Construction of a Conjugative Plasmid with Potential Use in Vaccines Against Heat-Labile Enterotoxin

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A conjugative plasmid with potential usefulness for vaccine strains was constructed. In the first step, a 5.9-kilobase DNA segment containing the two loci for the A and B subunits of heat-labile enterotoxin with a mutation in the gene for the A subunit was joined to the cloning vehicle pGA22, generating the nonconjugative plasmid pPMC4 with genes for resistance to tetracycline and chloramphenicol. In the second step, a segment of pPMC4 containing the genes for the A and B subunits, the gene for chloramphenicol resistance, and the replication genes of pGA22 was ligated to the genes for conjugal transfer of the F plasmid, generating the 54.9-kb plasmid pPMC5. Eleven porcine *Escherichia coli* isolates were tested as recipients for pPMC4 and pPMC5. For pPMC4, transformation and mobilization with a conjugative R plasmid were used to effect plasmid transfer. Only 1 of the 11 strains acted as a recipient in transformation. Mobilization with the R plasmid occurred with two strains, but the plasmids were altered during transfer. In contrast, pPMC5 was transferred with high frequency and unaltered to 9 of the 11 *E. coli* strains. Transconjugants from these nine matings produced high titers of the B subunit and no active heat-labile enterotoxin. Plasmid pPMC5 was stable in three porcine *E. coli* strains tested; plasmid pPMC4 was somewhat less stable in these strains. The method we describe for the construction of conjugative chimeric plasmids offers an opportunity for introducing genes with potential for immunization into bacterial strains that are suitable for colonizing the appropriate host sites.

Enterotoxigenic strains of *Escherichia coli* cause disease as a result of the production of heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST) or both and the possession of specialized adherence pili. Immunity can be generated by promoting antibody formation against the enterotoxins or against the colonization pili (for a review, see reference 10). We utilized these properties for the construction of live vaccine strains that protect piglets against diarrhea due to enterotoxigenic *E. coli* infections.

One of the immunogens, LT, consists of two subunits, A and B, with B being mainly responsible for immunogenicity. LT is very similar to cholera toxin (CT). The genes for LT and CT have been cloned in *E. coli* K-12 (15, 22), and for CT, mutants with a defect in the A subunit have been constructed with restriction enzymes (13). Such clones with a mutant LT gene are potentially useful for generating vaccines, but the host strain *E. coli* K-12 colonizes the intestine poorly, even when genes for adherence pili are present, and is thus not suitable as a live vaccine.

We describe here the isolation of a recombinant plasmid, containing the LT genes with a mutation in the A subunit, similar to the mutant clones isolated for CT (15). Since this plasmid is not conjugative, we attempted to transfer it to other porcine *E. coli* strains by transformation and mobilization with a conjugative plasmid. As will be shown, these attempts had limited success. We then constructed a chimeric plasmid by ligating a DNA segment containing the mutant LT gene and a gene for chloramphenicol resistance to a segment of the F plasmid containing the genes for conjugal transfer. We found this plasmid to be readily transmissible to other *E. coli* strains, and its B gene was

expressed in the new hosts as well as in the original *E. coli* K-12 strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used and their relevant genetic characteristics are listed in Table 1. *E. coli* K-12 C600 (*thr leu lac supE*) was used as the host for the recombinant plasmids. The bacterial strains used as recipients in conjugation were *E. coli* KL320 (*thi met pro trp his*) and 289-13 (*thi ilv pro trp his*) (12). The porcine isolates were obtained from C. Gyles and are described in Table 2.

Isolation of plasmids and DNA fragments. Plasmids were isolated by a Brij 58 lysis procedure as described previously (16). DNAs were digested with suitable restriction enzymes as recommended in a previous report (6). DNA length standards were obtained from Boehringer Mannheim Biochemicals, and electrophoresis was carried out as described previously (16). DNA fragments were purified from agarose gels by the procedure of Vogelstein and Gillespie (24).

Cloning procedures. Enzymes were obtained from New England Biolabs or Boehringer Mannheim Biochemicals. Restriction endonuclease digestion, conversion of fragments with protruding 5' ends to blunt ends, dephosphorylation of restriction enzyme-cleaved DNA fragments by calf intestinal alkaline phosphatase, ligation, and transformation were carried out as recommended previously (11). T4 DNA ligase was a gift from Chris Schindler.

Penicillin enrichment for tetracycline-sensitive cells. Tetracycline was added to a culture containing both tetracycline-sensitive and -resistant cells to a final concentration of 10 µg/ml. The culture was shaken at 37°C for 1 h to allow growth of tetracycline-resistant cells, and penicillin was then added to a final concentration of 1,000 U/ml. The culture was shaken for 30 min. Cells were collected by centrifugation, suspended in tryptone yeast extract (TYE) medium (19), and allowed to grow overnight. This procedure gave about 80% tetracycline-sensitive cells.

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TABLE 1. Plasmids used in this study

Plasmids	Relevant phenotype ^a	Reference or source
pWM2988	Ap ^r LT ST	8.9-kb LT ST <i>Bam</i> HI fragment of pCG86 cloned in pBR313 (12)
pBR322	Ap ^r Tc ^r	Cloning vehicle (3)
pGA22	Ap ^r Cm ^r Km ^r Tc ^r	Cloning vehicle (1)
pED100	Tra	Conjugative F plasmid derivative (25)
pPMC2	Ap ^s Tc ^r LT	5.9-kb LT <i>Pvu</i> I fragment of pWM2988 cloned in pBR322 (this study)
pPMC21	Tc ^r LTA ⁻ B ⁺	Frame shift mutant of pPMC2 at the <i>Xba</i> I site of <i>eltA</i> (this study)
pPMC4	Tc ^r Cm ^r LTA ⁻ B ⁺ Ap ^s Km ^s	5.6-kb LT <i>Xho</i> I- <i>Pvu</i> I fragment of pPMC2 cloned in pGA22 (this study)
pPMC5	Tc ^s Cm ^r LTA ⁻ B ⁺ Tra	43.5-kb <i>Bam</i> HI fragment of pED100 cloned in pPMC4 (this study)
R538	Cm ^r Sm ^r	19

^a Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; ST, heat-stable enterotoxin; Tra, conjugal transfer ability; ^r, drug resistant; ^s, drug sensitive.

PIH test. Goat antiserum to CT was kindly provided by R. K. Holmes and was diluted 50- to 100-fold for use. The LT antigen was prepared by the polymyxin release technique (7, 8), except that the strains were grown overnight on TYE slants containing 90 µg of lincomycin per ml, and the cell suspensions were adjusted to an optical density at 580 nm of 1.0 by emulsifying in Kolmer solution (0.85% NaCl, 0.01% MgCl₂). After treatment with polymyxin, the resultant supernatants were considered to be undiluted preparations of LT. Duplicate twofold dilutions were made for titration in the passive immune hemolysis (PIH) test. The procedures of the PIH test were similar to those described by Evans and Evans (7) and Serafim et al. (20). They provide a measure for the B subunit of LT or CT (7).

Plasmid stability assay. The methods of Sansonetti et al. (18) and Nordstrom et al. (14) were followed with modifications. Strains harboring plasmids pPMC4 or pPMC5 were grown in TYE broth with 25 µg of chloramphenicol per ml. Dilutions were plated onto TYE medium for determining viable counts. An inoculum of 1,000 cells per ml was grown in drug-free TYE broth at 37°C with aeration for about 20 generations (ca. 1.2×10^9 cells per ml) and then 10^{-7} dilutions were plated onto TYE medium. The resulting plates, containing a total of at least 1,000 colonies, were replicated onto TYE containing 25 µg of chloramphenicol per ml, and the percentage of chloramphenicol-sensitive colonies due to the loss of plasmid was calculated.

RESULTS

Construction of an LTA⁻ B⁺ plasmid. Previously, we reported the construction of pWM2988, an ampicillin-resistant plasmid which carries the genes for LT (*elt*) and STII (*estA*) of pCG86 on an 8.9-kilobase (kb) *Bam*HI fragment cloned into the *Bam*HI site of pBR313 (16). To study the *elt* genes independently of the *estA* gene, the *elt* genes carried on a 5.9-kb *Pvu*I fragment were subcloned into the *Pvu*I site of pBR322 to form a 10.3-kb tetracycline-resistant plasmid, pPMC2 (Fig. 1). This plasmid, isolated from transformed C600, yielded the expected fragments of 5.9 and 4.4 kb after digestion with *Pvu*I (Fig. 2, lane 4). The orientation of these

two *Pvu*I fragments was determined by digesting pPMC2 with *Eco*RI. Two fragments of 4.3 and 6.0 kb were found, thereby confirming the structure shown in Fig. 1. Cells of C600(pPMC2) were positive for LT in both the microtiter Y-1 adrenal cell assay (17) and the radial passive immune assay described by Bramucci and Holmes (4). Assays for STII in the intestinal loop assay (21) were negative.

Examination of the nucleotide sequences of *eltA* (23) and *eltB* (5) of P307, which apparently have complete homology with *eltA* and *eltB* of pCG86, showed the presence of a single *Xba*I recognition site near the NH₂ terminus of *eltA*. This was proved by double digestion of pPMC2 with *Pvu*I and *Xba*I which yielded three bands (Fig. 2, lane 6). The 4.4-kb band is the *Pvu*I fragment of pBR322, and the two smaller bands of 3.0 and 2.8 kb result from cleavage of the 5.9-kb *elt* *Pvu*I fragment by *Xba*I.

A mutation of this *Xba*I site would result in an *eltA*⁻ B⁺ plasmid that should produce biologically inactive, antigenically active LT. Cleavage of pPMC2 at the *Xba*I site followed by filling in of the single-stranded ends with the Klenow fragment of DNA polymerase I and subsequent blunt-end ligation resulted in covalently closed plasmid DNA (Fig. 1). The *Xba*I site was converted to 5'-TCTAGCTAGA-3' by a four-base insertion, producing a

FIG. 1. Construction of plasmid pPMC4. Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pvu*I; Xa, *Xba*I; Xo, *Xho*I; Ap, Cm, Km, Tc, resistance to ampicillin, chloramphenicol, kanamycin, and tetracycline, respectively; *elt*, heat-labile enterotoxin; *estA*, heat-stable enterotoxin II.

single base pair shift in the reading frame (Fig. 1). This frame shift generated a stop codon five codons downstream from the *Xba*I site.

Twenty colonies of C600 transformed to tetracycline resistance after in vitro mutagenesis of pPMC2 were negative in the Y-1 adrenal cell assay, and 17 of these were positive in the PIH assay. One colony was selected for further study, and its plasmid, pPMC21, was isolated and digested with *Pvu*I and *Xba*I. As expected, only two DNA fragments of 4.4 and 5.9 kb were generated (Fig. 2, lane 7).

To gain a more versatile *eltA*⁻ *B*⁺ plasmid, the *elt* genes were subcloned from pPMC21 into the vector pGA22 (1). Plasmid pPMC21, which has a single *Xho*I site (Fig. 2, lane 8) was doubly digested with *Pvu*I and *Xho*I. The larger 5.6-kb *Pvu*I-*Xho*I fragment (Fig. 2, lane 9), was isolated from an agarose gel and purified. Similarly, pGA22, which also has a single *Xho*I site, was doubly digested with *Pvu*I and *Xho*I. The larger 5.8-kb *Pvu*I-*Xho*I fragment (Fig. 2, lane 12) was isolated from an agarose gel, purified, and ligated to the 5.6-kb fragment of pPMC21. The resultant 11.4-kb plasmid transformed C600 to chloramphenicol and tetracycline resistance and was designated pPMC4 (Fig. 1). The single *Xho*I site was verified (Fig. 2, lane 11), and a *Pvu*I-*Xho*I digest of pPMC4 showed two bands of fragments of the expected sizes, 5.6 and 5.8 kb (Fig. 2, lane 10). As expected, the Y-1 and the pig intestinal loop assays were negative, and the PIH assay was positive for cells carrying this plasmid.

Construction of a transferable LTA⁻ B⁺ plasmid. The entire replication and transfer genes of the F plasmid are in a 55.9-kb plasmid, pED100 (25), that has a single *Hind*III site and two *Bam*HI sites that are in regions outside the *tra* genes. Cloning of these transfer genes into pPMC4 would provide a conjugally transferable multicopy, LTA⁻ B⁺, chloramphenicol-resistant plasmid. The construction of such a plasmid is shown in Fig. 3. pED100 was cleaved with *Bam*HI to yield two fragments of approximately 43.5 and 12.4 kb (Fig. 4, lane 7). This digest was then mixed and ligated with *Bam*HI-linearized pPMC4 DNA, which was treated with calf intestinal alkaline phosphatase to prevent ligation of the vector. The ligated mixture was used to transform *E. coli* C600. Since the *tra* gene fragment has no selectable marker and the *Bam*HI site in pPMC4 is located in the gene for tetracycline resistance, recombinants should be tetracycline sensitive and chloramphenicol resistant; transformed cells sensitive to tetracycline could be enriched by penicillin treatment. Accordingly, the transformation mixture was treated with penicillin as described above. For selecting transferable recombinants, mating was performed with the transformed cells as donors and chloramphenicol-sensitive KL320 cells as recipients, and chloramphenicol-resistant colonies were selected. Ten transconjugant colonies were obtained. Of the 10 transconjugants screened on agarose gels for the presence of plasmids by the method of Kado and Liu (9), 6 showed the presence of a large plasmid migrating with the same mobility as pED100, and 4 had a larger plasmid. All 10 transconjugants were negative in the Y-1 cell and the pig intestinal loop assays and were positive in the PIH assay, and they showed high transfer frequencies of the plasmid in matings. One of the six isolates was selected for further study, and its plasmid was designated pPMC5. A *Bam*HI digest of this 54.9-kb plasmid yielded two DNA fragments of 11.4 and 43.5 kb (Fig. 4, lane 3). The orientation of these fragments in pPMC5 (Fig. 3) was determined by consideration of the DNA fragment sizes produced by *Eco*RI digests of pPMC4 DNA (Fig. 4, lane 2), pPMC5 DNA (Fig. 4, lane 4), and pED100 (Fig. 4, lane 8).

FIG. 2. Restriction analysis of pWM2988, pBR322, pPMC2, pPMC21, pPMC4, and pGA22 by 0.75% agarose gel electrophoresis. Lane 1, Linear oligomers of λ dv-21 DNA; lane 2, pWM2988, *Pvu*I; lane 3, pBR322, *Pvu*I; lane 4, pPMC2, *Pvu*I; lane 5, pPMC2, *Xba*I; lane 6, pPMC2, *Pvu*I-*Xba*I; lane 7, pPMC21, *Pvu*I-*Xba*I; lane 8, pPMC21, *Xho*I; lane 9, pPMC21, *Pvu*I-*Xho*I; lane 10, pPMC4, *Pvu*I-*Xho*I; lane 11, pPMC4, *Xho*I; lane 12, pGA22, *Pvu*I-*Xho*I; lane 13, λ *Eco*RI/*Hind*III. Fragment sizes in kb refer to DNA standards.

Construction of potential vaccine strains. Strain C600 (pPMC5) was mated with 11 nonenterotoxigenic porcine strains, obtained from C. Gyles, that were surmised to colonize the small intestine (Table 2). These strains belong to serotypes that are usually enterotoxigenic. They were isolated from piglets with diarrhea, and in feeding experiments with piglets, they were found to be excreted for at least 3 days. Transfer of pPMC5 with selection for chloramphenicol resistance occurred with high frequency (about 1% of the donor input) with nine recipients. One transconjugant from each successful mating was examined for plasmid DNA and showed the presence of a plasmid considered to be pPMC5, since it had the expected size and was not seen in the recipient controls. The nine transconjugants were negative in the Y-1 assay and positive in the PIH test, with titers ranging from 1 to 32 times that obtained with strains carrying the parental plasmid pCG86 (Table 3).

Earlier we tried to introduce the mutant plasmid pPMC4 (Fig. 1) into the 11 porcine isolates by transformation but obtained transformants only with strain G58 and two tetracycline-sensitive derivatives, G58-1 and G58-6, isolated from it by the method of Bochner et al. (2). Subsequently, we attempted the transfer of pPMC4 by mobilization with the conjugative plasmid R538 (Table 1). Donor strain C600 (pPMC4, R538) was mated with two tetracycline-sensitive derivatives, G58-1 and W2954-1, and tetracycline-resistant transconjugants were selected. These were obtained with a moderate (ca. 0.1% of donor input) frequency, and 20 transconjugants from each mating were examined for plasmid DNA. They showed patterns of bands that were different from what we expected, suggesting that recombinations had occurred among the plasmids. Arrangement of the patterns

FIG. 3. Construction of plasmid pPMC5. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pvu*II; Xo, *Xho*I; Cm, Tc, resistance to chloramphenicol and tetracycline, respectively, *elt*, heat-labile enterotoxin; rep, replication region; *oriT*, origin of transfer; *tra*, transfer genes; AP, alkaline phosphatase.

FIG. 4. Restriction analysis of pPMC4, pPMC5, and pED100 by 0.75% agarose gel electrophoresis. Lane 1, pPMC4, *Bam*HI; lane 2, pPMC4, *Eco*RI; lane 3, pPMC5, *Bam*HI; lane 4, pPMC5, *Eco*RI; lane 5, *Eco*RI and *Hind*III; lane 6, linear oligomers of λ dv-21 DNA; lane 7, pED100, *Bam*HI; lane 8, pED100, *Eco*RI. Fragment sizes in kb refer to DNA standards.

into discrete types was difficult to achieve. All 20 transconjugants from the mating with G58-1 were positive in the PIH test, and only three of the transconjugants from the mating with W2954-1 were positive. The titers of the positive strains ranged from 4 to 64 times that of a pCG86-carrying strain. In contrast, the pPMC4 transformants had the expected plasmid profile and had titers that were uniformly 32 times that of a pCG86-carrying strain.

From these results it is clear that direct transfer of a conjugative plasmid carrying a mutant toxin gene is the most advantageous method for the construction of potential vaccines.

Stability of pPMC4 and pPMC5 in various hosts. An effective live oral vaccine should be stable. Therefore, this property was determined for strains G58, G58-1, and G58-6 harboring plasmids pPMC4 and pPMC5. After approximately 20 generations in drug-free medium after subculture from medium containing chloramphenicol, the loss of chloramphenicol-resistant cells from populations of the six strains was determined, and the results are presented in Table 4. The data indicate that pPMC5 is stably maintained in the three strains. Although the loss of pPMC4 was greater after 20 generations than that of pPMC5, the loss of this plasmid per generation is considered to be small.

TABLE 2. Prototrophic porcine strains used in this study

Strains	Serotype	Relevant phenotype ^a
G58	O101:K28	Tc ^r
G58-1 ^b	O101:K28	Tc ^s
G58-6 ^b	O101:K28	Tc ^s
W2954	O101:K30	Tc ^r Sp ^r
W2954-1 ^c	O101:K30	Sp ^r TC ^s
0919W	O101:K30	Tc ^r Km ^r
0919F	O101:K30	Tc ^r Km ^r
G59	O101:K30	Tc ^r Km ^r
6721	O138:K81	Tc ^r Km ^r
5686	O138:K81	Ap ^r Tc ^r
G24	O9:K103	Tc ^r

^a Ap, ampicillin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline, ^r, drug resistant; ^s, drug sensitive.

^b Tetracycline-sensitive derivative of strain G58.

^c Tetracycline-sensitive derivative of strain W2954.

DISCUSSION

The development of recombinant DNA technology has facilitated the isolation of mutants that have the potential for being vaccines, especially mutants that can generate antitoxic immunity. To illustrate this point, we have previously isolated, by nitrosoguanidine mutagenesis, mutants in the conjugative plasmid pCG86 that were of the A⁻ B⁺ type (21). However, pCG86 contains a gene for STII production, and the formation of this toxin would be undesirable in a vaccine strain. Although we were able to isolate a few STII⁻ mutants (21), we had difficulties in generating double mutants that were A⁻ B⁺ and STII⁻, and in which pCG86 had not lost its conjugative ability. Besides, the sites of the mutation in the nitrosoguanidine-induced mutants within the A gene are not known, whereas in the mutant described here, we know the precise nature and site of the mutation. Finally, the frame shift mutation induced here by recombinant DNA technology is presumably stable, whereas we have no information about the stability of our previously isolated mutants.

The vaccine strains we are trying to construct should induce localized immunity in the porcine small intestine. It is therefore important that such strains should colonize the small intestine. Since *E. coli* K-12 is a poor colonizer of the small intestine and since the construction of mutant plasmids is ordinarily carried out in *E. coli* K-12 with nonconjugative

TABLE 4. Plasmid loss after growth in drug-free medium

Strain	Loss of plasmid after 20 generations (% of population)
G58(pPMC4)	2.9
G58-1(pPMC4)	8.9
G58-6(pPMC4)	27.6
G58(pPMC5)	0.21
G58-1(pPMC5)	0.42
G58-6(pPMC5)	0.27

cloning vehicles, it is necessary to transfer the constructed plasmids to colonizing *E. coli* strains. In the present case, we found that transformation has serious limitations, since only one of eight *E. coli* strains could be transformed with our mutant plasmid. Mobilization with a conjugative plasmid was more effective, but we found that alterations occurred in the mutant plasmid during transfer, presumably as a result of cointegrate formation between the mutant plasmid and the mobilizing R plasmid. The best method of transfer was conjugation by the chimeric plasmid we had constructed by ligating part of the F plasmid to our mutant plasmid. Conjugal transfer of the F plasmid is high because there is no functional repressor for the *tra* operon in the F plasmid. Initially, we had difficulties in ligating the large fragment of the F plasmid to our mutant plasmid, but with the procedure developed here it is possible to obtain the desired plasmids, although at low frequencies.

Plasmid pPMC5 contains the transfer genes of the F plasmid and the replication genes of pPMC4. The latter plasmid is derived from pGA22, which has a higher copy number than the F plasmid (1). Presumably plasmid pPMC5 also has a higher copy number than the F plasmid, and this is a likely cause for the relatively high titers in the PIH test. Whatever the cause for this high titer may be, it should be advantageous for the strains in their ability to elicit antibody production.

The experiments reported here were reviewed by the Office of Recombinant DNA Research of the National Institutes of Health and were approved to be carried out at the P1 level of containment. The plasmid pPMC5 we have constructed contains a gene for chloramphenicol resistance, and this gene will be introduced into potential vaccine strains. A resistance gene of this type was necessary for the selection of transformants and transconjugants in the construction of pPMC5. In connection with the subsequent use of pPMC5-carrying strains as live vaccines, it should be noted that chloramphenicol is not used in animal feeds, and there is thus no undue selection for chloramphenicol resistance in farm animals. Moreover, many naturally occurring enteric strains contain transmissible plasmids with genes for chloramphenicol resistance. Finally, it is possible, once a suitable vaccine strain has been obtained, to modify the chloramphenicol resistance gene in order to render it inactive.

ACKNOWLEDGMENTS

We thank C. L. Gyles, N. Willetts, and J. Maule for supplying strains and C. L. Gyles for carrying out the intestinal loop assays for STII and LT.

This work was supported by grant PCM 8118494 from the National Science Foundation. W.K.M. is the holder of Public Health Service Research Career Award GM-15129 from the National Institute of General Medical Science.

TABLE 3. Y-1 cell assay and PIH test of strains containing mutant LT plasmids

Strain	Results of Y-1 cell assay	Titer in PIH test ^a
G58(pPMC5)	—	32
G58-1(pPMC5)	—	32
G58-6(pPMC5)	—	32
W2954(pPMC5)	—	8
0919W(pPMC5)	—	8
0919F(pPMC5)	—	8
6721(pPMC5)	—	16
5686(pPMC5)	—	1
G59(pPMC5)	—	4
Controls		
C600F(pPMC5)	—	8
289-1	+	1

^a Relative to the titer of strain 289-1, which carries plasmid pCG86.

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