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A broad-host-range vector of incompatibility group Q can work as a plasmid vector in *Neisseria meningitidis*: a new genetical tool

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Plasmid pHT128, a derivative of the broad-host-range IncQ vector pGSS33, was successfully introduced into *Neisseria meningitidis*. Under optimal conditions, pHT128 was transferred from *Escherichia coli* to *N. meningitidis* by triparental conjugation at a frequency of 10^{-5}–10^{-6}. The copy number of pHT128 in *N. meningitidis* was almost the same as in *E. coli*, in which the copy number of IncQ plasmids per chromosome is estimated to be 10. pHT128 was maintained as an episome in *N. meningitidis* in the presence of chloramphenicol, a marker of the plasmid. It was also shown that an *opc* or *pilE1* gene cloned on pHT128 could be expressed in *N. meningitidis* under control of the *tac* promoter and could complement a mutation of *opc* or *pilE1*, respectively. In addition, the conjugational introduction of pHT128 into *N. meningitidis* was demonstrated to be independent of natural transformation competence. All the results indicate that pHT128 is a useful vector for *N. meningitidis* as a new genetical tool.

Keywords: IncQ plasmid, triparental conjugation, restriction/modification systems, complementation

INTRODUCTION

Neisseria meningitidis is a Gram-negative diplococcus pathogen that colonizes the nasopharynx. It eventually spreads into the bloodstream, where it causes septicaemia and, furthermore, induces meningitis when it reaches the cerebrospinal fluid. Although some of the factors involved in pathogenesis, such as Opa (reviewed by Dehio *et al*., 1998; Nassif *et al*., 1999), Opc, pili (reviewed by Nassif *et al*., 1999) and the capsule (reviewed by Vogel *et al*., 1999), have been revealed, many problems still remain to be resolved (reviewed by Nassif & So, 1995; Nassif *et al*., 1999). A scarcity of genetic systems available for the meningococcus is one of the reasons hampering the development of genetic analysis (Kupsch *et al*., 1996).

Some R-plasmids in *Neisseria gonorrhoeae* have been used for the construction of vector plasmids (Stein *et al*., 1983; Piffaretti *et al*., 1988; Stein, 1989; Kupsch *et al*., 1996). Although it is well known that *N. gonorrhoeae* is genetically very closely related to *N. meningitidis* and a few plasmids were speculated to transfer from *N. gonorrhoeae* to *N. meningitidis* (Dillon *et al*., 1983; Ison *et al*., 1986; Knapp *et al*., 1988), most of the plasmids in *N. gonorrhoeae* were not applicable to *N. meningitidis* (Genco *et al*., 1984; Roberts *et al*., 1989). As far as we know, only two plasmids, a 4·5 Mb R-plasmid of *N. gonorrhoeae* and pMGC10, a derivative of shuttle vector pLES2, could be transferred to *N. meningitidis* (Ikeda *et al*., 1986; Nassif *et al*., 1991). On the other hand, some R-plasmids have been also found in *N. meningitidis* (Bhatti *et al*., 1981; Pintado *et al*., 1985; Rotger *et al*., 1986; Facinelli & Varaldo, 1987; Roberts *et al*., 1989). One of them was supposed to belong to the family of RSF1010, which is a broad-host-range vector of incompatibility group Q (IncQ) (Pintado *et al*., 1985).

IncQ plasmids such as RSF1010 and R300B (Sharpe, 1984) are found among Gram-negative bacteria (Barth *et al*., 1981). IncQ plasmids are not self-transmissible but mobile with the help of IncP plasmids. Since R300B is relatively small (8·68 kb; Meyer *et al*., 1982) and low in copy number (about 10 copies per *E. coli* genome; Barth & Grinter, 1974), it has been utilized for the construction of vectors (Sharpe, 1984). pGSS33 is one of

Abbreviations: Amp^r/Amp^s, ampicillin resistant/sensitive; Cml^r, chloramphenicol resistant; Erm^r, erythromycin resistant; Kan^r/Kan^s, kanamycin resistant/sensitive; Tet^r/Tet^s, tetracycline resistant/sensitive; Str^r/Str^s, streptomycin resistant/sensitive.

the derivatives of R300B and has been used as a cloning vector.

In this paper, we report that pHT128, a derivative of pGSS33, can be successfully introduced into *N. meningitidis*.

METHODS

Media and growth conditions. *N. meningitidis* was stocked in frozen solution (Suker *et al*., 1994) at −80 °C and routinely grown on GC agar plates, [GC medium agar (Becton-Dickinson) supplemented with 1% IsoVitaleX enrichment (Becton-Dickinson)], at 37 °C in 5% CO_2. Trypticase soy broth (TSB; Becton-Dickinson) supplemented with 1% IsoVitaleX enrichment was used for the isolation of the plasmids in *N. meningitidis*. *E. coli* was grown on L plates or in L broth liquid culture at 37 °C. When required, antibiotics were added at the following concentrations. For *N. meningitidis*, chloramphenicol at 5 μg ml^{-1}, erythromycin at 4 μg ml^{-1}, ampicillin at 1 μg ml^{-1}. VCN (V, vancomycin; C, colistin; N, nystatin) inhibitor (Becton-Dickinson) was added to a final concentration of 1% to select *N. meningitidis* transconjugants. For *E. coli*, chloramphenicol was added at 10 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, tetracycline at 15 μg ml^{-1}, ampicillin at 50 μg ml^{-1} and erythromycin at 150 μg ml^{-1}. IPTG was added at a final concentration of 1 mM. The growth and concentration of bacteria were measured by monitoring the OD_{600}.

Strains. Strains used in this study are listed in Table 1. HT1037 was constructed as follows. A 1·3 kb fragment containing the *opc* gene with *Bam*HI and *Eco*RI linkers was amplified from H44/76 chromosomal DNA by PCR. All the PCR reactions done in this study were performed in a Gene Amp PCR System 2400 (Applied Biosystems) using ExTaq DNA polymerase (Takara Shuzo). The resulting DNA fragment was digested with *Bam*HI and *Eco*RI, then cloned in the *Bam*HI and *Eco*RI sites of pUC118 to construct pHT3 (see also Table 2). A blunted 1·2 kb *Hin*dIII–*Hin*dIII fragment containing the *ermC* gene isolated from pHT24 was inserted into the blunted *Eco*T14I sites, located at 196 bp and 526 bp downstream from the start codon of the *opc* gene, to generate pHT148. pHT148 DNA (500 ng) linearized by digestion with *Sal*I, the site of which is located on the vector, was transformed into H44/76 and Erm^r clones were isolated, resulting in an *opc* deletion mutant of H44/76.

HT1033 was constructed as follows. A 1 kb fragment containing the *pilE1* gene with *Bam*HI and *Eco*RI linkers was amplified by PCR and cloned in the *Bam*HI and *Eco*RI sites of pUC119 to construct pHT65. A blunted 1·2 kb *Hin*dIII–*Hin*dIII fragment containing the *ermC* gene isolated from pHT24 was inserted into the *Bst*XI site, located at 135 bp downstream from the start codon of the *pilE1* gene, to generate pHT72. pHT72 (500 ng) linearized by digestion with *Eco*RI was transformed into NIID57 and Erm^r clones were isolated, resulting in a *pilE1* insertion mutant of NIID57.

Plasmids. Plasmids used in this study are listed in Table 2. A Kan^s Tet^s derivative of RP4 (Pansegrau & Lanka, 1987), pHT13, was constructed as follows. First, RP4 digested with *Hin*dIII and *Hpa*I was blunted with a DNA blunting kit (Takara Shuzo) and self-ligated by DNA ligation kit version 2 (Takara Shuzo) to generate pHT12 (Kan^s). In the next step, pHT12 digested with *Bgl*II and partially digested with *Stu*I was blunted, self-ligated and transformed to DH5α to select Amp^r Kan^s Tet^s clones. The resultant plasmid was renamed pHT13 and used as a helper for conjugation of a derivative of pGSS33 (Fig. 1a) from *E. coli* to *N. meningitidis*. pHT128 (Fig. 1b), a Str^s and Amp^s derivative of pGSS33, was constructed by removing the *Pst*I–*Sac*I region after blunting. pHT160 (see Fig. 4c) was constructed as follows. A 0·9 kb fragment containing the entire *opc* coding region with *Eco*RI and *Bam*HI linkers was amplified by PCR and cloned in the *Bam*HI and *Eco*RI sites of pTTQ18 (Stark, 1987) to construct pHT157. A 4·3 kb *Alw*NI–*Sca*I fragment of pHT157 containing both the *tac* promoter fused to the *opc* gene and the *lacI*^{q} gene was blunted and cloned in the *Pst*I and *Sac*I sites of pGSS33 after blunting. The resultant plasmid was pHT160. pHT161 (Fig. 4a) was constructed as follows. A 0·6 kb fragment containing the entire *pilE1* coding region was amplified by PCR with the corresponding primers, which contained the *Eco*RI linker tagged by six CAC tandem repeats (His_6) and a *Sma*I linker. The amplified fragment was cloned in the *Eco*RI and *Sma*I sites of pTTQ18 to construct pHT68. A 4 kb *Alw*NI–*Sca*I fragment of pHT68 containing both the *tac* promoter fused to the His_6-*pilE1* gene and the *lacI*^{q} gene was blunted and cloned in the *Pst*I and *Sac*I sites of pGSS33 after blunting. The resultant plasmid was pHT161.

All of the primers to amplify *N. meningitidis* genes used in this study were designed by reference to the TIGR *N. meningitidis* database (http://www.tigr.org/).

DNA transfer. For transformation by natural competence, *N.*

Table 1. Strains used in this study

Strain	Genotype, pedigree of strain	Source
E. coli		
DH5α	*supE44 ΔlacU169 (ϕ80dlacZΔM15) hsdR17 recA1 deoR endA1 gyrA96 thi-1 relA1*	Takara Shuzo
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	Promega
N. meningitidis		
H44/76	Serogroup B, a representative isolate of the Norwegian group B epidemic	National Institute of Public Health, Norway
NIID57	Serogroup B, a clinical isolate from a Japanese healthy carrier	This study
HT1037	H44/76 Δ*opc*::*ermC*	This study
HT1033	NIID57 *pilE1*::*ermC*	This study

Table 2. Plasmids used in this study

Plasmid	Genotype pedigree and markers	Source
pUC18	Cloning vector; Amp^r	Takara Shuzo
pUC118	Cloning vector; Amp^r	Takara Shuzo
pUC119	Cloning vector; Amp^r	Takara Shuzo
pTnMax2	Plasmid for *in vitro* mutagenesis; Erm^r	Hass *et al.* (1993)
pHT24	Derivative of pUC18 containing a 1·2 kb *Hin*dIII–*Hin*dIII fragment that carries the *ermC* gene of pTnMax2; Amp^r Erm^r	This study
pHT3	Derivative of pUC118 containing a 1·3 kb *Bam*HI–*Eco*RI fragment that carries the *opc* gene; Amp^r	This study
pHT148	Derivative of pHT3 in which the *ermC* gene was inserted in *Eco*T14I sites of the *opc* gene; Amp^r Erm^r	This study
pHT65	Derivative of pUC119 containing a 1 kb *Bam*HI–*Eco*RI fragment that carries the *pilE1* gene; Amp^r	This study
pHT72	Derivative of pHT65 in which the *ermC* gene was inserted in the *Bst*XI site of the *pilE1* gene; Amp^r Erm^r	This study
RP4	Broad-host-range IncP vector; Amp^r Kan^r Tet^r	Pansegrau & Lanka (1987)
pHT12	Kan^s derivative of RP4 deleting its *Hin*dIII–*Hpa*I region; Amp^r Kan^s Tet^r	This study
pHT13	Tet^s derivative of pHT12 deleting its *Bgl*II–*Stu*I region; Amp^r Kan^s Tet^s; a helper plasmid used for conjugation	This study
pGSS33	Broad-host-range IncQ vector; Str^r Amp^r Cml^r Tet^r	Sharpe (1984)
pHT128	Str^s Amp^s derivative of pGSS33 deleting its *Pst*I–*Sac*I region; Str^s Amp^s Cml^r Tet^r	This study
pTTQ18	Expression vector under control of the *tac* promoter; Amp^r	Stark (1987)
pHT157	Derivative of pTTQ18 containing the *opc* gene under control of the *tac* promoter; Amp^r	This study
pHT160	Derivative of pGSS33 containing a 4·3 kb *Alw*NI–*Sca*I fragment of pHT157; Cml^r Tet^r	This study
pHT68	Derivative of pTTQ18 containing the His-tagged *pilE1* gene under control of the *tac* promoter; Amp^r	This study
pHT161	Derivative of pGSS33 containing a 4 kb *Alw*NI–*Sca*I fragment of pHT68; Cml^r Tet^r	This study

meningitidis was transformed as previously described (Ryll *et al.*, 1997). Plasmids were linearized prior to transformation unless otherwise stated.

Electroporation for *Neisseria* was performed as follows. *N. meningitidis* grown on GC agar plates at 37 °C in 5 % CO_2 was scraped and suspended in 30 ml TSB supplemented with 1 % IsoVitaleX enrichment to adjust the supension to OD_{600} 0·1. The bacteria were cultured at 37 °C with shaking to OD_{600} of 0·6 and then the cells were chilled and harvested by centrifugation at 4 °C. The pellets were rinsed with 1 ml ice-cold 10 % (w/v) glycerol three times and the cells were resuspended to a final volume of 60 µl in ice-cold 10 % glycerol. A 20 µl sample of the cell suspension was mixed with up to 1 µg of plasmid DNA and electroporation was performed under the following optimized conditions: electric field strength, capacitance and resistance were 1·25 kV, 25 µF and 400 Ω, respectively, when using a 0·1 cm electrode gap cuvette (Bio-Rad) with the Gene Pulser (Bio-Rad). After being electroporated, cells were incubated in 1 ml TSB supplemented with 1 % IsoVitaleX enrichment, 10 mM $MgCl_2$ and 0·4 % glucose at 37 °C for 1 h. The cells harvested by centrifugation were spread on GC agar plates with 5 µg chloramphenicol ml^{-1} and incubated at 37 °C in 5 % CO_2 for 18 h.

For conjugation, *N. meningitidis* grown on GC agar plates at 37 °C in 5 % CO_2 was scraped and suspended in TSB to adjust the supension to OD_{600} 0·25. In order to transfer a cloned vector plasmid from *E. coli* to *N. meningitidis*, we used triparental conjugation. Overnight cultures of LE392 harbouring vector plasmid pGSS33 or pHT128, LE392 harbouring a helper plasmid pHT13 and *N. meningitidis* suspension prepared as described above were mixed at a volume ratio of 1:1:2 and then filtered through 0·45 µm membrane filters (Millipore) by use of a syringe. The filters were washed with 2 ml TSB, then incubated on GC agar plates at 37 °C in 5 % CO_2 for 8 h. Bacteria on the filters were washed out with 1 ml TSB. The bacteria, harvested by centrifugation, were then spread on GC agar plates containing 5 µg chloramphenicol ml^{-1} and 1 % VCN inhibitor and incubated at 37 °C in 5 % CO_2 for 24 h. Transconjugants of *N. meningitidis* were picked up and streaked on the same kind of plates to isolate a single clone.

Checking the maintenance of pHT128 in *N. meningitidis*. NIID57 harbouring pHT128 grown on a GC agar plate or *E. coli* strain LE392 harbouring pHT128 on an L agar plate was scraped and suspended in 10 ml TSB supplemented with 1 % IsoVitaleX enrichment at an OD_{600} of 0·2 and cultured at

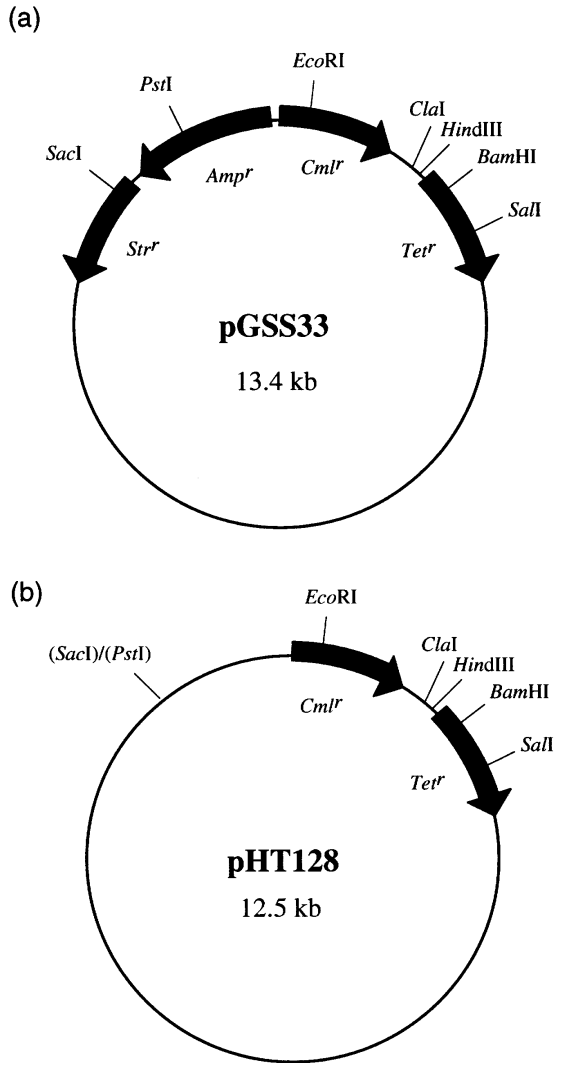

Fig. 1. Restriction maps of pGSS33 (a) and pHT128 (b). The sites of major restriction enzymes are shown. pHT128 was constructed as described in Methods.

37 °C with shaking for 24 h. One millilitre of the bacterial culture was added to 9 ml fresh medium and continuously cultured under the same conditions as described above. This step was repeated a total of four times. Each time, appropriate dilutions of the bacterial culture were spread on GC agar plates with or without 5 μg chloramphenicol ml^{-1} for NIID57 and L agar plates with or without 10 μg chloramphenicol ml^{-1} for LE392 to count the number of bacteria harbouring pHT128 and the total number of bacteria, respectively. The number of bacterial generations was determined graphically from the values of OD_{600}.

Isolation of DNA. Mini-preparation of plasmid from *E. coli* was carried out by Wizard Plus SV Minipreps DNA Purification systems (Promega). Isolation of plasmids from *N. meningitidis* transconjugants and *E. coli* transformants cultured in more than 1 ml of medium was essentially as described by Kado & Liu (1981).

For the isolation of total DNA, *N. meningitidis* was cultured in the same conditions as described in the preceding section except that chloramphenicol was added during the culturing. Total DNA from *N. meningitidis* transconjugants was isolated as described by Suker *et al.* (1994).

Southern blotting. One microgram of the total DNA of *N. meningitidis* was digested with *Cla*I and analysed on a 0·8% agarose gel. The gel was treated with 0·25 M HCl followed with 0·5 M NaOH and then transferred to Hybond-N+ membrane (Amersham). The resultant membrane was hybridized with the *cat* gene of pHT128 as a probe and signals were detected by ECL direct nucleic acid labelling and detection systems (Amersham) according to the manufacturer's protocol.

Preparation of antibody for pili. A peptide encompassing residues 41–55 (EGQKSAVTEYYLNHG) of pilin, a region recognized by the monoclonal antibody SM1 made by Virji and co-workers (Virji & Heckels, 1983; Virji *et al.*, 1989), was synthesized. An antiserum specific for the oligopeptide was obtained by immunization of rabbits with the peptide coupled to keyhole limpet haemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS).

Western blotting. The *N. meningitidis* cells were suspended in 1 × SDS buffer (2% SDS, 5% 2-mercaptoethanol, 62·5 mM Tris/HCl pH 8·0, 10% glycerol, 0·1% Bromophenol blue) and adjusted to OD_{600} 10. After boiling for 5 min, 2·5 μl samples were subjected to 12 or 15% SDS-polyacrylamide gel electrophoresis. Western blotting was performed with Sequi-Blot PVDF membrane (Bio-Rad) and Horizeblot (ATTO). The resulting membrane was soaked in PBS containing 0·1% Tween 20 (T-PBS) and 5% skim milk at room temperature for 1 h with gentle shaking. The membrane was incubated with 10^3-fold diluted anti-pili serum or 10^4-fold diluted monoclonal antibody against Opc (B306; Achtman *et al.*, 1988) at room temperature for 1 h. After washing the membrane in T-PBS for 10 min three times, it was incubated with 2000-fold diluted horseradish-peroxidase-conjugated anti-rabbit or anti-mouse antibody (Boehringer). After washing the membrane in T-PBS for 10 min three times, signals were detected by ECL detection kit (Boehringer) and ECL film (Boehringer).

RESULTS

Transconjugation of pHT128 from *E. coli* to *N. meningitidis*

To check growth inhibition by antibiotics, *N. meningitidis* strains H44/76 and NIID57 were incubated on GC agar plates containing various concentrations of antibiotics at 37 °C in 5% CO_2 for 18 h. Growth of both strains was completely inhibited by 5 μg chloramphenicol ml^{-1}, 1 μg ampicillin ml^{-1} and 4 μg erythromycin ml^{-1} but not clearly inhibited by 50 μg kanamycin ml^{-1} or 2 μg tetracycline ml^{-1} (data not shown). As penicillin has been used as a first choice of antibiotic for the treatment of meningococcaemia, we chose to use the chloramphenicol-resistance gene as a selective marker in this study.

We tried to introduce the IncQ plasmid pHT128 into *N. meningitidis* strain NIID57 by three methods: transformation by natural competence, electroporation and conjugation. Although no Cml^r colonies were obtained with transformation by natural competence or electroporation, some Cml^r colonies appeared when triparental conjugation was used to transfer pHT128 from *E. coli* to NIID57. To optimize the conditions of triparental

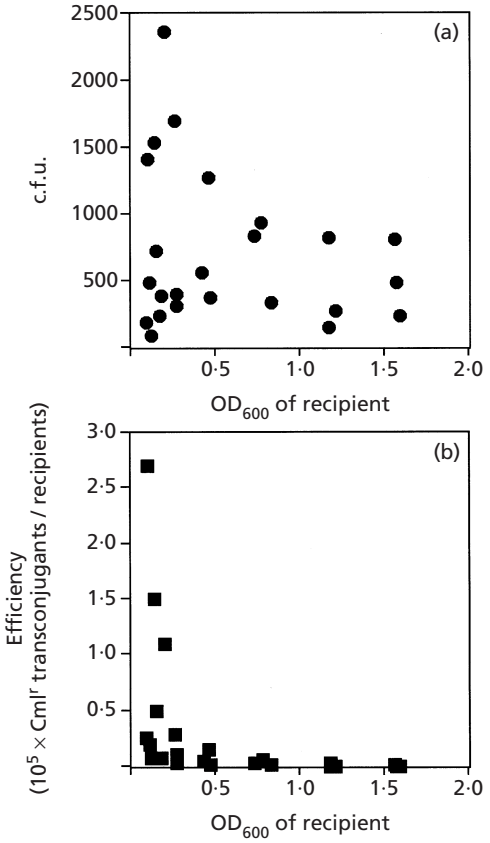

Fig. 2. Effect of concentration of the recipient on the efficiency of conjugation. (a) Number of Cml^r colonies appearing on plates versus OD_{600} of recipient. (b) Conjugation efficiency (number of Cml^r transconjugants/number of recipients) versus OD_{600} of recipient.

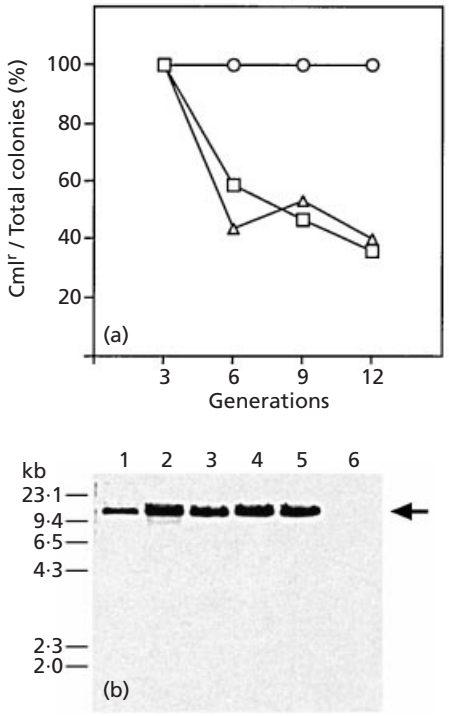

Fig. 3. Behaviour of pHT128 in *N. meningitidis*. (a) Stability of maintenance of pHT128 in *N. meningitidis* strain NIID57 (□, experiment 1; △, experiment 2) and in *E. coli* strain LE392 (○) in drug-free liquid culture. (b) Southern blotting using a *cat* gene as a probe. The bacteria were cultured under the same conditions as above except for the presence of chloramphenicol. DNA samples were isolated as described in Methods. Lanes: 1, *Cla*I-digested pHT128 as a positive control; 2, *Cla*I-digested total DNA isolated from bacterial culture at the third generation; 3, at the sixth generation; 4, at the ninth generation; 5, at the twelfth generation; 6, chromosomal DNA isolated from NIID57 as a negative control. The arrow indicates linearized pHT128.

conjugation, we changed the concentration of the recipient strain NIID57 and found that the efficiency of conjugation seemed to be highest at a density of OD_{600} 0·2–0·3, although the number of Cml^r transconjugants seemed to be almost equal (about 10^3 clones) independent of the concentration of the recipient strain (Fig. 2a). The efficiency of transconjugation was calculated to be about 10^{-5} (transconjugants/recipients) under the best conditions (Fig. 2b). As a similar result was obtained with *N. meningitidis* strain H47/76 as recipient (data not shown), pHT128 seems to be introduced into *N. meningitidis* serogroup B regardless of strain specificity.

Since no colonies appeared when transconjugants were selected on ampicillin-containing plates (data not shown), it was speculated that the IncP plasmid used as a helper plasmid could not be transferred or replicated in *N. meningitidis*.

Isolation of pHT128 from *N. meningitidis*

To confirm the existence of pHT128 in *N. meningitidis*, we isolated the plasmids from Cml^r *N. meningitidis* transconjugants. Plasmids the same size as pHT128 (12 kb) were isolated from all the Cml^r *N. meningitidis* colonies examined and the amount of plasmid DNA from *N. meningitidis* was as much as that in *E. coli* DH5α when examined by the density on agarose gel (data not shown). These results indicated that pHT128 could be conjugationally transferred from *E. coli* to *N. meningitidis* and existed at about 10 copies in *N. meningitidis*, as much as in *E. coli*.

Stability of pHT128 in *N. meningitidis*

To check the stability of pHT128 in *N. meningitidis*, we successively subcultured *N. meningitidis* harbouring pHT128 in liquid medium and counted total c.f.u. on GC agar plates with or without 5 μg chloramphenicol ml^{-1} as described in Methods. Although pHT128 was maintained at a frequency of 100% in *E. coli* strain LE392 even in drug-free liquid medium, the plasmid was gradually lost from *N. meningitidis* at a rate of about 7–9% loss per generation under the same conditions (Fig. 3a). This result indicated that maintenance of pHT128 in *N. meningitidis* was relatively unstable in the absence of selection by chloramphenicol.

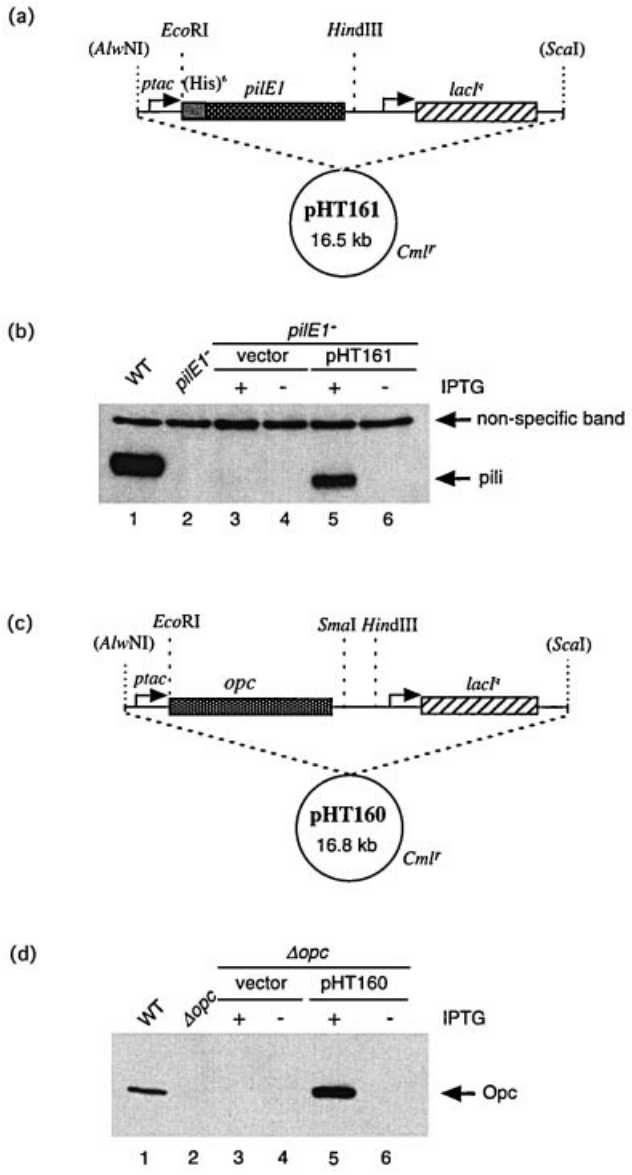

Fig. 4. Western blotting of *N. meningitidis* transconjugants. (a) Structure of pHT161. A histidine tag was attached to the 5′-terminus of the *pilE1* structural gene. (b) Western blotting using anti-pili serum. Lanes: 1, NIID57; 2, HT1033; 3, HT1033 harbouring pHT128 cultured in the presence of IPTG; 4, HT1033 harbouring pHT128 cultured in the absence of IPTG; 5, HT1033 harbouring pHT161 cultured in the presence of IPTG; 6, HT1033 harbouring pHT161 cultured in the absence of IPTG. The lower band corresponds to pili. The upper band is a non-specifically reacted band that was used as an internal control. (c) Structure of pHT160. (d) Western blotting using anti-Opc monoclonal antibody, B306. Lanes: 1, H44/76; 2, HT1037; 3, HT1037 harbouring pHT128 cultured in the presence of IPTG; 4, HT1037 harbouring pHT128 cultured in the absence of IPTG; 5, HT1037 harbouring pHT160 cultured in the presence of IPTG; 6, HT1037 harbouring pHT160 cultured in the absence of IPTG.

To further study whether pHT128 was maintained as an episome, we isolated total DNA from bacteria cultured in the presence of chloramphenicol and performed Southern blotting using the chloramphenicol acetyltransferase (*cat*) gene as a probe. A band corresponding to linearized pHT128 was detected from the samples at each cell generation tested and no other bands could be seen (Fig. 3b). This result showed that pHT128 was maintained in *N. meningitidis* as a plasmid.

The extraneous genes on pHT128 can be expressed in *N. meningitidis*

Next, we examined whether pHT128 vector is available for the complementation of mutations in *N. meningitidis*. A derivative of pHT128 carrying the His_6-tagged *pilE1* or the *opc* gene under the control of *tac* promoter, pHT161 or pHT160, respectively (Fig. 4a, c), was introduced into *N. meningitidis* strain HT1033 (*pilE1*) or HT1037 (Δ*opc*). Cell lysates of Cml^r transconjugants cultured in the presence and absence of IPTG were examined for the expression of pili and Opc protein by Western blotting with each antibody. As shown in Fig. 4(b, d), pili or Opc protein were expressed in *N. meningitidis* under the control of IPTG induction. These results confirmed that the foreign genes on pHT128 could be expressed in *N. meningitidis*.

Mobilization of the plasmids is independent of neisserial natural competence

To confirm that the mobilization of the plasmids observed in this study was independent of natural competence, we performed triparental conjugation using the *pilE1*-defective strain HT1033 as recipient, since the product of *pilE1*, pili, is essential for natural competence (Jyssum & Lie, 1965). The mean conjugation efficiency to the *pilE1*-defective strain was $6{\cdot}8 \times 10^{-6}$, which was not lower but rather higher than that to a wild-type strain ($3{\cdot}0 \times 10^{-6}$), for unknown reasons. This result showed that the conjugational transfer of plasmid from *E. coli* to *N. meningitidis* was not dependent on neisserial natural competence.

DISCUSSION

Genetical tools for *Neisseria* species have been mainly developed in *N. gonorrhoeae* (Stein *et al.*, 1983; Piffaretti *et al.*, 1988; Roberts, 1989) and there are few efficient genetic tools such as transposons and plasmids that work in *N. meningitidis*. Transposons derived from *Streptococcus faecalis*, Tn*916* (Kathariou *et al.*, 1990; Stephens *et al.*, 1991; Erwin & Stephens, 1995) and Tn*1545*-Δ*3* (Nassif *et al.*, 1991) were applied to *N. meningitidis* for random mutagenesis. In the case of plasmids, one R-plasmid in *N. gonorrhoeae* could be introduced into *N. meningitidis* by conjugation (Ikeda *et al.*, 1986), but no further information has been reported so far. pMGC10, a derivative of a shuttle vector between *E. coli* and *N. gonorrhoeae*, was successfully mobilized to *N. meningitidis* (Nassif *et al.*, 1991) and this was the only plasmid that was proved to work in *N. meningitidis*. However, this plasmid was constructed to introduce Tn*1545*-Δ*3* into *N. meningitidis* and has not been commonly used as a plasmid vector available in

N. meningitidis. Plasmids named pJS-A and pJS-B have been recently found in *N. meningitidis* (Hilse *et al*., 2000; Claus *et al*., 2001), but they might not be suitable for plasmid vectors in *N. meningitidis* because these plasmids, that were release-forms from the genome, seemed to be unstable as episomes. In this study, we showed that pHT128, a derivative of an IncQ broad-host-range vector, could be introduced into *N. meningitidis* at a considerable frequency and that it worked as a cloning vector in *N. meningitidis* in the presence of antibiotic. To our knowledge this is the first observation that an artificial plasmid can work well in *N. meningitidis*.

The difficulties of introducing R-plasmids into *N. meningitidis* may be partially attributed to some restriction/modification systems in *N. meningitidis* because naked DNA isolated from *N. meningitidis* transconjugants, but not from *E. coli*, could be easily transformed into *N. meningitidis* by electroporation (unpublished data). The influence of the restriction/modification systems on the efficiency of conjugation is still controversial (Stein *et al*., 1988; Butler & Gotschlich, 1991). However, mobilization of plasmid by conjugation from *E. coli* to *N. meningitidis* may circumvent the barrier of modification/restriction systems by some unknown mechanism(s) because pHT128 or pMGC10 (Nassif *et al*., 1991) was transferred at a considerable frequency.

The efficiency of plasmid DNA transfer in triparental conjugation was variable (Fig. 2). This might have been due to the difference of living state of *N. meningitidis* used for the preparation of recipient cells because neisseriae have rapid autolytic properties, even if cultured under steady conditions. In order to increase the transfer frequency, it would be critical to improve the recipiency of *N. meningitidis*.

To date, genetical analyses such as complementation tests in *N. meningitidis* have never been done because there were few useful plasmid vectors for *N. meningitidis*. We have shown here that pHT128 is applicable for complementation tests in *N. meningitidis*. We believe that our findings increase the possibilities for the molecular biological analysis of *N. meningitidis*.

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