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Novel *Pseudomonas aeruginosa* Gene That Suppresses Tolerance to Carbapenems

Kazuko Taniguchi,[1] Tsuneko Ono,[2]* Keiji Murakami,[1] Darija Viducic,[1] Sizuo Kayama,[1] Katsuhiko Hirota,[1] Ken Nemoto,[1] and Yoichiro Miyake[1]

Department of Microbiology, Tokushima University School of Dentistry, Tokushima 770-8504,[1] and Major in Laboratory Science, Tokushima University School of Health Science, Tokushima 770-8509,[2] Japan

A biapenem-tolerant mutant of *Pseudomonas aeruginosa* was isolated by Tn*1737*KH insertion. The survival of the mutant 3 h after the addition of biapenem was about 1,000 times greater than that of the wild type. The mutant was also tolerant to other biapenems, such as imipenem, panipenem, and meropenem.

Pseudomonas aeruginosa infections are difficult to eradicate with antibiotic treatment. It is well-known that biofilm bacteria are more resistant to the killing effect of antibiotics than planktonic bacteria. It has previously been reported that adherent bacteria on a solid surface were already tolerant to antibiotics even before biofilm formation (5). It seems that one of the mechanisms of tolerance is related to a stress response by adherent cells to attachment stimuli on a solid surface. In that case, an antibiotic-tolerant mutant(s) which cannot regulate the gene involved in survival during attachment on a solid surface will probably be obtained among the planktonic cells. We have searched for the gene(s) involved in bacterial survival in the presence of antibiotics and, as a result, isolated a mutant more resistant to the killing by biapenem than the wild type from planktonic cells of *P. aeruginosa*. Here we report a gene, termed *tcp* in this study, that has a suppressive role in antibiotic tolerance.

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. DNA manipulations were performed by standard procedures (6). The MIC and minimal bactericidal concentration (MBC) of each antibiotic were determined by the broth microdilution method as previously described (5) with the following modification: the bacterial suspensions at a density of 0.5×10^6 to $\sim 1 \times 10^6$ cells/ml were incubated in Luria-Bertani (LB) broth.

Susceptibility to antibiotics was also determined by the killing test. The cultures were grown overnight in LB broth supplemented with the appropriate antibiotics, and the bacterial cells were resuspended in 10 ml of LB broth. The suspension was incubated for 4 to 10 h at 37°C in the presence of each carbapenem, at a concentration ranging from 8 to 32 µg/ml. The number of live cells was determined by colony counting.

The transposon insertion mutant(s) was constructed as follows. *Escherichia coli* strain CT726 harboring Tn*1737*KH in plasmid pMT6121 (14), which is a temperature-sensitive plasmid for replication, was mobilized to *P. aeruginosa* SM7 cells (9). The kanamycin- and mercury-resistant and temperature-

sensitive transconjugant was isolated. The transconjugant *P. aeruginosa* KMX78 strain was grown at 30°C for 17 h, and then the cells were transferred to 96-well microtiter plates. The plates were incubated at 42°C for 24 h, and temperature-

TABLE 1. Bacterial strains, plasmids and primers used in this study

Strain, plasmid, or primer	Genotype, description, or sequence	Reference or source
Plasmids		
pACΩGm	pACYC184 derivative carrying Ω fragment, Gm^r	7
pMOB3	Km^r Cm^r, 5% sucrose sensitive	8
pGEM-T Easy	Ap^r and TA cloning vector	Promega
pMT6121	*oriT*, *sacB*, and Hg^r Km^r in Tn*1737*KH	M. Tsuda (14)
pTK01	*tcp* in pGEM-T Easy	This study
pTKG01	*tcp*::Gm^r in pGEM-T Easy	This study
pTKM01	*tcp*::Gm^r and *mob* in pGEM-T Easy	This study
pCR2.1	Ap^r Km^r and TA cloning vector	Invitrogen
pTKM02	*tcp* and *mob* in pCR2.1	This study
pTKM03	*tcp*, *orf2*, and *mob* in pCR2.1	This study
pTKM04	*tcp* and *mob* in pCR2.1	This study
Strains		
P. aeruginosa		
SM7	Sm^r derivative of PAO1	This study
SM21	Sm^r derivative of PAO1	This study
KMX78	Km^r Hg^r, SM7 derivative harboring pMT6121::Tn*1737*KH	This study
KMX7803	SM7 *tcp*::Tn*1737*KH(Km^r Hg^r)	This study
TKP011	SM21 *tcp*::Gm^r	This study
TKP02	KMX7803 derivative harboring pTKM02	This study
TKP03	KMX7803 derivative harboring pTKM03	This study
TKP04	TKP011 derivative harboring pTKM04	This study
E. coli		
CT726	DH1(pMT6121::Tn*1737*KH)	M. Tsuda (14)
S17-1	*thi endA recA hsdR* with RP4-2Tc::Mu-Km::Tn7 integrated in chromosome	10
Primers		
galk-s	5′-CGCAGAACAGGCAGCAGAGCGT TT-3′	This study
galk-a	5′-GCAGCAGAGGCGGATAAAAGT GCG-3′	This study
PAO561-s1	5′-GTGAAGGAAAGCGAGCACAACA AGGCGT-3′	This study
PAO561-a	5′-CGGGGTTAGAATGCCTCGCTC CCA-3′	This study
PAO561-s2	5′-GTGATTGCCACTGTCCCACTCCG CTCAT-3′	This study

* Corresponding author. Mailing address: Major in Laboratory Science, Tokushima University School of Health Science, Tokushima 770-8509, Japan. Phone: 81-88-633-9061. Fax: 81-88-633-9061. E-mail: ono@medsci.medsci.tokushima-u.ac.jp.

TABLE 2. Antibiotic susceptibility of planktonic cells

Strain	Biapenem		Cefoperazone		Cefepime		Ofloxacin		Gentamicin	
	MIC[a]	MBC[a]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
SM7[b]	1	1	4	8	2	2	1	1	0.25	0.5
KMX7803[c]	1	16	4	8	2	2	1	1	0.5	0.5

[a] MICs and MBCs given in micrograms per milliliter.
[b] *tcp*+ wild-type strain.
[c] SM7 *tcp*::Tn*1737*KH.

resistant mutants with the Tn*1737*KH insertion in their chromosome were selected. Strains harboring the chromosomal Tn*1737*KH insertion were first inoculated into LB broth supplemented with biapenem (8 μg/ml) in 96-well plates. Cultures that showed inhibited growth were collected from the wells and plated on LB agar containing kanamycin and mercury chloride.

The antibiotic susceptibility of strains obtained was determined by the broth microdilution method, and the biapenem-tolerant mutant *P. aeruginosa* KMX7803 strain was isolated. The Tn*1737*KH insertion site was determined by inverse PCR amplification using primers galk-s and galk-a and the Dye

Terminator Cycle Sequence method. The Tn*1737*KH transposon had been inserted into the open reading frame (ORF) of 1,150 bp referred to as PA0561 (GenBank accession no. AE004492) in the *Pseudomonas* Genome Project. No gene showing high homology to the ORF was found. The gene corresponding to this ORF was named *tcp* (named tcp for tolerance to carbapenem in planktonic cells).

In order to obtain a *tcp* gene knockout mutant, first, we amplified the *tcp* gene by PCR using primers PAO561-s1 and PAO561-a (GenBank accession no. AE004492; protein identification no. AAG03950.1) and PAO1 DNA as a template. Plasmid pTK01 was constructed by insertion of the *tcp* gene into a pGEM-T Easy vector (Promega). The gentamicin resistance gene of pACΩGm (7) was then inserted into the HindIII site of pTK01 to form pTKG01. Plasmid pTKM01 was constructed by ligation of a NotI fragment containing *tcp*::Gm^r from pTKG01 and a NotI fragment containing the *mob* cassette from pMOB3 (10), and it was transformed into *E. coli* S17-1 (10). After conjugal transfer of pTKM01 into *P. aeruginosa* SM21, we selected gentamicin-resistant strain TKP011, which contained the *tcp*::Gm^r insertion in place of the *tcp* gene, and confirmed the insertion site by DNA sequencing analysis. For complementation testing, plasmids pTKM02, pTKM03, and pTKM04 were constructed (Fig. 1), and complemented *P. aeruginosa* TKP02, TKP03, and TKP04 strains were obtained.

Whereas the MIC of biapenem for *P. aeruginosa* strain KMX7803 was exactly the same as the MIC for the wild type, the MBC for the strain was 16 times higher than that for the wild type (Table 2). The number of mutant cells 3 h after the addition of biapenem (32 μg/ml) was about 1,000 times greater than that of the wild type (Fig. 2A), while the growth curve for KMX7803 was the same as that for the wild type (Fig. 2B). MICs of cefoperazone, cefepime, gentamicin, and ofloxacin for KMX7803 were exactly the same as those for the wild type,
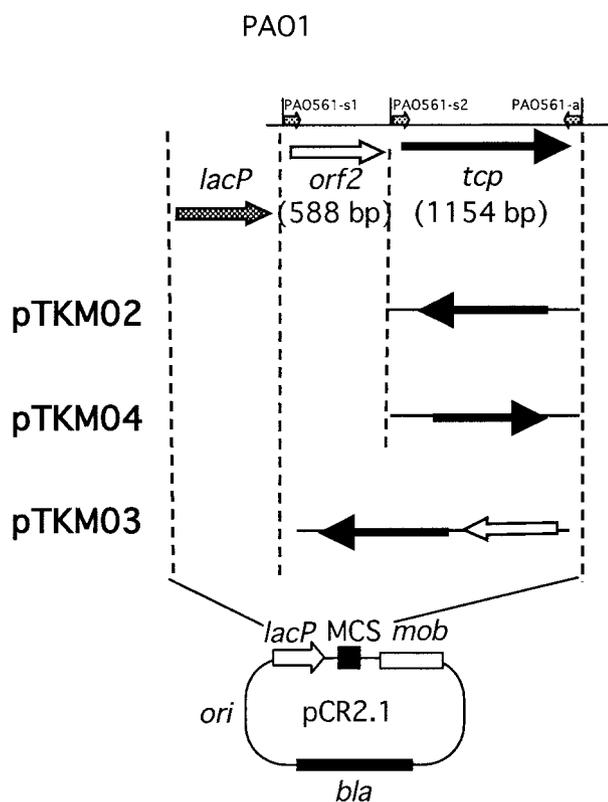
FIG. 1. Scheme showing the construction of plasmids for complementation testing. The primers (▧) for PCR were as follows: primers PAO561-s2 and PAO561-a for the *tcp* structural region only and primers PAO561-s1 and PAO561-a for the *tcp* structural gene and the upstream region of *orf2*. Plasmid pTKM02 carried the *tcp* structural region in the orientation opposite that of the *lac* promoter in the pCR2.1 vector. Plasmid pTKM03 carried the *tcp* and the *orf2* in the reverse direction, and pTKM04 carried the *tcp* structural gene in the forward direction. The *tcp* gene [➤], *orf2* gene [⇨], and *lac* promoter [➡] are shown. Drawings are not to scale. MCS, multiple cloning site.

TABLE 3. Carbapenem susceptibility of *tcp* knockout mutants, complemented strain, and parent strains

Strain	Biapenem		Imipenem		Panipenem		Meropenem	
	MIC[a]	MBC[a]	MIC	MBC	MIC	MBC	MIC	MBC
SM7[b]	1	1	1	2	8	16	1	2
SM21[b]	1	1	1	2	4	8	1	2
KMX7803[c]	1	16	2	4	8	16	1	2
TKP011[d]	0.125	8	2	4	4	16	0.5	4
TKP04[e]	0.5	4	4	16	8	16	4	4

[a] MICs and MBCs given in micrograms per milliliter.
[b] *tcp*+ wild-type strain.
[c] SM7 *tcp*::Tn*1737*KH.
[d] SM21 *tcp*::Gm.
[e] TKP011(pTKM04 *tcp*+).
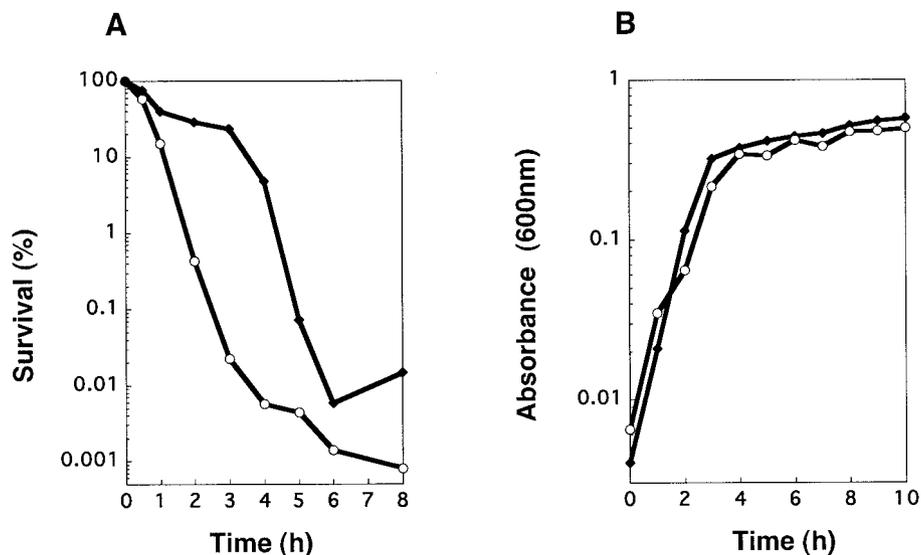
FIG. 2. (A) Killing curves for biapenem (32 μg/ml) on *P. aeruginosa* SM7 and KMX7803 and (B) growth curves of *P. aeruginosa* SM7 and KMX7803. For panel A, assuming that the survival at zero time (1×10^9 to 2×10^9 CFU/ml) was 100%, the number of CFU was changed to a percentage. Symbols: —○—, SM7 (wild type); —◆—, KMX7803 (*tcp*::Tn*1737*KH).

except for the MIC of gentamicin, which was two times higher than the MIC for the wild type. The MBCs of cefoperazone, cefepime, gentamicin, and ofloxacin for KMX7803 were the same as those for the wild type (Table 2).

The MBC of biapenem for the *P. aeruginosa tcp*::Gm^r strain TKP011 was 64 times higher than the MIC. The MBC/MIC ratios of the other carbapenems for the mutant were not as high as that of biapenem (Table 3). It appears that MBC is not a suitable or reliable measure of tolerance, because it is defined as the endpoint survival of more than 99.9% cells. Ac-

cordingly, cell viability in the presence of antibiotic was determined to clarify whether the *tcp* gene affects only biapenem susceptibility. The survival rate of *P. aeruginosa* TKP011 after incubation in the presence of biapenem at 8 μg/ml was as high as that of *P. aeruginosa* KMX7803 (Fig. 3A). The survival rates of TKP011 to imipenem, meropenem, and panipenem were 10 to 100 times higher than those of the wild type (Fig. 4). Moreover, the survival rate of TKP04, complemented strain harboring pTKM04 (Fig. 1), was as low as that of the wild type (Fig. 3B). The survival of another complemented strain, TKP03, was
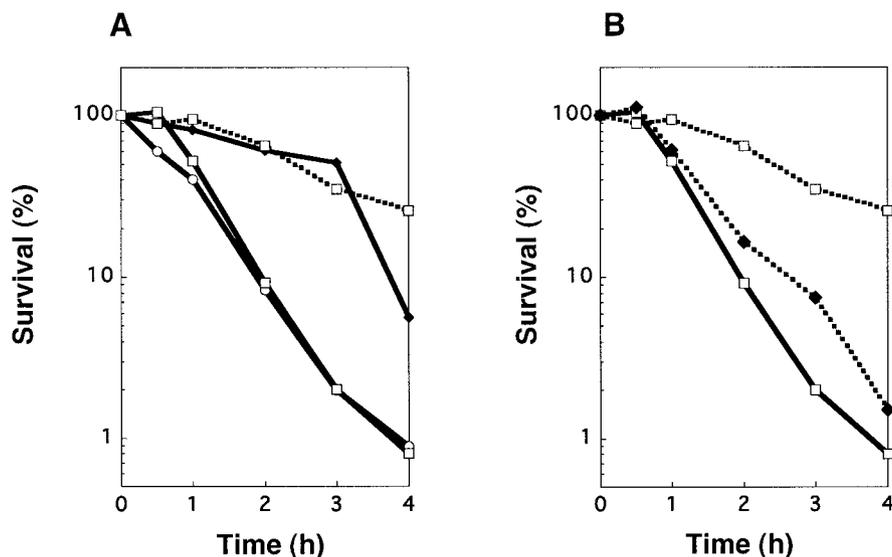
FIG. 3. Killing curve of *tcp* mutants in the presence of biapenem at 8 μg/ml. Assuming that the survival at zero time was 100% (1×10^9 to 2 $\times 10^9$ CFU/ml), the number of CFU was changed to a percentage. (A) Killing curve of *tcp* knockout mutant. [—○—], *P. aeruginosa* SM7 (parent strain for KMX7803); [—◆—], *P. aeruginosa* KMX7803 (*tcp*::Tn*1737*KH); [—□—], *P. aeruginosa* SM21 (parent strain for TKP011); [····□····], *P. aeruginosa* TKP011 (*tcp*::Gm^r) (B) Complementation of *tcp* gene. [—□—], *P. aeruginosa* SM21; [····□····], *P. aeruginosa* TKP011 (*tcp*::Gm^r); [····◆····], *P. aeruginosa* TKP04 (*tcp*::Gm^r, pTKM04).
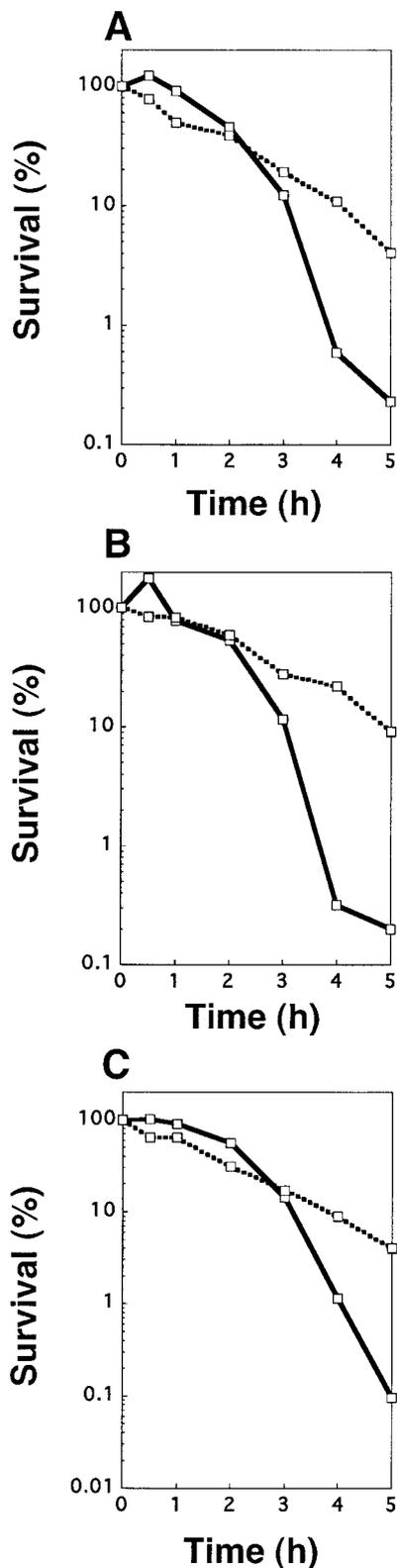
FIG. 4. Killing curves of *P. aeruginosa* SM21 and TKP011 in the presence of carbapenems. Assuming that the survival at zero time was 100% (1×10^9 to 2×10^9 CFU/ml), the number of CFU was changed to a percentage. Samples were incubated in the presence of imipenem at 16 µg/ml, panipenem at 8 µg/ml, or meropenem at 12 µg/ml for 5 h. After that, the number of CFU were counted. [—□—], SM21; [••••□••••], TKP011.

also equally low as that of the wild type (data not shown). In addition, the survival of TKP02, possibly lacking the *tcp* promoter, was shown to be as high as that of KMX7803 (data not shown). Therefore, constructed plasmids pTKM03 and pTKM04 could complement the mutation in strains KMX7803 and TKP011, respectively, but pTKM02 could not do so.

The results of this study show that the number of live cells of the *tcp* mutant was higher than that of the wild-type strain in the presence of carbapenem. We found no known motif sequence of penicillin-binding proteins in the Tcp protein and no homology between penicillin-binding proteins and Tcp. Autolysis in *tcp*-defective mutants was not affected by the *tcp* gene (data not shown). However, it cannot be concluded that the *tcp* gene is entirely unrelated to the autolytic process in *P. aeruginosa* (12). Biofilm and stationary-phase cells are more tolerant than planktonic cells (1–4, 13, 16). Tolerance to antibiotics in stationary-phase or biofilm cultures is largely dependent on the presence of persister cells, according to Spoering and Lewis (11). After biapenem addition, survival of adherent cells of the *tcp* mutant on a plastic surface was higher than that of planktonic cells (preliminary data). We are not sure whether the *tcp* gene affects the biapenem tolerance of adherent cells of *Pseudomonas aeruginosa*. It was reported that viability of attached bacteria was very high and that physiological adaptations occurred during the early phases of attached growth (15). For full understanding of the role of the *tcp* gene in the mechanism of tolerance to antibiotics, further study is needed to identify the Tcp protein and to elucidate the regulation of the *tcp* gene.

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