signal and phoA without its signal peptide. It was constructed in three steps. A PCR product amplified by using Med-AP-U (5′-GTCGAATTCTCCATTCTCAAAAGAAGAGCAGAAA-3′) and Med-AP-D (5′-GTCGATATCTCAGGAAAGGAGCATGCGCA-3′) as primers and the total DNA of CU74115 was digested with PvuII and EcoRI and cloned into pJv211 treated with EcoRV and EcoRI. The plasmid thus constructed was digested with HindIII, and the fragment containing med-phoA was cut out from an agarose gel and cloned into the HindIII-digested pMA5.18) The resultant plasmid was cut with SacI and self-ligated to generate pMed-PhoA1. To introduce mutations causing a change of cysteine to proline or alanine into pMA5 carrying the med-phoA fusion, a Quick-change site-directed mutagenesis kit (Stratagene, CA, U.S.A.) and oligonucleotide pairs, Med-CP-U (5′-ATTGAGTGGACCTGGACAAAAGAAGAGCAGAAA-3′) and Med-CP-D (5′-AGTTTGTCCAGTCCACTCAAT-3′), and, Med-CA-U (5′-ATTGAGTGAGCTGGACAAAAGAAGAGCAGAAA-3′) and Med-CA-D (5′-AGTTTGTCCAGTCCACTCAAT-3′) were used, respectively. The resultant pMA5 derivatives were put through the same procedures used for construction of pMed-phoA1. All the PCR-derived regions of the cloned DNA were sequenced using a 377 DNA Sequencer and Dye Terminator Cycle Sequencing Kit (Perkin Elmer). Oligonucleotides were commercially prepared by Espec Oligo Service (Ibaraki, Japan).

The AP assay was done as follows. One ml of the culture was taken at hourly intervals. After centrifugation the pellets were washed by the same volume of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and resuspended in 1 ml TE buffer. The suspension thus obtained was designated as an intact-cell fraction. Next, to obtain a protoplast fraction cells were centrifuged, resuspended in the same volume of TE buffer containing 20 mM MgCl2 and 20% sucrose with lysozyme (1 mg/ml; Sigma Co.) and incubated at 37°C for 30 min, followed by a wash with the same volume of the TE buffer. The protoplasts were directly resuspended with the buffer for the AP assay. Detection of AP activities in intact cells, protoplasts, and culture supernatant was done as described in Dartois et al.16) AP activity (units) was calculated by the following equation.

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\text{units} = \frac{1000 \times \text{dilution factor} \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550})}{(\text{time (minutes)} \times \text{OD}_{600} \text{(growth)})}
\]

We first examined the AP activity in the intact cell fraction of the wild-type Med-AP fusion. Activity higher than the background level was detected although the overall activity was very low for an unknown reason (data not shown). Thus, we next did the AP assay using the protoplast fraction. As shown in Fig. 2A, an AP activity from the Med-AP fusion was observed, indicating that the fusion was specifically localized at the outer region of the cell membrane. In addition, considerable amounts of the AP activity were also detected in the culture supernatant (Fig. 2A). This is consistent with the previous observation that lipoprotein of Gram-positive bacteria can be released from the cell surface into the medium.7) In a lgt disruptant, the activity of the fusion was barely detected in the protoplast fraction but considerably in the culture supernatant (Fig. 2A), suggesting that the fusion could not be efficiently localized at the cell surface in the lgt strain. It is speculated that the attachment of the diacylglycerol moiety to the cysteine residue of the Med-AP fusion and removal of the signal peptide would not occur in the lgt strain as in the case of a lipoprotein, PrsA,9) resulting in diffusion of the fusion into the medium. However, we do not know the reason for this phenomenon. Next, we introduced mutations that replace the cysteine residue within the lipo-modifica-

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**Fig. 1. Western Analysis of ComK in the Mutants.**

OGM100 (WT), OGM101 (med), and OGM104 (comK) cells were grown in 100 ml of modified competence (MC) medium and harvested at T0 and T2 relative to the end of the vegetative growth (T0). After cell disruption by a French press and measurement of the protein concentration by a Bio-Rad Protein Assay Kit, samples were mixed with SDS-PAGE sample buffer and put onto a gel. Western analysis was done using a PVDF membrane (Boehringer Manheim) and BM Chemiluminescence Western Blotting Kit (Rabbit/Mouse) (Boehringer Manheim Co.).
Fig. 2. Alkaline Phosphatase-fusion Showed That a Certain Amount of Med is Localized at the Outer Surface of the Cell Membrane.

Cells were grown in sporulation medium and samples were taken at hourly intervals. AP activities of protoplast and culture supernatant were measured (see Text). Experiments were done three times independently and the average values with standard deviations are shown. Numbers on the x axis represent the growth time in hours relative to the end of the vegetative growth (T0). Below the panels are shown the relevant constructs in the plasmids carrying \( \text{phoA} \) with (pMed-phoA1) or without (pJV211) the N-terminal region of \( \text{med} \). Arrows indicate the promoter on the expression vector, pMA5. The short and long open boxes show the N-terminal region of \( \text{med} \) and truncated \( \text{phoA} \) without the signal peptide. The \( \text{lgt} \) mutation was derived from JH16724 (\( \text{trpC2 pheA1 phoA::Cmr phoB::mlsr} \)). A. OM201 (\( \text{phoA phoB} \)) carrying pMed-PhoA1, ●; OM203 (\( \text{phoA phoB lgt} \)) carrying pMed-PhoA1, □; OM201 carrying pJV211, ●; OM203 carrying pJV211, ○. B. OM201 (\( \text{phoA phoB} \)) carrying pMed-PhoA2 (Cys to Ala mutation), □; OM201 (\( \text{phoA phoB} \)) carrying pMed-PhoA3 (Cys to Pro mutation), ○.

Fig. 3. Western Analysis of Med.

CU741 (\( \text{trpC2 leuC7} \)) and OMM43 (\( \text{trpC2 leuC7 med} \)) were grown in MC medium and 250 µl of the sample was taken at T0. S, PS, M, and C indicate the culture supernatant, protoplast supernatant, membrane and cytoplasmic fractions, respectively. The protoplast supernatant fraction contained both the supernatant and wash fractions obtained during the protoplast formation procedure for the AP assay. Protoplasts were disrupted by three cycles of freezing and thawing, and then centrifuged to prepare membrane and cytoplasmic fractions. Equivalent amounts of each fraction were put onto the gel. Procedure for Western analysis is described in legends to Fig. 1.
from the Med protein. The Western results are consistent with the observation that the Med-AP fusion is localized at the outer surface of the cell membrane. Med was not detected in the culture supernatant (Fig. 3), although the experiment using the AP fusion showed a considerable level of activity in the medium fraction (Fig. 2A). The apparent discrepancy between the locations of the AP fusion and the Med protein itself may be due to the possibility that the Med protein might have a signal(s) that enables it to be trapped in the cell wall, but the Med-AP fusion might lack such a signal(s). However, this notion is speculative since well-known cell-wall targeting signals such as LPXTG are not detected in the amino acid sequence of Med. From these results we concluded that Med is localized at the outer surface of the cell, probably through lipo-modification, although direct evidence, such as incorporation of radiolabeled palmitic acid into Med, may be required.

Based on a computer search Med was shown to have a similarity to various substrate-binding lipoproteins. Figure 4 shows an alignment of Med to an aliphatic amino acid-binding protein of *E. coli*, LivJ. Med has a part of the consensus of the aliphatic amino acids-binding motif. In addition, the region of Med showing sequence similarity to the consensus for aliphatic amino acids-binding motif could align partially with the consensus for oligopeptide binding. Med might be an orphan substrate-binding protein since the med operon lacks the genes coding for potential subunits to constitute an ABC-transporter. These observations suggest that Med might function as a substrate-binding protein to incorporate some unknown extracellular factor regulating competence development.

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**References**


