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A Transferable 20-Kilobase Multiple Drug Resistance-Conferring R Plasmid (pKL0018) from a Fish Pathogen (*Lactococcus garvieae*) Is Highly Homologous to a Conjugative Multiple Drug Resistance-Conferring Enterococcal Plasmid▽

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***Lactococcus garvieae*, the causative agent of lactococcosis, has evolved strains that are highly resistant to antibiotics. Here, the 20,034-bp sequence of *L. garvieae* conjugative plasmid pKL0018 was determined. It contained two *erm*B genes and one *tet*S gene and a backbone more than 96% identical to that of pRE25, an *Enterococcus faecalis* plasmid from dry sausage.**

Lactococcus garvieae, a gram-positive bacterium that causes lactococcosis, has caused serious economic damage to fish aquaculture worldwide (11). The appearance of *L. garvieae* strains resistant to macrolides and tetracycline following antibiotic treatment practices has compounded the problem. The newly acquired resistance was attributed partly to a transferable R plasmid(s) carried by the resistant strains, based on studies done as early as 1990 (1).

Of 170 strains of *L. garvieae* isolated from cultured *Seriola* species (yellowtail, amberjack, and kingfish) from nine prefectures in Japan in 2002, most have been reported to have high frequencies of erythromycin (EM), lincomycin (LCM), and oxytetracycline resistance, and all isolates possessed *erm*B and *tet*S genes (6). Moreover, the yellowtail-derived *L. garvieae* isolates appeared to be homogenous and very different from isolates obtained from other fish, terrestrial animals, and food plants (4, 5, 6). Recently, of 146 *L. garvieae* strains isolated from 1999 to 2006 from yellowtail farms in three prefectures in Japan, 46 strains had high levels of resistance to EM, LCM, and tetracycline and were found to be carrying transferable R plasmids that carry *erm*B and *tet*S genes, as evidenced by conjugation, Southern blotting, and PCR methods (7).

In this study, the complete nucleotide sequence of R plasmid pKL0018 from a multiple-drug-resistant, gram-positive *L. garvieae* strain isolated from yellowtail was determined and analyzed.

L. garvieae strains were cultured in Todd-Hewitt broth (Difco) and 2% NaCl at 25°C. *Enterococcus faecalis* strain OG1SS was used as recipient cells of R plasmid. The cells were cultured in Todd-Hewitt broth (Difco) at 37°C. R plasmid pKL0018, isolated from EM-, LCM-, and tetracycline-resistant *L. garvieae* strains from Kagoshima, Japan, in 2000, was sequenced using cloning vector pBluescript (Stratagene).

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R plasmid was transferred in mixed culture with *E. faecalis* OG1SS for 18 h at 37°C and was selected by culture in medium containing 150 μg/ml of streptomycin and 100 μg/ml of EM. Purified plasmid DNA was randomly fragmented by sonication, cloned into the sequencing vector pBluescript (Stratagene), and transformed into *Escherichia coli* JM109 by electroporation. Clones were then sequenced using a Thermosequenase sequencing kit (Amersham-Biotech) with an LC4200 (Li-Cor) automated DNA sequencer. Data were assembled and analyzed using the ATSQ program in GENETYX v. 7.0 (SDC Software Development Co.), BLASTX of NCBI (http://www.ncbi.nlm.nih.gov), ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/), and BLAST 2 sequences (http://ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Sequence of R plasmid pKL0018. The R plasmid pKL0018 contains 20,034 bp (GenBank accession no. AB290882) and contains 21 open reading frames (ORFs) initiated by 18 ATG and 3 TTG start codons, with a computed total G+C content of 32.7% (Table 1).

pKL0018 is generally divided into two regions bounded by two replication proteins. The first region (about 12 kb) is identical at the nucleotide level to two corresponding regions in pRE25: a 3,577-bp block at 96% and a 8,349-bp block at 99.6% (Fig. 1A). Consequently, the ORFs in this region are also identical to each other in amino acid sequence, location, and orientation. Following annotations for pRE25, putative proteins identified in this area include a theta mechanism replication protein, a resolvase, a type I topoisomerase, a macrolide-lincosamide-streptogramin B (MLS) leader peptide, a macrolide resistance protein (23S rRNA methyltransferase [ErmB1]), and 10 hypothetical proteins (Fig. 1B). The second region (about 8 kb) contains a rolling circle replication protein, a transposase, a hypothetical protein, a tetracycline resistance protein [TetS], a duplicate macrolide resistance protein (23S rRNA methyltransferase [ErmB2]), and a copy number control protein (CopS). Also found in this region are a consensus *ori*T sequence located in between ORF14 and ORF15 and two 10-bp inverted repeats flanking the transposase [*tet*S] genes (Fig. 1B).

As mentioned above, the drug resistance genes consist of

TABLE 1. Amino acid and nucleotide sequence identities of putative proteins encoded by pKL0018 (GenBank accession no. AB290882) with other proteins

ORF	Locus position (bp)[a]	Length (bp)	Protein identification (organism with highest homology)	% aa[b] identity	% nt[c] identity	Start codon	% GC content	GenBank accession no.
ORF1	1–1491	1,491	RepS protein of pRE25 (*E. faecalis*)	98	97	ATG	35.44	CAC29162
ORF2	1839–2009	162	ORF7 of pRE25 (*E. faecalis*)	100	100	ATG	31.25	CAC29163
ORF3	2022–2640	519	Resolvase of pRE25 (*E. faecalis*)	95	91	ATG	34.97	CAC29164
ORF4	2640–4784	2,145	Type I topoisomerase of pRE25 (*E. faecalis*)	97	97	ATG	35.85	CAC29165
ORF5	5208–5291	84	MLS leader peptide of pRE25 (*E. faecalis*)	100	100	ATG	31.33	CAC29168
ORF6	5416–6153	738	23S rRNA methyltransferase (ErmB) of pRE25 (*E. faecalis*)	100	99	ATG	33.39	CAC29169
ORF7	6158–6289	132	ORF15 of pRE25 (*E. faecalis*)	100	100	ATG	37.64	CAC29170
ORF8	6686–7636	951	ORF16 of pRE25 (*E. faecalis*)	100	100	ATG	31.59	CAC29171
ORF9	7675–7889	215	ORF17 of pRE25 (*E. faecalis*)	100	100	TTG	34.03	CAC29172
ORF10	7907–8179	273	ORF18 of pRE25 (*E. faecalis*)	100	100	ATG	29.39	CAC29173
ORF11	8181–9044	864	ORF19 of pRE25 (*E. faecalis*)	100	100	ATG	37.45	CAC29174
ORF12	9436–9801	366	ORF20 of pRE25 (*E. faecalis*)	100	100	ATG	30.70	CAC29175
ORF13	10326–10832	507	ORF21 of pRE25 (*E. faecalis*)	100	100	ATG	31.72	CAC29176
ORF14	11450–11193	258	ORF22 of pRE25 (*E. faecalis*)	100	100	ATG	23.73	CAC29177
ORF15	11752–11453	300	ORF23 of pRE25 (*E. faecalis*)	100	100	ATG	22.92	CAC29178
ORF16	13044–12322	723	Rep protein (*Lactobacillus curvatus*)	59	79	ATG	31.09	CAA78602
ORF17	13938–13252	687	Transposase (*Lactococcus lactis*)	100	99	ATG	37.40	CAA63524
ORF18	13974–14219	246	ORF13 (*Clostridium perfringens*)	53	NS[d]	TTG	28.10	AAK17951
ORF19	15048–16988	1,941	Tetracycline resistance protein TetS (*Enterococcus faecium*)	100	100	ATG	33.32	ABB97394
ORF20	18187–18924	738	23S rRNA methyltransferase (ErmB) of pRE25 (*E. faecalis*)	100	99	ATG	33.23	CAC29169
ORF21	19396–19644	249	CopS of pRE25 (*E. faecalis*)	58	NS	TTG	34.17	CAC29195

[a] Nucleotide positions are given.
[b] aa, amino acid.
[c] nt, nucleotide.
[d] NS, no significant identity.

two macrolide resistance genes and one tetracycline resistance gene (Table 1 and Fig. 1B). Both macrolide resistance genes were classified as *erm*B genes, differing only in 2 amino acid residues, i.e., residue 75 (Thr to Ile) and residue 99 (Ser to Asn). The tetracycline resistance gene was classified as *tet*S.

This is a first report of the sequence of a gram-positive bacterium-derived plasmid from a fish pathogen. The nearly identical nucleotide sequences of the 12-kb-region backbone of pKL0018 and the corresponding region in pRE25 clearly suggest that, at the functional level, the two plasmids behave similarly in many aspects (9, 10). The resolvase protein (ORF3) of pKL0018 has 95% homology with the resolvase protein (ORF8) of pRE25 and would therefore function as a site-specific recombinase. The type I topoisomerase (ORF4) in pKL0018 is complete and should fully function for plasmid stability, in contrast to its truncated topoisomerase counterparts in pRE25, which have been suggested to be inactivated. ORF8 to ORF11 of pKL0018 are identical to ORF16 to ORF19 of pRE25, which are believed to be responsible for partitioning and the prevention of the appearance of plasmid-free segregates. CopS (ORF21) of pKL0018 is related to CopS (ORF40) of pRE25, which is suggested to be a regulator of plasmid copy number.

In pRE25, the ORF9 (truncated topoisomerase gene)-ORF10 (*cat*)-ORF11 (*rep*) region was reported to have been integrated as a block, with the accompanying insertion elements disappearing with the passage of time (9). This is supported by our findings, since the equivalent topoisomerase (ORF4) is present in full in pKL0018 and since the ORF9-ORF10-ORF11 pRE25 block and possible insertion sequences are missing in pKL0018.

The replication protein (ORF1) of pKL0018 is the same as the replication protein (ORF6) of pRE25, which has been shown to be of the theta mechanism type and is responsible for the replication of pRE25. However, in pKL0018, ORF1 may not be the dominant replicating mechanism, because of the presence of another replication protein (ORF16) at the start of the second 8-kb region of the rolling circle type, which, in small, unstable gram-positive bacterium-derived plasmids, is said to be the mechanism of preference (2, 9). The presence of an *ori*T consensus sequence upstream of ORF16 further supports the rolling circle replication type of plasmid similar to what has been observed for many transferable plasmids in gram-positive bacteria (3).

*erm*B1 is located next to an MLS leader peptide, which is known to regulate *erm* gene expression in the absence of EM (8), in the first 20-kb region, while the *erm*B2 gene is located in the plasmid's second 12-kb region downstream of the tetracycline resistance gene *tet*S. The fact that both of the *erm*B genes of pKL0018 are identical to that of pRE25 suggests that both genes were originally part of the pKL0018 plasmid. In contrast, the *tet*S gene is flanked upstream by an insertion sequence transposase as well as by two inverted repeats, strongly suggesting that the tetracycline resistance gene has been integrated into pKL0018.

Finally, it is surprising and remarkable that the 12-kb regions in pKL0018 and pRE25 are identical, since the former is isolated from a fish pathogen while the other originated from *E.*

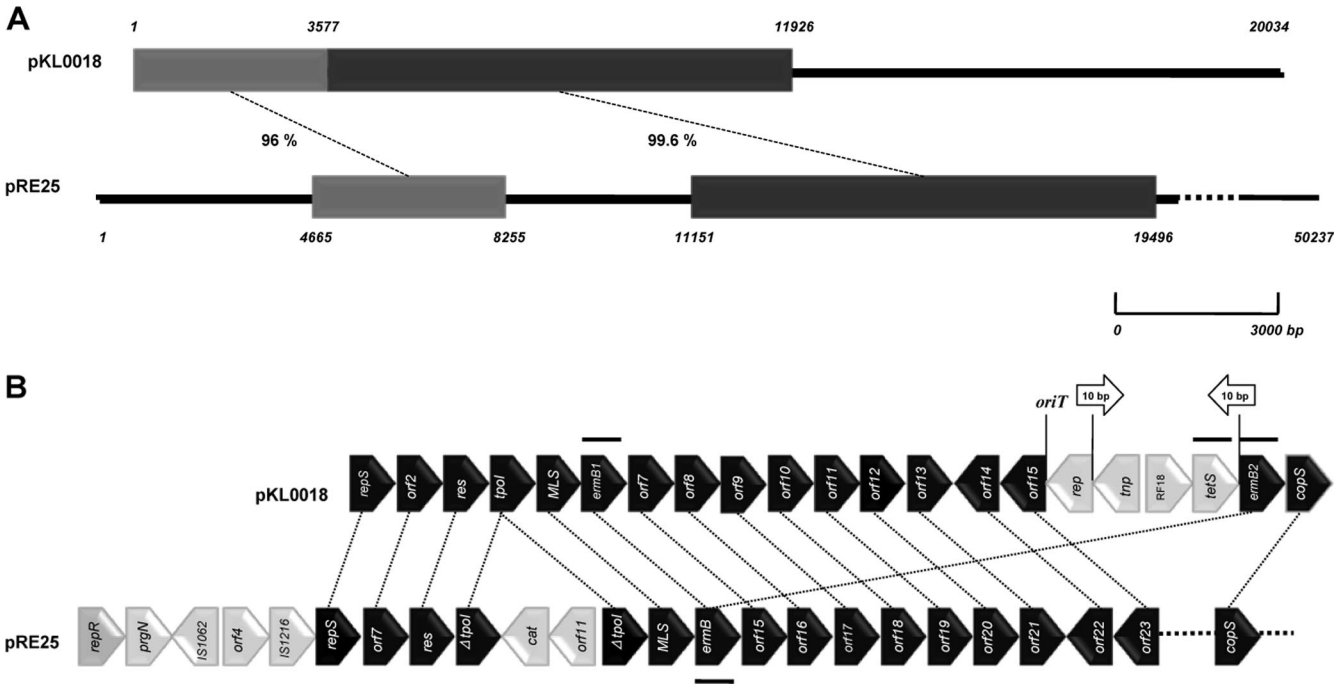

FIG. 1. Comparison of transferable pKL10018 and pRE25 plasmids. (A) Nucleotide sequence alignment. The 3,577-bp regions (gray boxes) are 96% identical, and the 8,349-bp regions (black boxes) are 99.6% identical. (B) Synteny alignment of ORFs. Putative proteins that are 97 to 100% identical (black arrowheads connected by dotted lines), nonidentical putative proteins (gray arrowheads), inverted 10-bp repeats (open arrows), *ori*T (as labeled), and drug resistance genes (horizontal bars) are shown.

faecalis found in raw fermented sausage (9, 10). This is clear evidence that the two plasmids are directly related and have been propagated by a common plasmid backbone of 12 kb. This further suggests that this plasmid backbone has a broad host range that is capable of disseminating mobile genetic elements containing antibiotic resistance genes in the environment.

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