We isolated temperature-sensitive mutants of the *Escherichia coli* bamD gene, which is essential for the assembly of β-barrel outer membrane proteins. As their multicopy suppressor, we identified a novel yiaD gene encoding a putative lipoprotein, YiaD. Mutations of its OmpA domain, which is required for interaction with peptidoglycan, affected suppression, suggesting that interaction with peptidoglycan is important to YiaD function.

**Key words:** *Escherichia coli*; outer membrane; peptidoglycan

The outer membrane proteins of *E. coli* are classified into two classes: β-barrel proteins and lipoproteins. Both are amphipathic proteins, and their mode of transport across the periplasmic space, the hydrophilic area between the outer and inner membranes, has been investigated. The transport systems for lipoproteins and β-barrel proteins have been identified as the Lct and Bam (β-barrel assembly machine) systems respectively.\(^1\)\(^2\) The Bam system is composed of one β-barrel protein, BamA (formerly YaeT), and four lipoproteins, BamB, -C, -D, and -E (formerly YfgL, NlpB, YfiO, and SmpA respectively).\(^3\)\(^4\) In this study, we genetically identified a novel lipoprotein, YiaD, as an accessory factor of the essential BamD protein. We suggest that its interaction with peptidoglycan is important to its function.

We isolated four temperature-sensitive (ts) mutants of *bamD* (*bamD1*, *bamD2*, *bamD3*, and *bamD5*) by plasmid shuffling using a mini-F plasmid.\(^5\)\(^6\)\(^7\) Because depletion of BamD led to drastic reductions, in the levels of OmpA and OmpF/C in the membrane fraction,\(^8\) we prepared a membrane fraction from the *bamD*\(^{ts}\) mutants grown under a non-permissive temperature and fractionated by sucrose gradient centrifugation\(^9\) (Fig. 1A, B). The levels of OmpA and OmpF/C fell, which indicates that the assembly of the β-barrel proteins in the *bamD*\(^{ts}\) mutants was defective.

To identify the factors involved in the function of BamD, we introduced an *E. coli* genome library into the *bamD*\(^{ts}\) mutants. From the *bamD2* mutant, eight temperature-resistant strains were obtained, and PCR analysis showed that four of them were *bamD* plasmids. The other four plasmids were extracted and introduced again into the *bamD2* mutant. The transformants became temperature resistant, indicating that temperature resistance is to be ascribed to the plasmids. For one of these plasmids, both junction sequences between vector and cloned chromosomal regions were determined, and we found that this plasmid carried the *yiaD* and *yiaE* genes. To determine the gene responsible for temperature resistance, we subcloned the *yiaD* and *yiaE* genes and introduced them into the *bamD2* mutant (Fig. 2A). Only the *yiaD* plasmid eliminated the temperature sensitivity for growth. Thus the *yiaD* gene was identified as a multicopy suppressor for the *bamD2* mutant. The *yiaD* plasmid was introduced into the other three *bamD*\(^{ts}\) mutants and the transformants became temperature resistant, indicating that multicopy suppression is not allele-specific. The *bamD* deletion mutant was not obtained in the presence of the *yiaD* plasmid, suggesting that the multicopy of the *yiaD* gene does not suppress the *bamD* null-type mutation.

To determine whether phenotypic suppression was involved, we prepared a membrane fraction from *bamD*\(^{ts}\) mutants having the *yiaD* plasmid (Fig. 1C). In comparison with the protein profile of the control strain (the *bamD*\(^{ts}\) mutants having the vector plasmid, Fig. 1D), the levels of OmpA and OmpF/C clearly recovered. This suggests that YiaD is involved in the function of BamD.

To determine only the multicopy of the *yiaD* gene affected the *bamD*\(^{ts}\) mutations, we constructed a disruptant of the chromosomal *yiaD* gene and examined its effects on the growth of the *bamD*\(^{ts}\) mutants. The growth of the *yiaD* disruptants was slower than that of the parental strain (Fig. 2B). We also constructed the MG1655 Δ*yiaD* strain and examined its growth. The *yiaD* disruptant grew as well as the parental strain. These results suggest that the involvement of YiaD in the function of BamD is not specific to overproduction of this protein.

Analyses of the amino acid sequence of the YiaD protein suggest that it is an outer-membrane lipoprotein. This protein has an OmpA domain, and might interact with peptidoglycan.\(^10\) To investigate the role of the OmpA domain in multicopy suppression, we constructed six plasmids with a *yiaD* gene with one missense mutation (F116A, G150A, D153A, G156A, L164A, or R168A) for the conserved amino acid residues of the OmpA domain.\(^10\) Each of these plasmids was intro-
The **bamD** mutants were isolated by plasmid shuffling. First, to construct a complementing plasmid, the **bamD** DNA fragment was amplified by PCR using oligonucleotides 346-8 (5' - ccAgATC-TAAAATCagcTAggATc-3') and 346-9 (5' - ccAgcTTAggcTaCtAcg-3') as primers and then digested with BglII and HindIII. The resulting fragment was cloned into the BamHI-HindIII site of mini-F vectors pJK286 (Km') and pJK286 (Ap') to construct **bamD** complementing plasmids pl52.53-1 (Ap') and pl52.53-1 (Km') respectively. Next, the **bamD** chromosome disruptant was constructed using the SD system. A linear DNA fragment encoding the Cm' gene was generated by PCR using the 40 bp regions flanking the **bamD** coding region, and b2595-N (5' - AgATcTcctCGATTACITCGGTGAAAAgCTcCTc-3') and b2595-C (5' - cggcAgcTaAggcCTggcCTgtTcCAGgtTcAgcAC-3') oligonucleotides as primers. This fragment was introduced into E. coli strain MG1655 red with the **bamD** complementing plasmid pl52.53-1 (Km') by electroporation, and Cm' recombinants were isolated. Finally, to isolate mutants, the **bamD** DNA fragment was prepared by error-prone PCR in the presence of MnCl₂ using oligonucleotides 346-8 and 346-9 as primers, and was cloned into mini-F vector pJK286 (Ap'). The resulting plasmids were introduced into E. coli strain MG1655 **ΔbamD**:Cm' recA/pL52.53-1 (Km'). The Ap' Km' transformants were isolated at 30 °C, and ts mutants were selected at 42 °C. For membrane fractionation, 1 mL of overnight culture was added to 200 mL of LB medium containing 0.5% NaCl. The cells were incubated for 1 h at 30 °C and then for 2.5 h at 42 °C, harvested, suspended in 200 μL of buffer (20% sucrose, 30 mM Tris–HCl pH 8.0, and 5 mM EDTA), and incubated for 1 h at 0 °C after the addition of 50 μL of lysosome solution (5 mg/mL of 5 mM EDTA), 50 μL of DNase solution (5 mg/mL), and 50 μL of RNase solution (5 mg/mL). Three milliliters of 5 mM EDTA was added for osmotic shock, and the mixture was sonicated. Crude membrane fractions were prepared and subjected to sucrose step centrifugation. Fractions were collected from the bottom of the tube and numbered no. 1 being the densest fraction. Aliquots from the fractions were run on SDS PAGE gels and stained with Coomassie Blue. (A) **bamD**., (B) **bamDS**. (C) **bamD5** with pACYC184-**yiaD**; (D) **bamDS5** with pACYC184 vector. Lane M shows mass markers (phospholipase B, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa).

**Fig. 1.** Membrane Fractionation and Protein Profile of **bamD** Mutants with or without the **yiaD** Multicopy Plasmid.

The **bamD** mutants were isolated by plasmid shuffling. First, to construct a complementing plasmid, the **bamD** DNA fragment was amplified by PCR using oligonucleotides 346-8 (5' - ccAgATC-TAAAATCagcTAggATc-3') and 346-9 (5' - ccAgcTTAggcTaCtAcg-3') as primers and then digested with BglII and HindIII. The resulting fragment was cloned into the BamHI-HindIII site of mini-F vectors pJK286 (Km') and pJK286 (Ap') to construct **bamD** complementing plasmids pl52.53-1 (Ap') and pl52.53-1 (Km') respectively. Next, the **bamD** chromosome disruptant was constructed using the SD system. A linear DNA fragment encoding the Cm' gene was generated by PCR using the 40 bp regions flanking the **bamD** coding region, and b2595-N (5' - AgATcTcctCGATTACITCGGTGAAAAgCTcCTc-3') and b2595-C (5' - cggcAgcTaAggcCTggcCTgtTcCAGgtTcAgcAC-3') oligonucleotides as primers. This fragment was introduced into E. coli strain MG1655 red with the **bamD** complementing plasmid pl52.53-1 (Km') by electroporation, and Cm' recombinants were isolated. Finally, to isolate mutants, the **bamD** DNA fragment was prepared by error-prone PCR in the presence of MnCl₂ using oligonucleotides 346-8 and 346-9 as primers, and was cloned into mini-F vector pJK286 (Ap'). The resulting plasmids were introduced into E. coli strain MG1655 **ΔbamD**:Cm' recA/pL52.53-1 (Km'). The Ap' Km' transformants were isolated at 30 °C, and ts mutants were selected at 42 °C. For membrane fractionation, 1 mL of overnight culture was added to 200 mL of LB medium containing 0.5% NaCl. The cells were incubated for 1 h at 30 °C and then for 2.5 h at 42 °C, harvested, suspended in 200 μL of buffer (20% sucrose, 30 mM Tris–HCl pH 8.0, and 5 mM EDTA), and incubated for 1 h at 0 °C after the addition of 50 μL of lysosome solution (5 mg/mL of 5 mM EDTA), 50 μL of DNase solution (5 mg/mL), and 50 μL of RNase solution (5 mg/mL). Three milliliters of 5 mM EDTA was added for osmotic shock, and the mixture was sonicated. Crude membrane fractions were prepared and subjected to sucrose step centrifugation. Fractions were collected from the bottom of the tube and numbered no. 1 being the densest fraction. Aliquots from the fractions were run on SDS PAGE gels and stained with Coomassie Blue. (A) **bamD**., (B) **bamDS**. (C) **bamD5** with pACYC184-**yiaD**; (D) **bamDS5** with pACYC184 vector. Lane M shows mass markers (phospholipase B, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa).

**Fig. 2.** Growth of the **bamD** Mutants with the **yiaD** Multicopy Plasmids and the **bamD** **ΔyiaD** Mutant.

(A) The **bamD** mutant, in which the **ΔbamD**:Cm' was converted to **ΔbamD**:Sm' by homologous recombination with the Sm' PCR fragment, was transformed with an E. coli DNA library constructed with pACYC184 vector, temperature resistant (transf) transformants were selected, and the **yiaD** **ΔyiaD** plasmid (pTT2) was obtained from the tr transformants. The pACYC184-**yiaD** plasmid was obtained by ligation of the NOT digested PCR fragments, which were prepared with oligonucleotide 432-22 (5' - TTggcgcTgcAggATACAgcAggTcAcg-3') and 432-23 (5' - TTggcgcTgcAggATACAgcAggTcAcg-3') as primers and pTT2 as template. The pACYC184-**yiaE** plasmid was obtained by ligation of NOT-digested PCR fragments, which were prepared with oligonucleotide 432-20 (5' - TTggcgcTgcTgcAcTcTcAcg-3') and 432-21 (5' - TTggcgcTgcTgcAcTcTcAcg-3') as primers and pTT2 as template. pACYC184-**yiaD**, pACYC184-**yiaE**, and pACYC184 vectors were introduced into the **bamD** mutant, and Cm' transformants were selected on Antibiotic medium 3 plates containing Cm at 42 °C. (B) The **yiaD** chromosome disruptant was constructed using the SD system. A linear DNA fragment encoding the Cm' gene was generated by PCR using 40 bp regions flanking the **yiaD** coding region and in1D-Cm' with pACYC184-**yiaE** plasmid as template. pACYC184-**yiaD**, pACYC184-**yiaE**, and pACYC184 vectors were introduced into the **bamD** mutant, and Cm' recombinants were isolated. The result **ΔyiaD**:Cm' chromosomal region was introduced into the **bamD** mutant by P1 transduction. The **bamDS** and **bamD5** **ΔyiaD**:Cm' mutants were streaked onto the Antibiotic medium 3 plate containing Cm and incubated at 30 °C.
lipoprotein that is anchored to the outer membrane through an N-terminal lipid attachment and is non-covalently linked to the peptidoglycan layer through a periplasmic domain. Our results suggest that the interaction of YiaD with peptidoglycan is important to YiaD function. YiaD is not essential for cell growth and is an accessory protein of BamD. Because BamD interacts directly with BamA and stabilizes the BamA complex, YiaD may function as a structural support for BamD and may contribute to stabilization of the BamA complex. Alternatively, the level of BamD in the outer membrane may be increased by YiaD overproduction. Further analysis is necessary to clarify specific role.

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