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Identification and characterization of the *Escherichia coli envC* gene encoding a periplasmic coiled-coil protein with putative peptidase activity

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Abstract

PM61 is a chain-forming *envC* strain of *Escherichia coli* with a leaky outer membrane. It was found to have an oversized penicillin-binding protein 3, which was the result of an IS*4* insertion in the *prc* gene. The other properties of PM61 were caused by the *envC* mutation. We cloned the *envC* (*yibP*) gene and identified the mutation site, causing a single residue substitution H366Y, in the PM61 *envC* allele. The gene product was predicted to be a periplasmic protein having coiled-coil structure in the N-terminal region and homology to lysostaphin in the C-terminal region. Overexpression of *envC* inhibited cell growth, and overexpression of *envC61* caused cell lysis. Disruption of the chromosomal *envC* caused the same defects as *envC61*, indicating the gene is dispensable for growth but important for normal septation/separation and cell envelope integrity.

1. Introduction

PM61 is a non-conditional, morphological mutant of *Escherichia coli* that forms chains of cells of irregular lengths [1]. It carries the only known mutant allele of *envC*. In this strain septation is partially blocked so that some cells become longer than normal, and cell separation is delayed with daughter cell poles remaining fused to each other for several generations so that cells form chains. PM61 also shows hypersensitivity to dyes, leakage of periplasmic proteins and many other defects indicating perturbation of the outer membrane [2]. The *envC* mutation was mapped very close to *cysE* at min 81.5 [3].

In early 1990s a DNA fragment that corrected the dye sensitivity of PM61 was cloned on a multicopy plasmid [4,5]. It contained two genes, both necessary for correction of the sensitivity, which were named *envC* and *envD*. The latter encodes a multidrug resistance efflux protein and the former an inner-membrane lipoprotein associated with the efflux protein [6]. In the recent *E. coli* linkage map [7] they are renamed *acrE* and *acrF*, respectively. The *acrEF* operon was mapped at min 73.5 [8], far from the position where the *envC* mutation had been mapped, and thus most likely is a multicopy suppressor distinct from the true *envC*. In

this report we cloned the *envC* gene whose allele in PM61 contained a point mutation and characterized it.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strains used in this study are listed in Table 1. The *envC*-carrying plasmids constructed in this study are illustrated in Fig. 2. pHR499 was constructed by cloning the *envC*-containing *Eco*RI-*Hin*cII fragment of pHR489 into the *Eco*RI-*Aat*II region of pBR322 after blunting the *Aat*II-cut end and by inserting the *Hae*II fragment containing the chloramphenicol resistance (*cat*) gene of pACYC184 into the *Eco*T14I site after blunt-ending. pHSK11 is a kanamycin-resistant, multiple cloning site (MCS)-carrying derivative of low-copy number plasmid pHS1 whose replication is thermosensitive [9]. The MCS was introduced by replacing the 3.3-kb *Eco*RI-*Hpa*I fragment containing the tetracycline resistance (*tet*) gene of pHS1 with a partially digested 1.6-kb *Eco*RI-*Dra*I fragment containing the MCS and the ampicillin resistance (*bla*) gene of pUC18. Then the 0.9-kb *Pvu*I fragment containing most of the *bla* gene was replaced with the 1.5-kb *Hae*II fragment containing the kanamycin resistance (*kan*) gene of pUC4K after blunt-ending.

2.2 Genetical and recombinant DNA procedures

PCR primers used in the cloning of the *prc* mutant allele were 5′-AAGCACCTCGCGAA-GAACAGCACA-3′ (corresponding to nucleotides [nt] 77-100 of DNA database entry under accession no. D00674) and 5′-CATTACGGCCAGGTTCGTTAGCAG-3′ (nt 2,571-2,548). Primers for the *envC* mutant allele were 5′-ACAACTGCTGATCACCGCTGACCA-3′ (nt 3,998-4,021; accession no. AE000439) and 5′-ATCAGCGTGCTCTGGCTGATGCTT-3′ (nt 6,412-6,389). Primers for the cloning of *envC* and *envC61* into pTrc99A [10] were 5′-AGG-GGAAAGGCGATTAATACCATG-3′ (starting with the second codon) and 5′-AACAAAgCT-

*TA*TCTTCCCAACCACGGCTGTGG-3′ (containing the termination codon [italicized] and a single base substitution [lower case] introduced to create a *Hin*dIII site [underlined]). The PCR products were inserted into the *Nco*I (blunt-ended)-*Hin*dIII region of pTrc99A, and the constructs, pHR648 and pHR651, were verified by sequencing. The *Escherichia coli* Gene Mapping Membrane was purchased from Takara Shuzo. The *envC*::*cat* allele was introduced into the chromosome using a replication-thermosensitive plasmid [11]. After pHR480, a pHSK11 derivative carrying *envC*::*cat*, was integrated at 42°C into the chromosome of MG1655 and resolved at 30°C, the disrupted allele retained on the chromosome was P1-transduced into JE7989 harbouring pSN5 by selecting for Cys^+ and then screening for chloramphenicol resistance.

2.3. Radioactive labelling and analysis of proteins

PBPs were assayed with [*phenyl*-4(n)-^{3}H]benzylpenicillin (Amersham) [12]. Clavulanic acid was used when necessary to inactivate the plasmid-coded β-lactamase [13]. Maxicells [14] were prepared from CSR603 harbouring pHR499 and pHR506, labelled with Pro-mix L-[^{35}S] (Amersham) and fractionated as described [15]. Radioactive protein bands in sodium dodecyl sulfate (SDS)-polyacrylamide gels were detected by fluorography or with the BAS phosphoimaging system (Fuji Photo Film Co.).

3. Results and discussion

*3.1. An IS*4 *insertion in the* prc *gene of PM61*

Penicillin-binding protein 3 (PBP 3) is an essential enzyme required for septation. Mutants conditionally defective in PBP 3 are blocked in septation under restrictive conditions [12,16]. Since the morphological defect of PM61 was related to completion of septation, we examined the strain for its PBP 3. PBP assay showed that PBP 3 of PM61 was larger in molecular size than the normal PBP 3 present in the parent strain P678 and other wild-type

strains. P1 transductions of *cysE*::Tn*5* (tightly linked to *envC* [3]) from CBK286 and of *leu*$^+$ (>50% cotransducible with *ftsI* at min 2.0) from W3110 gave transductants all of which had the oversized PBP 3, indicating that the PBP 3 anomaly was not caused by the *envC* mutation nor by a mutation in *ftsI*, the structural gene for PBP 3. When *ruv*::Tn*10* (linked to *prc* at min 41.2) was transduced from N2058, some transductants had normal PBP 3. Introduction of pHR164, a pBR322 derivative carrying *prc*$^+$ [17], resulted in normal PBP 3. Hence PM61 carries a mutation allelic to *prc*. The *prc* gene encodes a processing enzyme that cleaves a C-terminal peptide from the PBP 3 precursor [17-20]. *Pst*I-digested chromosomal DNA was examined by Southern hybridization using a radiolabelled 2.9-kb *Eco*RI fragment containing the *prc* gene as a probe. The fragments from PM61 differed in size and indicated the presence of an additional *Pst*I site. PCR amplification of the whole *prc* region from PM61 gave a product longer than the wild type by 1.4 kb. Cloning and sequencing of the PCR product revealed the presence of an insertion sequence IS*4* [21] in the midst of the *prc* gene, at nt 1,618 [17]. IS*4* is 1,426-bp long and has a *Pst*I site in it. Following the IS*4* sequence was a duplication of the 12 nucleotides of 1,607-1,618. The sequence in and around the duplication showed a weak similarity to the previously published target sequences [22].

The *prc* function is inactivated in PM61, and the oversized PBP 3 is a precursor form with the C-terminal extension peptide unprocessed. The *prc* mutants grow as normal rod cells under the condition where PM61 forms cell chains, although cells become elongated at 42°C in salt-free L broth [17].

3.2. Properties of the envC *mutant*

The *prc* null mutation causes a thermosensitive growth defect on a salt-free L agar medium [17,23], and PM61 showed this Ts(-NaCl) phenotype. The *ruv*::Tn*10 prc*$^+$ transductants of PM61 were Ts(-NaCl). When a *cysE*::Tn*5* derivative of P678 (JE7935) was transduced to Cys$^+$ using a P1 lysate prepared on PM61, more than 80% of the transductants were Ts(-NaCl). All of these thermosensitive transductants showed a morphological defect very similar to that of PM61 whereas the thermoresistant transductants showed the wild-type

morphology (Fig. 1A-D). The Ts(-NaCl) transductants should have received the *envC* mutation from PM61. PM61 was reported to leak periplasmic proteins to the culture media [2] as the *prc* mutant did [17]. All of the Ts(-NaCl) transductants showed this leaky phenotype when tested on RNase indicator plates [24]. The *envC* mutation alone, even when segregated from the *prc* mutation, caused thermosensitivity at low osmolarity and a permeability barrier defect. A transduction experiment using a *cysE*::Tn*5* derivative of MG1655 (JE7989) as a recipient gave essentially the same result (Fig. 1E-G), indicating the irrelevance of the *fic* (filamentation induced by cAMP) mutation at min 75.2 in P678 [25,26]. All the *envC* transductants were inhibited by 10 μg ml^{-1} crystal violet. This property was utilized when we cloned the *envC* gene in the next subsection.

Cell morphology of the transductants that received the *envC* mutation from PM61 indicated that the mutation was responsible for the defect in septation and separation. But the morphology varied slightly from that of PM61: similarly long chains of cells were formed but with a tendency toward fewer septa and longer individual cells. Transduction of a *prc* deletion/insertion allele [23] into *envC* strain JE7987 appeared to give a slight bias toward shorter cells but did not completely reproduce the morphology of PM61. For explanation of the subtle difference, we may have to assume an additional mutation(s) in PM61 making a minor contribution to the morphological defect as suggested previously [3]. PM61 was isolated by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine treatment [1], which is known to often cause multiple mutations.

When incubated in salt-free L broth at 42°C, JE7987 showed a heavier septation defect than in regular L broth at 37°C, resulting in formation of long filaments with no apparent septum.

3.3. Cloning of the envC *gene and identification of the mutation site*

The results described above confirmed that the *envC* mutation, largely responsible for the characteristic morphology of PM61, was located very close to *cysE* at min 81.5. The *acrE* gene at min 73.5, once reported as *envC* [4], cannot be allelic to *envC*. So we decided to clone

the true *envC* gene. *Eco*RI-digested chromosomal fragments of W3110 were inserted into the *Eco*RI site of low-copy number vector pGB2 [27] and introduced into JE7987. The transformants that formed colonies in the presence of 10 μg ml^{-1} crystal violet were selected and screened for the complemented leaky phenotype on RNase indicator plates. Two such transformants were isolated, and they were complemented also for the morphological defect and Ts(-NaCl). Both plasmids thus obtained carried a 3.5-kb *Eco*RI fragment of the same restriction pattern. When one of them, named pSN5 (Fig. 2), was tested, it complemented the defects of PM61 as well (Fig. 1HI). Probing the dot blots of Kohara miniset library [28] with digoxigenin-labelled pSN5 gave positive signals with clones 574 and 575 derived from the min 81-82 region. The *cysE* gene is in the middle of clone 575.

We determined the nucleotide sequence of the *Eco*RI fragment. It perfectly matched nt 3,388-6,916 of the DNA database entry under accession no. AE000439. Two ORFs, named as *yibP* and *yibQ* in this database entry, are in the fragment (Fig. 2). The *Bcl*I fragment carrying *yibP* only was subcloned into the *Bam*HI site of pGB2 (pHR488 and pHR489; Fig. 2) and found to complement the *envC* mutation for Ts(-NaCl), leaky phenotype, dye sensitivity and morphological defect (see Fig. 1LM). The *yibP* gene seems to have its own promoter within the *Bcl*I fragment, because the complementation was observed even when cloned in the orientation opposite to the *aadA* promoter on the vector.

Next, the PCR-amplified *yibP* region of JE7987 was cloned and sequenced. A substitution C→T was found at nt 5,337, causing an amino-acid substitution His→Tyr at the 366th position of the gene product (see below). We conclude that *yibP* is the *envC* gene mutated in PM61 and responsible for the pleiotropic phenotype. We will refer to the mutant allele as *envC61*.

3.4. EnvC protein

In the DNA sequence database, *yibP* is annotated as encoding a 427-residue polypeptide with a predicted molecular weight of 47,464. The initiation codon is assigned to an AUG codon at nt 4,242-4,244. In its immediate upstream region, however, no appropriate sequence

corresponding to a ribosome-binding site can be found. Twenty-four nucleotides downstream is a second AUG codon, which is preceded by a potential ribosome-biding site, AAGG, at nt 4,251-4,254. The gene product starting with this second AUG codon would be a 419-residue polypeptide of 46,592 Da. We don't know at present which AUG codon is actually used as an initiation codon. In this report we use the residue numbering according to the DNA database.

The N-terminal 42 residues of the deduced amino acid sequence show a feature commonly found for signal peptides. Cleavage of the 42 residues would yield a mature product of 42,923 Da, which is considerably hydrophilic.

When proteins were radiolabelled in maxicells harbouring pHR499, in which *envC* and *cat* were cloned on pBR322 without disrupting *bla* or *tet* on the vector (Fig. 2), and analyzed by SDS-gel electrophoresis, two protein bands of about 47 and 43 kDa (the former in a much smaller amount) were detected in addition to antibiotic resistance gene products (Fig. 3). These two bands disappeared when pHR506, in which a 2 bp deletion was introduced to the unique *Sac*II site within *envC* in pHR499 by an exonuclease reaction of T4 DNA polymerase followed by ligation (Fig, 2), was subjected to a maxicell experiment. This disruption should truncate the gene at the 253rd codon and add 10 extra codons, resulting in the reduction of the product size by 17.8 kDa. A faint band of about 30 kDa was probably the truncated form of the 47 kDa product, and the truncated form of the 43 kDa product may have overlapped Cat protein (25.7 kDa). When the maxicells harbouring pHR499 were converted to spheroplasts and centrifuged, the 43 kDa product, but not the 47 kDa product, was recovered in the supernatant, which contained periplasmic proteins including Bla although there was a small leakage of the cytoplasm from broken spheroplasts as judged from the distribution of Cat protein. The 43 kDa product was also found, together with the 47 kDa product, in the pelleted spheroplasts. Although further fractionation of the spheroplasts by freeze-thawing, centrifugation and solubilization of the inner membrane by *N*-lauroylsarcosinate was imperfect and the fractions were contaminated with each other, both the 47 and 43 kDa products were distributed to the fractions in a way very similar to Tet, an inner-membrane protein. The 47 and 43 kDa proteins are most probably the primary and processed products, respectively, of the *envC* gene. The mature EnvC protein seems to be a periplasmic protein with an affinity, higher at least than Bla

protein, to the cytoplasmic membrane, although the overproduction in maxicells may have affected the localization.

The N-terminal half of the mature form is composed mostly of the sequences with a repetitive pattern characteristic of coiled-coil structure. The Paircoil program [29] predicted three long heptad repeats with short interruptions: residues 59-133 (at 100% probability for coiled coil), 168-201 (99.0%) and 207-252 (78.1%). The COILS program [30] gave essentially the same prediction. This structure is possibly involved in the oligomerization of EnvC protein or the interaction with another protein(s) [31].

The C-terminal 99 residues show a high homology to the C-terminal regions of *E. coli* NlpD and hypothetical YebA proteins (41 and 33 identical residues, respectively). As described for NlpD [32], this region has a homology to lysostaphin, an extracellular peptidase that degrades Staphylococcal peptidoglycan, and may have a hydrolytic function in cell wall formation or maintenance. The 366th His, which is mutated in *envC61*, is located in this region and conserved among EnvC, NlpD and YebA but not in lysostaphin.

3.5 Overexpression of envC *and* envC61

When the *envC* gene was cloned under the *trc* promoter on multicopy plasmid pHR648 (Fig. 2) and its expression was induced in DH5 by addition of isopropyl-thio-β-D-galactoside, the growth was inhibited in an hour (Fig. 4A). The cell length became irregular, and some swollen cells and ghosts were observed (Fig. 4BC). This might be due to the hydrolytic function of the C-terminal region. Overexpression of the *envC61* allele cloned on pHR651 caused a similar morphological aberration and cell lysis (Fig. 4AD). Thus, the mutant protein was unlikely to be very unstable. It exerted an even more deleterious effect than the wild-type protein in spite of the mutated C-terminal region. It might interact with the chromosomally coded EnvC protein through their N-terminal coiled-coil regions and lead to uncontrolled hydrolytic function.

3.6. Insertional inactivation of the chromosomal envC *gene*

The *envC* gene was disrupted at the *Pvu*II site by inserting the blunt-ended *Hae*II fragment containing the *cat* gene of pACYC184 (pHR480; Fig 2) and crossed into the chromosome of a strain harbouring pSN5 as described in Materials and methods. The inserted *cat* gene was in the same orientation as the *envC* gene and unlikely to cause a polar effect [16] on the downstream *yibQ* gene. The insertion introduced a termination codon immediately after the 142nd codon in the midst of the coiled-coil region. A P1 phage lysate prepared on the pSN5-harbouring *envC*::*cat* strain was used to transduce JE7989 with or without pSN5 to Cys^+. Chloramphenicol resistance was cotransduced at almost the same frequency of about 80% irrespective of the presence or absence of pSN5, indicating dispensability of the *envC* gene for viability. However, the *envC*::*cat* transductants without pSN5 showed all the defects described above for *envC61*, namely, Ts(-NaCl), leakage of periplasmic RNase, dye sensitivity and morphological defect (Fig. 1JK), which were complemented by pHR488 and by pHR489 (Fig. 1LM). This suggests that EnvC protein is important for normal septation/separation and cell envelope integrity and that the *envC61* mutation abolishes this important function as does the null mutation. Genes encoding homologues of EnvC are present in many Gram-negative bacteria, and their products possibly play a similar important role.

The *envA1* mutant is another chain-forming strain showing defects similar to those of the *envC* mutant [33]. The *envA* gene encodes an enzyme required for lipid A biosynthesis [34]. Thus, a defective outer membrane interferes not only with the permeability barrier but also with septation/separation. A defect in EnvC, a putative lysostaphin-like peptidase, which causes a delay in cell separation, may secondarily affect outer membrane assembly at the division site. This suggests a close interdependence between cell separation and outer membrane assembly during the division process.

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Figure legends

Fig. 1. Cell morphology of the *envC* strains. Cells were grown at 37°C to an early log phase in L broth containing 0.5% NaCl and 10 mM glucose and examined under phase-contrast microscopy. Under this culture condition, PM61 showed its typical cell morphology at an early to mid log phase and rather healthier morphology at a late log to stationary phase. A: PM61. B: JE7935 (P678 *cysE*::Tn*5*). C: JE7987 (a *cysE*$^+$ *envC* transductant). D: JE7988 (a *cysE*$^+$ *envC*$^+$ transductant). E: JE7989 (MG1655 *cysE*::Tn*5*). F: JE7991 (a *cysE*$^+$ *envC* transductant). G: JE7990 (a *cysE*$^+$ *envC*$^+$ transductant). H: PM61 harbouring pGB2. I: PM61 harbouring pSN5. J: JE7992 (*envC*::*cat*). K: JE7994 (*envC*$^+$, isogenic to JE7992). L: JE7992 harbouring pHR488. M: JE7992 harbouring pHR489. Bar, 10 μm.

Fig. 2. Plasmids carrying the *envC* gene. Only the cloned chromosomal fragments are shown (filled bars). An open square in pHR506 is a 2-bp deletion. A gray bar is the *cat*-containing fragment inserted in pHR480 . At the top are map position (min) and genomic address (kb). Orientation and approximate sizes of the genes are shown by open arrows. Open arrowheads denote the direction of the promoters on the vectors. pHR499 and pHR506 carry the *cat* gene in addition to the fragments shown here. Only relevant restriction sites are indicated: B, *Bcl*I; E, *Eco*RI; P, PvuII; S, *Sac*II.

Fig. 3. Identification and localization of the *envC* gene product in maxicells. Radiolabelled maxicells harbouring pHR499 (W) and pHR506 (Δ) and the fractionated samples of the pHR499-harbouring maxicells were analyzed by SDS-gel electrophoresis with 10% polyacrylamide. Maxicells were incubated with lysozyme in a hypertonic buffer and centrifuged. The supernatant was the periplasmic fraction (P). The precipitated spheroplasts were disrupted by freeze-thawing and separated by centrifugation into the supernatant (cytoplasmic fraction, C) and the precipitate (membrane fraction, M). The latter was treated with 1% *N*-lauroylsarcosinate and centrifuged to obtain the solubilized inner membrane fraction (I). Plasmid-coded products are shown on the right. preEnvC, the precursor form of EnvC.

Protein bands in the lower parts of lanes probably include the product of the *yibQ* gene truncated at the *BclI* site within it (16.3 kDa) and Rop protein encoded by pBR322 (7.2 kDa).

Fig. 4. Overexpression of *envC* and *envC61*. A: Exponentially growing DH5 harbouring pTrc99A (circles), pHR648 (squares) and pHR651 (triangles) were incubated in buffered L broth-glucose-thymine [16] containing 50 μg ml^{-1} ampicillin in the presence (closed symbols) and the absence (open symbols) of 0.5 mM isopropyl-thio-β-D-galactoside. The growth was monitored with a Klett-Summerson colorimeter equipped with a no. 66 filter. B-D: Phase-contrast micrographs of DH5 harbouring pTrc99A (B), pHR648 (C) and pHR651 (D) incubated for 1.5 hr in the presence of the inducer. Bar, 10 μm.

Table 1

Bacterial strains

Strain	Relevant genotype or comment	Source or reference
W3110	Wild type	Laboratory collection
MG1655	Wild type	GSRC[a]
DH5	*recA1 endA1 hsdR17*	Laboratory collection
CSR603	*recA1 uvrA6 phr-1*	[14]
P678	*leuB6 fic*	[1,25,26]
PM61	P678 *envC61 prc*::IS*4*	[1], this work
CBK286	*cysE*::Tn*5*	[35], GSRC[a]
N2058	*ruv-59*::Tn*10*	[36]
JE7934	W3110 Δ*prc*::*neo*	[23]
JE7935	P678 *cysE*::Tn*5*	P1(CBK286) × P678
JE7987	P678 *envC61*	P1(PM61) × JE7935
JE7988	*envC*$^+$, isogenic to JE7987	P1(PM61) × JE7935
JE7989	MG1655 *cysE*::Tn*5*	P1(CBK286) × MG1655
JE7991	MG1655 *envC61*	P1(PM61) × JE7989
JE7990	*envC*$^+$, isogenic to JE7991	P1(PM61) × JE7989
JE7992	MG1655 *envC*::*cat*	P1 transduction from a pSN5-harbouring *envC*::*cat* strain to JE7989; see text
JE7994	*envC*$^+$, isogenic to JE7992	as above; see text

[a]GSRC, Genetic Strains Research Center of the National Institute of Genetics, Mishima, Japan.

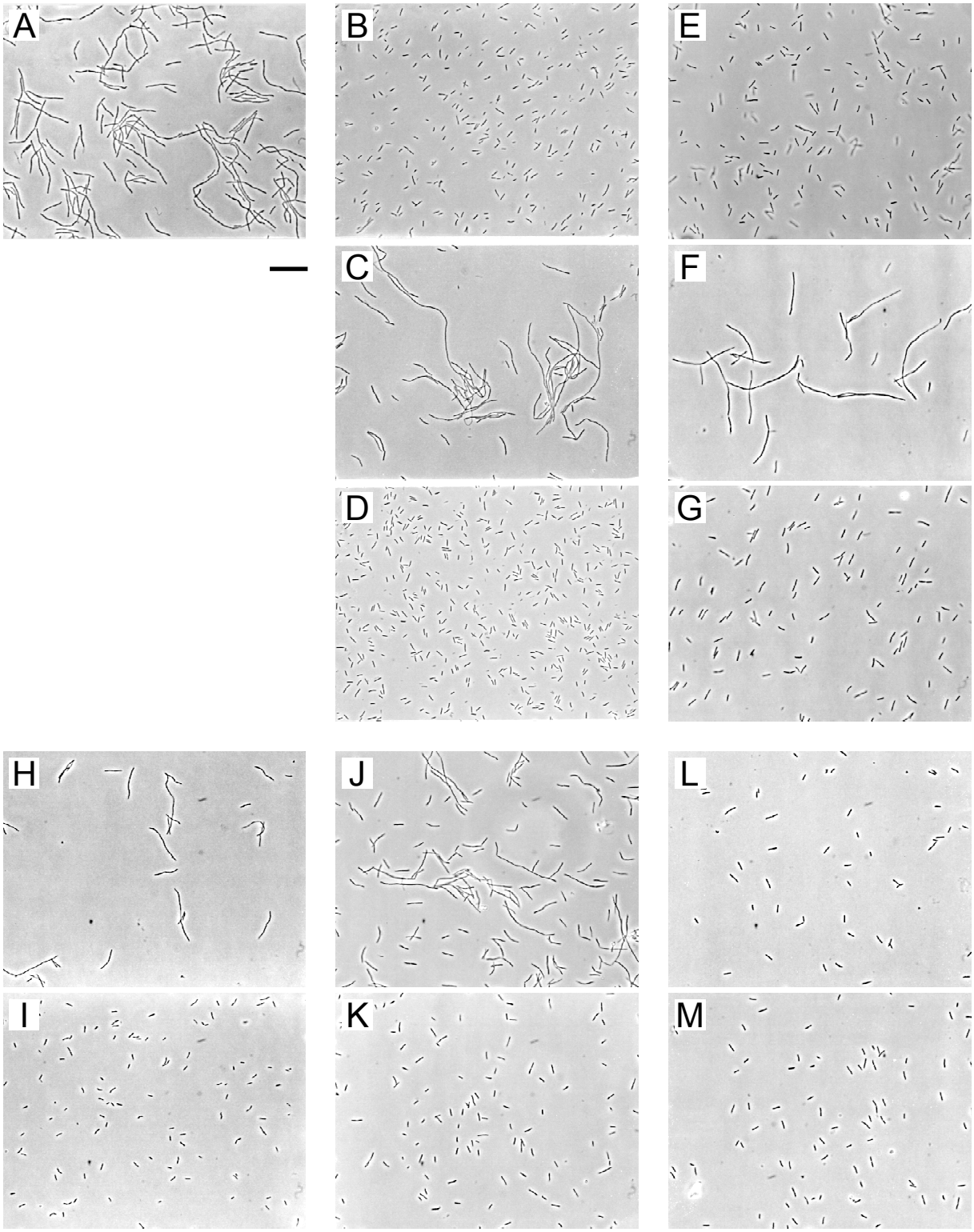
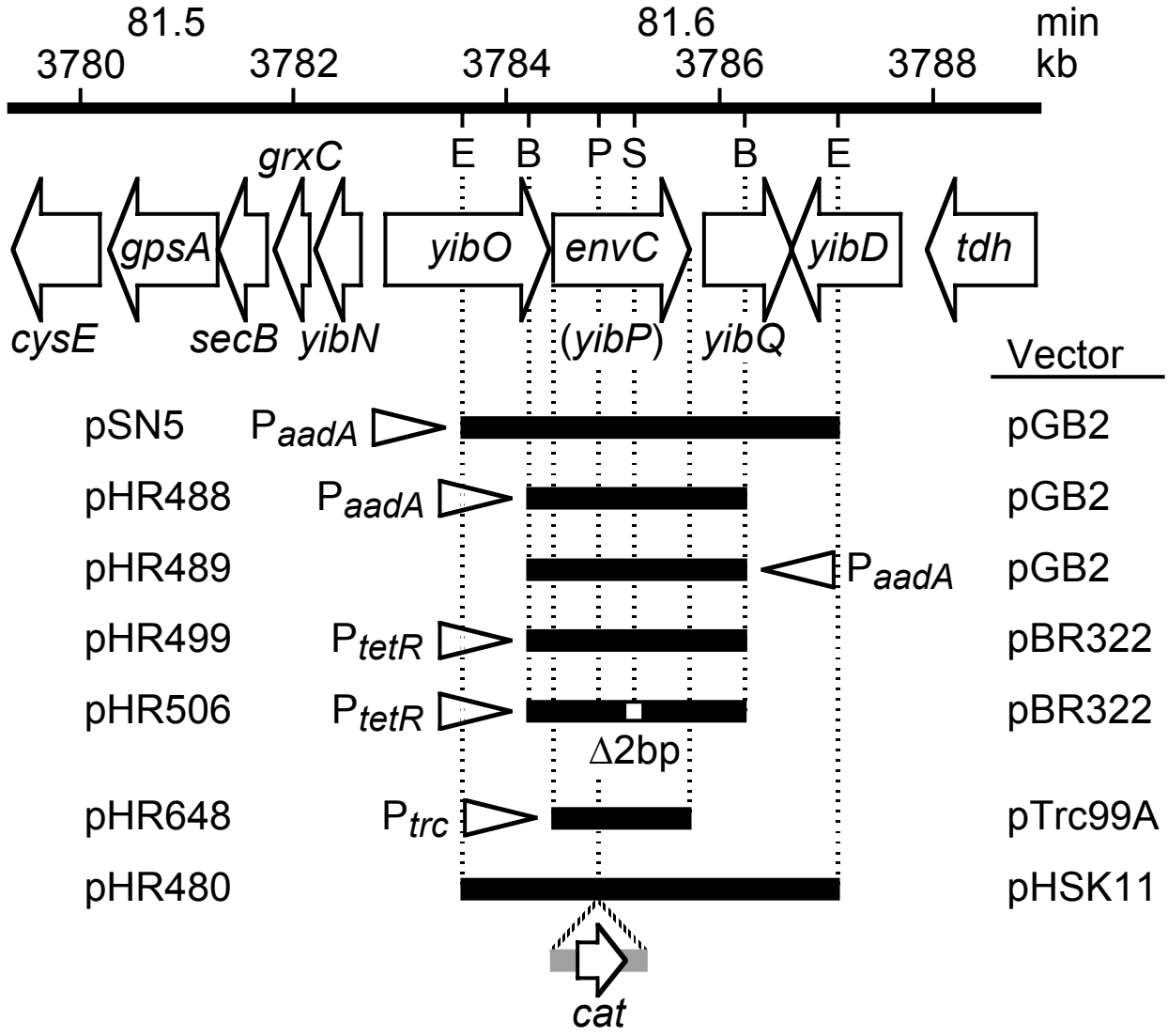


Fig. 1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81.6
min
3780
3782
3784
3786
3788
kb
E
B
P
S
B
E
grxC
gpsA
yibO
envC
yibD
tdh
cysE
secB
yibN
(yibP)
yibQ
Vector
pSN5
P aadA
pGB2
pHR488
P aadA
pGB2
pHR489
P aadA
pGB2
pHR499
P tetR
pBR322
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Δ2bp
pHR648
P trc
pTrc99A
pHR480
pHSK11
cat

Fig. 2

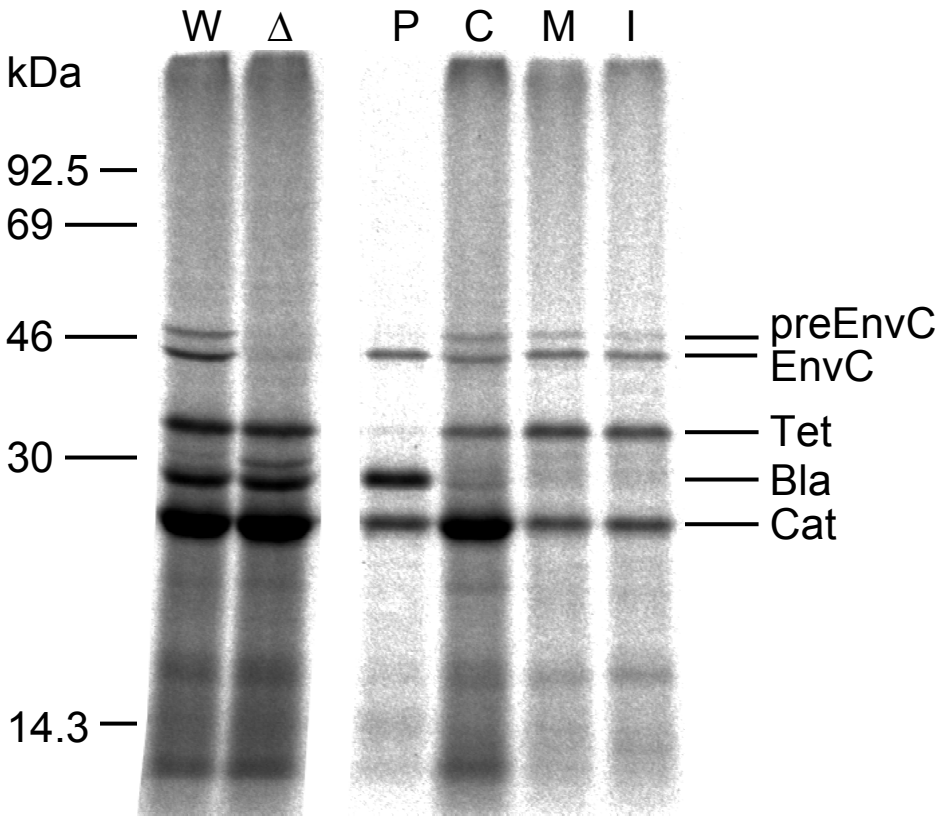

Fig. 3

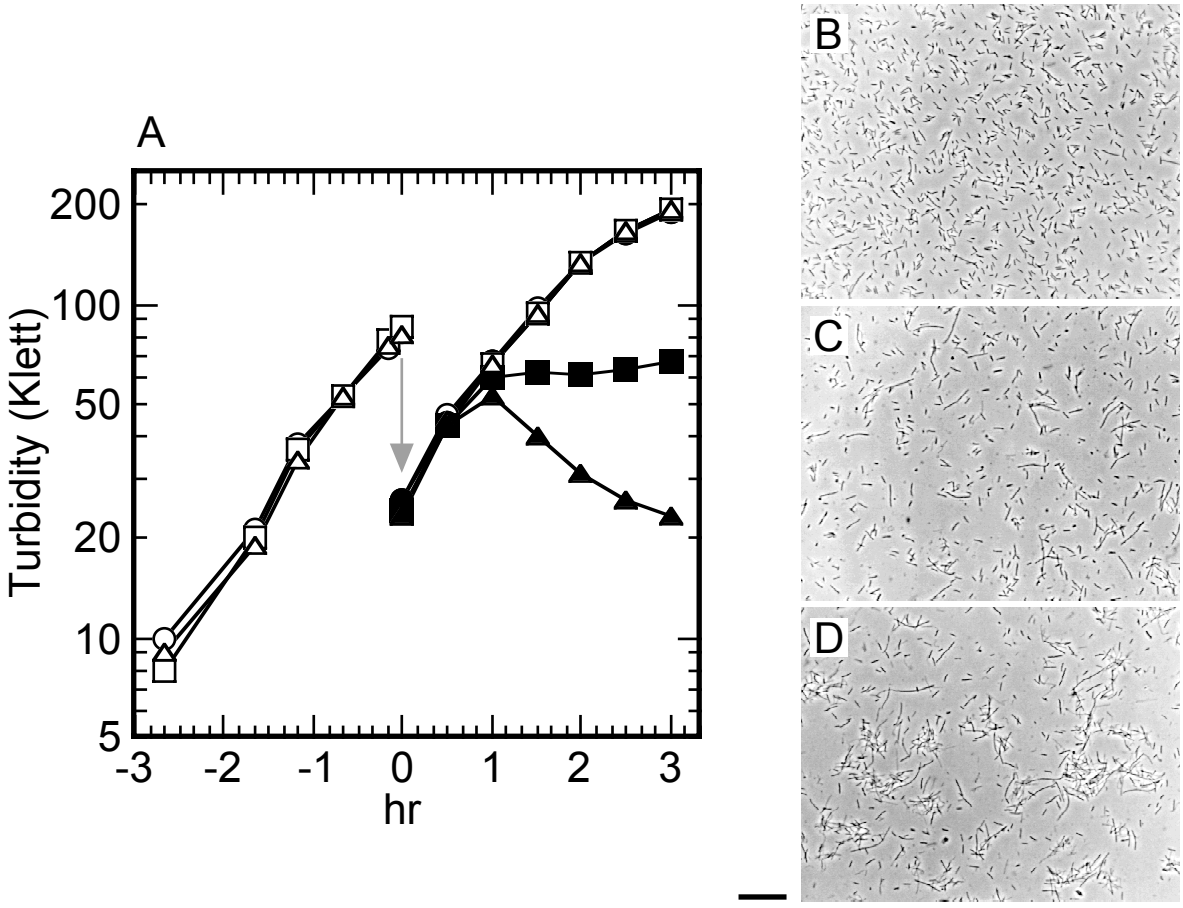

Fig. 4