THE TFP ATPASE OF *HAEMOPHILUS INFLUENZAE* AND DNA UPTAKE

by

MINGHUI YANG

B.Sc., Shandong University (P. R. China), 1985

M.Sc., Shandong University (P. R. China), 1988

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

October 2005

ABSTRACT

Haemophilus influenzae Rd is a Gram negative bacterium capable of taking up exogenous DNA and undergoing genetic transformation through natural competence. As in most naturally competent bacteria, *H. influenzae* proteins related to type IV pili are required for natural competence. The *pil* operon of *H. influenzae* is composed of four open reading frames, *pilA*, *pilB*, *pilC* and *pilD*, coding for homologues of proteins involved in the assembly and function of type IV pili.

pilB is the second gene of the *pil* operon. It codes for the only *H. influenzae* homologue of *Pseudomonas aeruginosa* PilB and PilT, which are required for Type IV pili assembly and retraction, respectively. The deduced protein sequence of *H. influenzae* PilB was analyzed and nucleotide-binding motifs conserved in the VrbB/GspE family ATPases were identified. Alignment with the homologues in other naturally competent bacteria identified the most conserved regions in the C-termini of proteins in this family that are essential for ATPase activities. However, little is known with respect to the specific role of PilB in natural competence and DNA uptake. To find out *H. influenzae* PilB's function in DNA uptake, a *pilB* knockout RR1150 was constructed by an insertion at the *BclI* site using a Tn903 Kan^R cassette. To allow expression of the downstream genes *pilC* & *pilD* in this strain, an additional CRE (Competence Regulatory Element) regulatory sequence and promoter were placed in the knockout RR1150, right before *pilC*. Natural competence was eliminated in RR1150, indicating that *pilB* is essential for natural competence. DNA binding and uptake assays showed that the defect of competence in RR1150 is at the

level of DNA uptake. To further investigate the specific function of *pilB*, the *P. aeruginosa* PA01 homologues *pilB* and *pilT* were introduced into the above *H. influenzae pilB*-background. The two *P. aeruginosa* genes were strongly expressed in *H. influenzae pilB* and the sequences have no mutations, as indicated by real time PCR and DNA sequencing. However, DNA binding and uptake in *H. influenzae pilB* was not restored. The experimental data and the recently available evidence in related studies suggest that complementation failures could be attributed to either the species-specific interactions of Tfp proteins, or to the different ways by which the Tfp assembly/function systems work. Furthermore, since PilT-like ATPases were not found in any Pasteurellaceae species possessing type IV pili by BLAST search and sequence analysis, I suggest that the energetics of Pasteurellaceae Tfp is different from the PilB-PilT system of *P. aeruginosa* Tfp, and that an as-yet-uncharacterized protein provides the driving force required for Tfp retraction in Pasteurellaceae.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Amp	ampicillin
BHI	brain heart influsion (rich culture medium)
cDNA	complementary DNA
Cam	Chloramphenicol
cpm	counts per minute
dATP	deoxyadenosine 5′-triphosphate
dCTP	deoxycytocine 5′-triphosphate
dGTP	deoxyguanine 5′-triphosphate
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTPs	deoxyribonucleoside 5′-triphosphate
dTTP	dioxythymidine 5′-triphosphate
EDTA	ethylenediaminetetraacetic acid
E value	expected value
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
LB	luria Bertani broth
MIV	"M-four", starvation medium for *H. influenzae* competence induction
mRNA	messenger RNA
NAD	nicotinamide adenine allele
NOV	Novobiocin
PA01	*Pseudomonas aeruginosa* PA01
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
sBHI	BHI supplemented with hemin and NAD
Tfp	Type IV pili
Tris	Tris(hydroxymethyl)aminomethane
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor, Dr. Rosemary Redfield, for her encouragement, guidance and financial support. Her expertise, vast knowledge, and work attitude added considerably to my graduate experience. I also appreciate her understanding, patience and assistance in writing reports (*i.e.* proposals, progress reports and this thesis). I would like to thank the other members of my committee, Dr. Patricia Schulte and Dr. Rachel Fernandez, for their counsel and assistance.

Special thanks goes out to Andrew Cameron, for his numerous help and the constructive discussions. He was always a source of information, a listening ear as well. I want to thank all of the current and former members of the Redfield lab for their help and friendship over the last few years. I had a great time with them.

I am grateful to my family, which is a great source of courage and venting of frustration during my graduate program. This study could not have been finished without their support.

Chapter 1: Introduction

Haemophilus influenzae has evolved the ability to take up DNA from the extracellular environment using a genetically regulated process called natural competence. As in most naturally competent bacteria (6, 12, 26, 58), proteins related to cell-surface filaments called type IV pili (Tfp) are required for DNA uptake in *H. influenzae* (23). The *pil* operon of *H. influenzae* contains genes coding for homologues of important Tfp proteins (Fig. 1.1), and previous studies have shown that this operon is essential for natural competence (23). However, the specific functions of these proteins in DNA uptake remain to be elucidated.

This study focused on the second gene of the *pil* operon, *pilB*. The objective was to investigate the specific function of *H. influenzae* PilB in DNA binding and uptake. This is important because: (1) Expression of type IV pili has not been demonstrated in *H. influenzae* Rd, despite the presence of Tfp in another *H. influenzae* strain 86-028NP (7). Type IV pili have been identified in many Gram negative bacteria including competent and non-competent bacteria. Most naturally competent bacteria express Tfp on the cell surface (except *A. actinomycetemcomitans*) (2, 26, 58). The presence of Tfp proteins but no protruding type IV pili suggests that the *H. influenzae pil* operon may have evolved to encode a DNA uptake apparatus. (2) Unlike *Pseudomonas aeruginosa, Pseudomonas stutzeri,* and *Nesseria gonorrhoeae*, whose Tfp systems are well studied, PilB is the only Tfp-related ATPase of the GspE family proteins in *H. influenzae* (4, 17, 32, 37, 66, 98). Studies on PilB's function may provide insight into the specific mechanism of *H.*

influenzae's DNA uptake, and help as well to understand the evolutionary role of the Tfp-like machinery in *H. influenzae*.

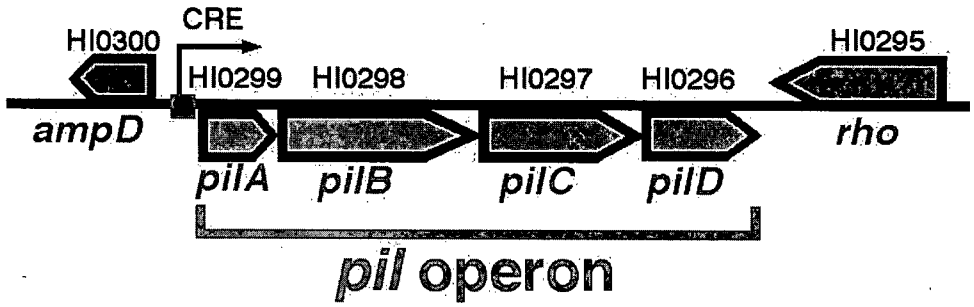

Fig. 1.1 The *H. influenzae pil* operon.

1.1 Natural competence and transformation

Natural competence, defined as the ability to take up exogenous DNA from the extracellular milieu using a genetically programmed process, plays a central role in bacterial horizontal gene transfer. As a general consequence of competence, natural transformation happens when DNA uptake is followed by recombination of the incoming DNA into the bacterial chromosome, and may result in a heritable change of genotype. Naturally transformable bacteria have been found in at least 40 species, distributed through all taxonomic groups (12).

The mechanism of natural competence has been intensively studied in some model organisms, including the Gram-negative bacteria *Neisseria gonorrhoeae, Pseudomonas*

(6, 12). *N. gonorrhoeae* and *H. influenzae* and their relatives share the property that they preferably take up DNA containing a specific uptake signal sequence (USS) that is distributed in their genomes in high frequency (20, 27, 92). Other transformation systems do not display such specificity.

The process of natural transformation can be envisaged as several sequential steps (Fig. 1.2): (1) DNA binds to the cell surface. (2) taken up into the periplasm, where DNA is inaccessible to exogenous DNase I (known as the DNase I resistant state), (3) passing through the inner membrane and, (4) sometimes foreign DNA recombining with any chromosomal homologue on the chromosome (71).

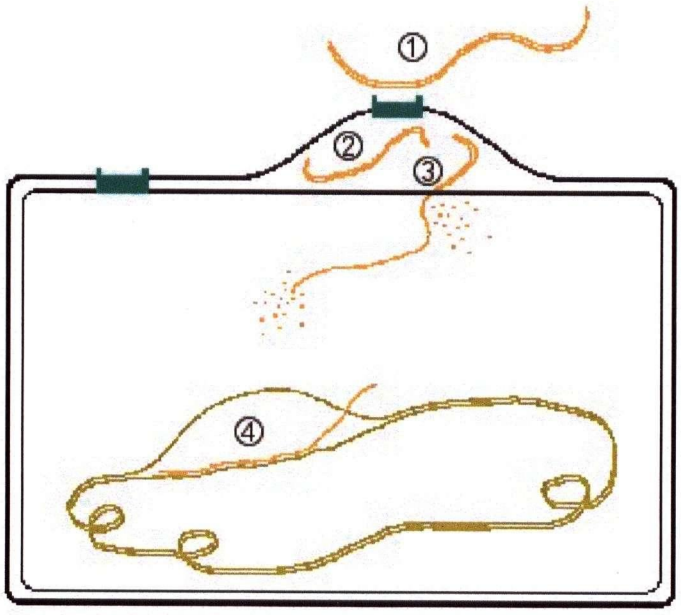

Fig. 1.2 An overview of natural competence and transformation. ① DNA binding to the putative receptor. ② Initial uptake across outer membrane. ③ DNA translocation across inner membrane. ④ Chromosomal displacement through homologous recombination.

1.2 Type IV pili and natural competence

Type IV pili are filamentous appendages constituted of pilin subunits on the surface of bacterial cells, mediating adherence and twitching motility, a flagella-independent bacterial translocation over moist surfaces (58, 62). Twitching motility is achieved through the extension, tethering, and then retraction of type IV pili into the bacterium, thereby pulling the cells along. A schematic overview of a *P. aeruginosa* type IV pilus is shown in Fig. 1.3.

Tfp and Tfp-related proteins can be assigned to distinct groups including pilin and pilin proteins, ATPases, prepilin peptidases, secretins and other assembly factors (6, 12) (Table 1.1).

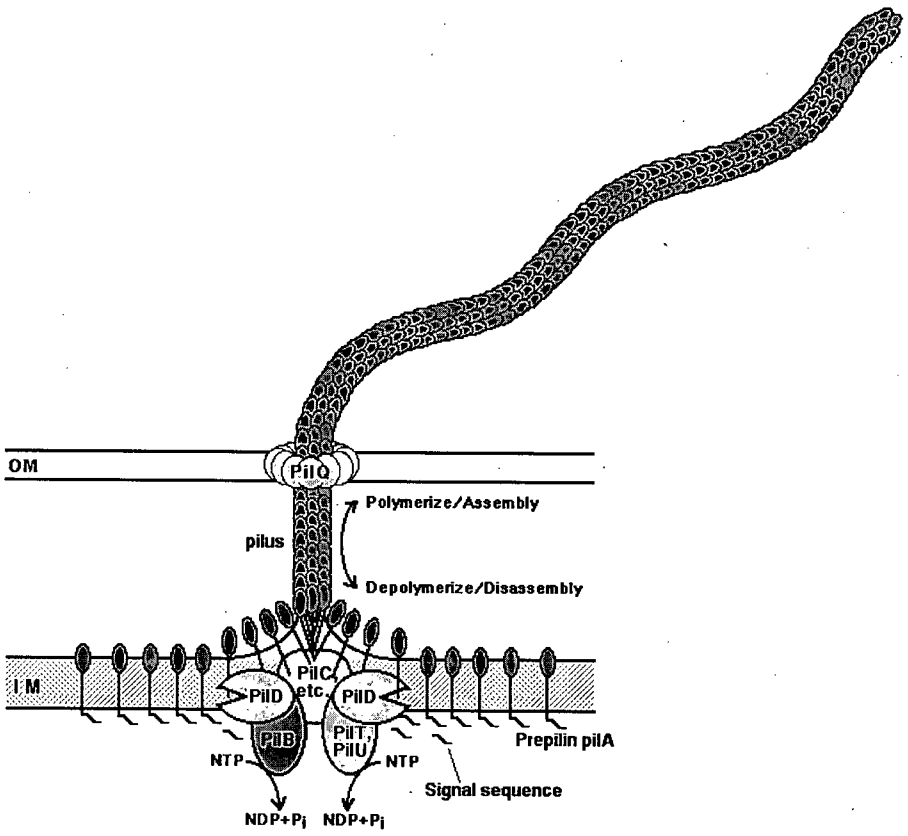

Fig. 1.3 Type IV pili of *P. aeruginosa.* After processing by the pre-pilin peptidase PilD, the PilA subunits associate via their hydrophobic stems to form a pilus with the help of PilB and possibly other assembly factors. Thereby, the core of the pilus forms a continuous hydrophobic layer with the inner membrane. The assembled pili penetrate the outer membrane through a gated pore formed by the multi-subunit complex of PilQ. The retraction is driven by PilT via depolymerization of the pilus fibers.

Table 1.1 Names given to Tfp & Tfp related proteins in different bacteria

Tfp proteins	***Neisseria gonorrhoeae***	***Haemophilus influenzae***	***Bacillus subtilis***	***Pseudomonas aeruginosa***
Pilin (pili subunit) & Pilin like proteins (various functions)	PilE, ComP, PilE1, PilV, etc.	PilA	ComGC, ComGD, ComGE, ComGG (Minor similarity to Pilin subunits)	PilAI, PilAII
Nucleotide binding proteins (generate forces)	(1) PilF (for Tfp biogenesis) (2) PilT (for Tfp retraction) (3) PilU (doesn't affect competence)	PilB	ComGA	(1) PilB (for Tfp biogenesis) (2) PilT (for Tfp retraction) (3) PilU (required for twitching motility)
Prepilin peptidase (prepilin processing)	PilD	PilD	ComC	PilD
Tfp assembly factor	PilG	PilC	ComGB	PilC
Secretin (forms gated pore on outer membrane)	PilQ①	ComE (different from comE1)	None	PilQ
Pili tip protein (adhesion)	PilC②	None	?	?
Other Tfp assembly factors	PilM, N, O, P	ComA, B, C, D		PilM, N, O. P

Pilin proteins are the subunits of the actual type IV pilus (or pseudopilus). A type IV pilus is primarily made up of the major pilin, a single small protein subunit, usually termed PilA or pilin (58). The common feature of Type IV pili from a wide range of Gram negative bacteria is the sequence similarities among the major pilins (29), which are highly conserved in the N-terminal region including a short positively charged leader sequence and a hydrophobic domain. In addition to the major pilin, some Tfp systems have several pilin-like proteins called minor pilins, which might be incorporated as well in the pilus fiber (12, 58, 63).

Pilins are all synthesized as prepilins, whose leader sequence is removed by the prepilin peptidase PilD to produce pilins. PilD is a bifunctional enzyme carrying out both this cleavage and *N*-methylation of the pilin subunit (85). It also cleaves a wide variety of substrates with prepilin-like leader sequences, including those involved in protein secretion (52).

The nucleotide-binding proteins are ATPases that use energy from ATP hydrolysis to drive the assembly or retraction of Tfp. They belong to a large superfamily of proteins, the VirB/GspE family, which function not only in the Tfp system but also in Type II and Type IV protein secretion (30, 49, 63). Proteins in this family are characterized by their nucleotide-binding motifs in the C-terminal region and by their peripheral (*e.g.* not integral) membrane localization. Multiple Tfp-related ATPases have been identified in most Tfp systems, including the well-studied Tfp systems of *P. aeruginosa* and *N. gonorrhoeae*. In *P. aeruginosa*, 3 Tfp related ATPases have been identified. PilB powers assembly of the pilus (90), while PilT is required for pilus retraction (94). Both

are essential for DNA uptake in naturally competent bacteria. The third ATPase, PilU, is required for twitching motility, but PilU mutants are competent for DNA uptake in *N. gonorrhoeae* (66).

Secretin proteins (PilQ) form the polymeric gated pore on the outer membrane, allowing passage of Tfp (41). Electron microscopic analyses revealed that PilQ forms a dodecameric ring with a central cavity of 5-6.5 nm in diameter, which is wide enough for passage of an intact type IV pilus (~ 6 nm in diameter) and could easily accommodate the DNA double helix (~2.4 nm) (6, 10, 12, 16). Therefore current model postulates that transforming DNA is taken up through the secretin pore left by depolymerization of the Type IV pilus (6, 26).

Other outer or inner-membrane proteins are also required for Tfp assembly and functions (*e.g.* PilC, PilM) (62). But little is known about their specific functions.

Tfp related functions are mainly mediated by the process of pilus elongation and retraction. Elongation is by subunit addition to the base of the helical array of pilin subunits, and retraction is thought to involve subunit removal (58, 62). In *P. aeruginosa* (Fig. 1.3), after the prepilin signal peptides are cleaved by the prepilin peptidase PilD, the PilA subunits associate with each other to form a pilus through interactions between the conserved N-terminal α-helices, with the help of PilB and other assembly factors (47). A continuous hydrophobic core is formed in the pillus filament and maximizes contact between subunits to provide extreme mechanical strength (18). The assembled pili penetrate the outer membrane through the gated pore formed by the multi-subunit

complex of secretin PilQ (10). The retraction is driven by the ATPase PilT via depolymerization of the pilus fibre at its base (60).

Type IV pili systems are evolutionally related to type II secretion systems (30, 63, 78). A subset of the components of type II secretion system share extensive sequence similarity with Tfp pilins and other Tfp proteins required for biogenesis of the type IV pilus (including PilB, PilC, PilD, PilQ) (63). The pseudopilins of type II secretion systems are homologous to Tfp pilins, and are thought to function in secretion by forming a periplasmic pseudopilus, which might work as a piston, pushing specific substrates from the periplasm through the secretin pore in the outer membrane (41, 72). When the genes of the *Klebsiella oxytoca* pullulanase (Pul) secreton (a type II secretion system) were expressed in *E. coli* K12 cells at high levels, a bundled pilus-like structure composed of the pseudopilin PulG could be formed on the cell surface (81). Furthermore, in *P. aeruginosa*, the secretin PilD is shared between type II secretion and type IV piliation system (84). Because of the similarities of the two systems, knowledge of Tfp systems have been extrapolated to understand the structure and function of Type II secretion systems, and vice versa (63, 67).

Significant involvement of Tfp-related proteins in DNA binding & uptake has been found in most of the naturally competent bacteria studied so far (6, 12, 58), except *Helicobacter pylori*, which takes up DNA through an unrelated type IV secretion system (82), and the related *Campylobacter jejuni*, whose DNA uptake uses a type II secretion system (95). In the bacteria requiring Tfp proteins for competence, mutants failing to express Tfp

homologues are unable to bind or take up DNA (26). The different names used for Tfp related proteins in different bacteria are listed in Table 1.1.

1.3 Possible mechanism of Tfp mediated DNA binding and uptake

Tfp proteins and homologues appear to function in the first steps of transformation, *e.g.* DNA binding and uptake. Experimentally this is measured by the amount of DNA bound to the cell surface and taken up into a DNase I resistant state, respectively (2, 26).

The specific role of Tfp in DNA uptake remains to be elucidated. However, based on the structure model of Tfp (X-ray diffraction of pilus assembly, electrostatic surface analysis) and the experimental data obtained from the model Tfp systems of *N. gonorrhoeae* and *P. aeruginosa* (18, 19, 47, 91), possible mechanisms of Tfp-mediated DNA binding and uptake in Gram negative bacteria have been proposed as follows (taking the *P. aeruginosa* Tfp proteins as an example):

Electrostatic surface analysis of modeled pilus fibers of *P. aeruginosa* pilin monomers suggested that a band of positive charge may be a common feature of all type IV pili (18, 47, 91). These positively charged patches that coil around the Tfp fibres provide high affinity for double-stranded DNA to bind non-specifically to the cell surface (2, 47, 65). Recently, van Schaik *et al.* showed that *P. aeruginosa* Tfp can bind DNA non-specifically and that the DNA binding appeared to be a function of the intact pilus (91). In *N. gonorrhoeae* and *H. influenzae,* whose DNA uptake prefer DNA with USS, an as-yet-unidentified sequence-specific receptor is also required (12). In *N. gonorrhoeae*, sequence-specific binding of DNA to the cell can be modulated by the expression of

different minor pilins (1, 2). Once DNA is selected by the sequence-specific receptor, the secretin channel is opened allowing passage of DNA. Since the predicted structure of Type IV pilus does not have space for a DNA channel in the center of the pilus (~ 10Å) (47), the transforming DNA is thought to be bound to the surface and taken up through the secretin pore as the pilus retracts by pilus depolymerization (26). Retraction of pili is powered by PilT, which provides energy through hydrolysis of ATP (33, 39, 54, 60). However, PilT may not be directly involved in DNA uptake (12, 37).

DNA uptake also involve the periplasmic DNA receptor (12), known in *N. gonorrhoeae* as ComE (Table 1.2) (13), which has homologues or partial homologues in both Gram positive and negative bacteria including *B. subtilis* and *H. influenzae* (43, 74). ComE is a periplasm protein with DNA binding activity. Chen and Dubnau proposed that binding of DNA to the cell surface triggers the opening of the secretin channel in the outer membrane and ComE could also mediate this process (12). Once the transforming DNA is taken up into the periplasm, ComE delivers the DNA to the channel in the inner membrane and DNA is then translocated to the cytoplasm.

Table 1.2 The homologues of the putative non-specific DNA binding protein in different bacteria

Natural competent bacteria	***Neisseria gonorrhoeae* (*meningitidis*)**	***Haemophilus influenzae***	***Bacillus subtilis***
putative non-specific DNA binding protein	ComE	ComE1	ComEA

1.4 Natural competence of *H. influenzae*

H. influenzae becomes competent either when the cells enter stationary phase (spontaneous competence) or when cells are transferred from rich medium into starvation medium (induced competence) (35, 45, 71). The natural competence of *H. influenzae* is a tightly regulated progress. Most of the competence genes identified so far are under the transcriptional control of the Competence Regulatory Element (CRE), which promotes transcription in response to depletion of energy or nucleotide pools (74).

1.5 *H. influenzae pil* operon and Tfp homologues

Like most naturally competent bacteria, *H. influenzae* encodes proteins homologous to type IV pili (23). Since Tfp-mediated DNA uptake has been identified in most naturally competent bacteria, this raises the question whether *H. influenzae*'s DNA uptake mechanism is similar to that of other bacteria.

The *pil* operon contains four open reading frames (*pilA, pilB, pilC, pilD*), which code for Tfp homologues (Fig.1.1). Like most *H. influenzae* competence induced genes, a CRE consensus sequence is found 61.5 bp upstream of the transcription start of the operon (53). A BLAST search showed that PilA is a prepilin peptidase dependent protein homologous to the *P. aeruginosa* & *N. gonorrhoeae* Tfp subunits PilA (PilE) (See Table 1.1 for different names of Tfp proteins). All these three proteins contain a signal sequence and a conserved region at the N-terminal (22, 59, 83, 99, 100). PilB, a protein with nucleotide binding motifs, is a homologue of both PilB (PilF) and PilT, paralogous *P. aeruginosa* & *N. gonorrhoeae* proteins with putative ATPase functions (32, 60, 98).

The other two proteins, PilC and PilD, are homologues of *P. aeruginosa*& *N. gonorrhoeae* Tfp biogenesis factors PilC (PilG) and prepilin peptidase PilD respectively (25, 48, 64, 89).

Previous studies showed that the *H. influenzae pil* operon is essential for natural competence. Insertions into the upstream regulatory region were found to eliminate competence (23). In addition, all the four genes of the *pil* operon are induced 100 – 150 fold as other competence genes do when the cells become competent (74). Mutations have been constructed in our lab by insertion of a mini-Tn*10* transposon into *pilA* and into the CRE binding site of the operon (Lab data: CM#889, CM#893). Transformation experiments showed that the *pilA* mutation eliminated natural competence, and insertion into the CRE site reduced the transformation frequency to background level.

In addition to the four Tfp genes in the *pil* operon, *H. influenzae* also has genes coding for homologues of most other Tfp-like proteins identified in other bacteria so far, including *comA, comB, comC, comD, comE* and *HI0366* (coding for homologues of *P. aeruginosa* PilM, PilN, PilO, PilP, PilQ and PilF) (31, 55, 56). *P. aeruginosa, pilQ* codes for the secretin protein that forms oligomeric rings in the outer membrane allowing passage of Tfp. The other genes *pilM, pilN, pilO, pilP* and *pilF* of *P. aeruginosa* are also required for biogenesis of Type IV pili (56, 93), although the specific functions of these genes are not known. In *H. influenzae,* Mini-Tn*10* insertions in the *pilM* and *pilQ* homologues *comA* and *comE* (different from *comE1*) prevent DNA binding & uptake and abolish transformation (88). These findings suggest that *H. influenzae* may share similar

mechanisms in DNA binding and uptake with other Gram negative bacteria that are naturally competent.

1.6 *H. influenzae* Tfp and competence

Despite the existence of a general model for DNA binding & uptake in Gram negative bacteria, comparison with the Tfp and competence of *N. gonorrhoeae* and other bacteria reveals some atypical features of *H. influenzae* Tfp and competence.

The first is that no visible type IV pili have been identified on the cell surface of *H. influenzae*, unlike most Gram negative bacteria capable of DNA uptake, including *N. gonorrhoeae, N. meningitidis, P. stutzeri* and *Acinetobacter* sp. BD413. I hypothesize that in *H. influenzae* the Tfp-like machinery is maintained specifically for DNA uptake. Previous studies have shown that DNA uptake in *N. gonorrhoeae* requires only small amounts of pilin rather than long pilus fibers (33, 51). Furthermore, substantial increases in transformation efficiency over an isogenic, nonpiliated mutant *N. gonorrhoeae* were observed when limited amounts of three of the pilin variants were expressed (51). It was proposed that, although Tfp proteins are required in DNA uptake, fully assembled type IV pili may not be necessary (12). Therefore I hypothesize that, the Tfp proteins in *H. influenzae* form Tfp-like assemblies or pseudopili within the periplasm, as was also proposed by Chen and Dubnau (12). The pseudopili that serve as the DNA uptake machinery would need to be long enough to penetrate the outer membrane through the pore constituted by ComE (PilQ in *Neisseria*) on the outer membrane (Fig. 1.4). The

retraction of the pilus-like fiber within the periplasm would then allow the translocation of DNA across the outer membrane.

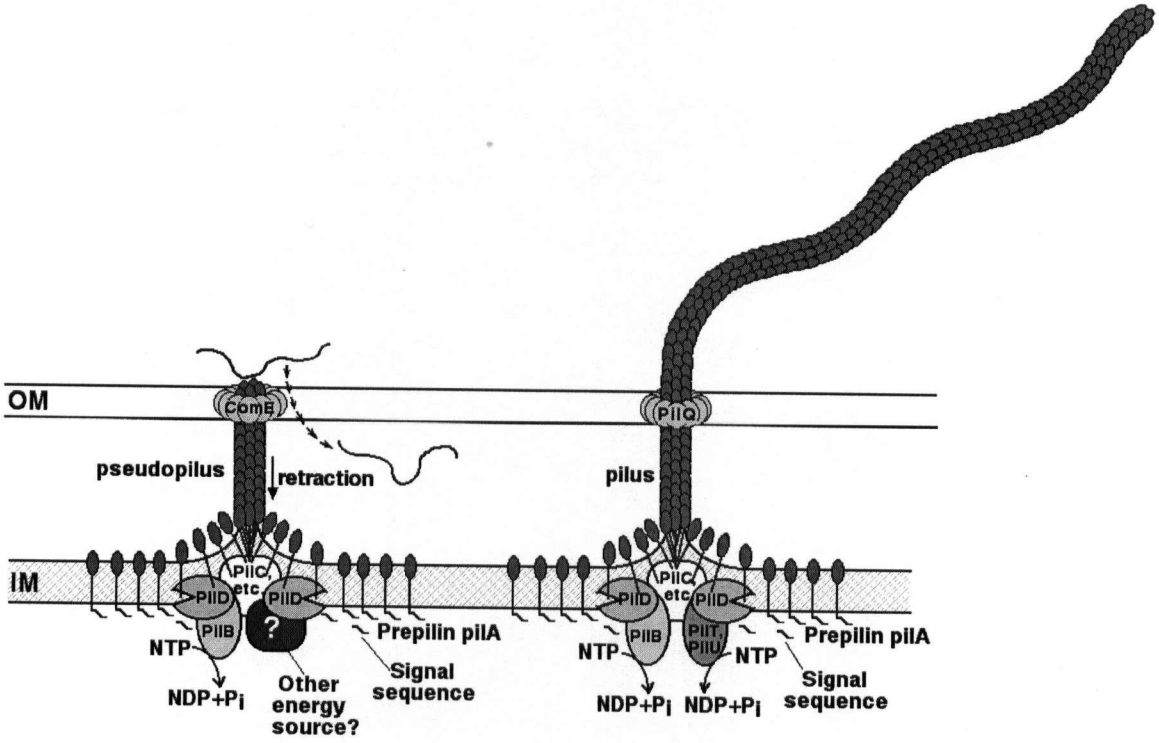

Fig. 1.4 Proposed model of the Tfp-like DNA uptake apparatus in *H. influenzae*, in comparison with *P. aeruginosa* Tfp. After processed by the pre-pilin peptidase PilD, the PilA subunits associate with their hydrophobic stems to form a pilus-like fibre with the help of PilB and possibly other assembly factors. The assembled pili-like fibres of *H. influenzae* are only long enough to penetrate the outer membrane through a gated pore formed by the polymeric complex of ComE. Driven by PilB, or perhaps another as yet unidentified protein, the retraction of the fibre through depolymerization of the PilA subunits, allows DNA to be taken up into the periplasm.

The second unusual feature of *H. influenzae*'s Tfp genes is that only a single gene *pilA* encodes a Tfp prepilin protein (26). Multiple pilin proteins have been identified in most naturally competent bacteria containing Tfp. The pilus is primarily composed of the major pilin, while minor pilins might also be present in the pilus assembly (12, 26). In naturally competent bacteria, minor pilins have various effects on DNA uptake. In *N. gonorrhoeae,* the Tfp structural protein PilE (major pilin) is required for non-specific DNA binding. A homologous protein, ComP, is involved in sequence-specific DNA binding (99). A third protein, PilV, whose mutation causes a hypertransformable phenotype, acts as an inhibitor of ComP function (1). *P. stutzeri* also has a major pilin gene *pil*VI that is required for DNA uptake and a minor pilin gene *pil*VII that is antagonistic to transformation (36). In *H. influenzae*, however, the only other two pilin-related genes (HI0938, HI0939) code for PulG-like proteins that are more similar to the pseudopilins in Type II secretion systems, than to Tfp pilins (61).

The third unusual feature of *H. influenzae*'s competence genes is the role of ComE1, the protein thought to be functionally equivalent to *N. gonorrhoeae* ComE. As mentioned in Section 1.2.3, *Neisseria* ComE and its homologues are periplasmic proteins capable of DNA binding and have been proposed to be the periplasmic DNA receptor (12, 26). The first identified protein of this family, *B. subtilis* ComEA, is essential for DNA binding (43). In *N. gonorrhoeae*, a 4×10^{-4} fold reduction in transformation frequency was reached when all four copies of the *comE* genes were deleted (13). In *H. influenzae*, however, ComEA only shares homology at the C-terminal region with *Neisseria* ComE,

as indicated by a BLAST search, and deletion of *comEA* only resulted in a 10 fold decrease in transformation frequency (lab data CM#957).

The absence of a second Tfp related GspE-like ATPase is the fourth unique feature of *H. influenzae*'s competence. As mentioned in Section 1.2, three Tfp-related ATPases PilB, PilT and PilU have been found in the well studied Tfp systems of *N. gonorrhoeae, P. aeruginosa* and *P. stutzeri* (17, 32, 37, 60, 66). Both PilB and PilT are required for DNA uptake in *N. gonorrhoeae*. PilB homologues are known to function in Tfp biogenesis (32, 90). PilT is responsible for Tfp retraction/depolymerization, through which DNA is thought to be taken up in naturally competent bacteria (98). Our BLAST searches with the above well-characterized Tfp-related ATPases showed that PilB is the only Tfp-related homologue of this family in *H. influenzae*. When searching *H. influenzae*'s genome with *P. aeruginosa* PA01 PilB and PilT, no other protein was found with E-values lower than 1.1 and 0.36. The possible explanations are discussed below.

Taken together, although Tfp proteins are required for natural competence in *H. influenzae*, the detailed mechanism of Tfp-mediated DNA uptake is unknown. Since there is only one homologue of the Tfp-related ATPase PilB in *H. influenzae*, the possible explanations would be that either PilB is required only for Tfp assembly, or functions for both Tfp biogenesis and DNA uptake via Tfp retraction. Therefore in the former case, an as-yet-uncharacterized protein, other than a PilT homologue, would power the retraction of the Tfp-like machinery, using either ATP or other energy source (*e.g.* proton motive force) or both.

1.7 Objective and approaches

Based on the above considerations, *pilB* was chosen as the focus of this study. The predicted protein sequence of PilB was analyzed and conserved nucleotide-binding motifs in the VrbB/GspE family ATPases were identified (49). The sequence was aligned with PilB, and PilT homologues, showing the most conserved C-terminal region in this family (see Section 1.2). To experimentally investigate the specific function of PilB in DNA uptake, a *pilB* knockout was constructed by insertion of a Tn*903* Kan^R cassette at the *Bcl*I site. To examine the polarity of the knockout, a wild type *pilB* was introduced into the strain. The transformation capacity of the complemented *pilB* were tested. Real time PCR was used to examine the expression of the downstream genes. Subsequently, transformation assays and DNA binding & uptake experiments were performed to test the effect of the knockout on competence. To investigate *H. influenzae* PilB's specific function, plasmids containing either *P. aeruginosa* PA01 *pilB* or *pilT*, or both, under control of the CRE and the promoter of the *H. influenzae pil* operon, were constructed and introduced into the *H. influenzae pilB* knockout. The sequence and the expression of *P. aeruginosa pilB* and *pilT* in the knockout cells were verified by DNA sequencing and real time PCR, respectively. The complemented *pilB* knockout strains, thus constructed, were used to carry out transformation tests and DNA binding & uptake assays.

Chapter 2: Material and methods

2.1 General methods

2.1.1 Strains, media and plasmids

The bacterial strains used in this study are listed in Table 2.1. All *H. influenzae* strains are descendants of the original Rd strain (3, 96). *H. influenzae* strains were routinely grown in sBHI medium (Brain Heart Infusion supplemented with 10 μg/ml hemin and 2 μg/ml NAD) at 37°C and shaken at 200 rpm (70). To promote aeration, the culture volume did not exceed 20% of the flask capacity. When growing strains with plasmids or cassettes, sBHI was supplemented with novobiocin (Nov, 2.5 μg/ml), kanamycin (Kan, 7.0 μg/ml), or chloramphenicol (Cam, 1.0 μg/ml). MIV starvation medium was used for preparation of competent cells (40). Transformed *H. influenzae* cells were plated on sBHI agar with or without addition of appropriate antibiotics. The *E. coli* DH5α, Top10 (Inv itrogen) and GM2163 (NEB) strains, grown in LB, were used for propagating plasmids. When necessary, the following antibiotics were used for *E. coli*: ampicillin (Amp) 100 μg/ml; chloramphenicol (Cam) 20 μg/ml; kanamycin (Kan) 10 μg/ml. *P. aeruginosa* PA01 was grown at 37°C in low salt LB (0.05% NaCl).

The plasmids used in this work are listed in Table 2.2. pGEM-T easy vector (Promega) was used for cloning of *H. influenzae pilB*. pWJC3 is the source of the Tn*903* Kan^R cassette (14). pSU20 was used as a shuttle vector between *E. coli* and *H. influenzae* (57).

Table 2.1 Bacterial strains used in this study

Strain	Genotype	Source or Reference
H. influenzae		
KW20	wild-type	(3)
RR1137	*pilB::Kan*^{R}	This study
RR1136	KW20, pSUPILB	This study
RR1138	*pilB::Kan*^{R}, pSUPILB	This study
RR1150	Δ*pilB::Kan*^{R}*/CRE/pilC*	This study
RR1151	Δ*pilB::Kan*^{R}*/CRE/pilC*, pSUPILB	This study
RR1156	Δ*pilB::Kan*^{R}*/CRE/pilC*, pPAPT (Pa *pilT*)	This study
RR1157	Δ*pilB::Kan*^{R}*/CRE/pilC*, pPAPB (Pa *pilB*)	This study
RR1158	Δ*pilB::Kan*^{R}*/CRE/pilC*, pPAPBT (Pa *pilB*, Pa *pilT*)	This study
RR1159	Δ*pilB::Kan*^{R}*/CRE/pilC*, pPAPT2 (Pa *pilT*)	This study
RR1196	Δ*pilB::Kan*^{R}*/CRE/pilC*, pPAPBT2 (Pa *pilB*, Pa *pilT*)	This study
E. coli		
DH5α	*supE44 recA1*	(38)
GM2163	*dam*$^-$ *dcm*$^-$	NEB
Top10	*lacZ*ΔM15, *end*A1, *deo*R, *hsd*R, *mcr*A, *rec*A1	Invitrogen

Pa: *P. aeruginosa* PA01

Table 2.2 Plasmids used in this study.

Plasmid	Vector	Insert	Application	Source or Reference
pPILB	pGEM-T easy	P_{pi}+ *pilA* + *pilB* + part of *pilC*	Construction of RR1137	This study
pPILB::Kan^R	pGEM-T easy	P_{pil} + *pilA* + *pilB* with Kan^R + part of *pilC*	Construction of RR1137	This study
pSUPILB	pSU20	P_{pil} + *pilA* + *pilB* + part of *pilC*	Polarity examination	This study
pPILC	pGEM-T easy	P_{pil} + *pilC* + part of *pilD*	Construction of RR1150	This study
pBKan(CREC)$_5$	pGEM-T easy	P_{pil} + *pilA* + truncated *pilB* with Kan^R + (P_{pil} + *pilC*)$_5$	Construction of RR1150	This study
pPAPB	pSU20	Hi P_{pil} + Pa *pilB*	Complementation of RR1150	This study
pPAPT	pSU20	Hi P_{pil} + Pa *pilT* (Starts at ATG)	Complementation of RR1150	This study
pPAPBT	pSU20	Hi P_{pil} + Pa *pilB* + Hi P_{pil} + Pa *pilT* (Starts at ATG)	Complementation of RR1150	This study
pPAPT2	pSU20	Hi P_{pil} + Pa *pilB* (Starts at TTG)	Complementation of RR1150	This study
pPAPBT2	pSU20	Hi P_{pil} + Pa *pilB* + Hi P_{pil} + Pa *pilT* (Starts at TTG)	Complementation of RR1150	This study
pWJC3	N/A	Kan^R	Source of Tn*903* Kan^R cassette	(14)
pSU20	N/A	N/A	Shuttle vector between *H. influenzae* and *E. coli*	(57)
PGEM-T easy	N/A	N/A	Cloning vector	Promega

N/A: Not applicable.
P_{pil}: CRE promoter of *H. influenzae pil* operon.
Hi: *H. influenzae*
Pa: *P. aeruginosa*

2.1.2 *H. influenzae* transformation

2.1.2.1 Natural transformation of *H. influenzae* with double-stranded linear DNA

Standard procedures for *H. influenzae* natural transformation have been described by Goodgal (35). The cells were grown as described above. At an OD_{600} nm of 0.2-0.3, 10-12 ml of culture were washed with and transferred to MIV medium in which they were shaken for 100 min at 37°C at 100rpm to induce competence (40, 97). MAP7 DNA, the genetically marked DNA from the strain MAP7, was used for standard transformation assays (71). After addition of 1.0 μg/ml MAP7 DNA or other DNA needed to 1 ml of cells, the cells were shaken at 37°C for 15-30 min, then diluted and plated on sBHI agar. Novobiocin was used as the antibiotic marker in transformation tests unless otherwise noted. When selecting for chloramphenicol resistance, the transformation mixture was diluted with 2 volumes of sBHI and incubated at 37°C for 90 minutes to allow expression of the resistance gene (71). Transformation frequency was defined as the number of transformants per milliliter divided by the total number of cells per milliliter and was measured using an excess of chromosomal DNA (1μg to 1 ml of competent cells).

2.1.2.2 Natural transformation of *H. influenzae* with plasmid DNA

The method for transforming a plasmid into *H. influenzae* cells was described by Stuy (86) and performed with minor modifications. After incubation of 1-3 μg plasmid DNA with 1 ml of MIV-induced competent cells for 30 min at 37°C, glycerol was added to a final concentration of 30-32%. The cells were incubated at room temperature for 10 min, collected by centrifugation, resuspended in 1.5 ml of sBHI, and plated as usual. For

chloramphenicol selection, the cells were incubated at 37°C for 90 minutes before plating (71).

2.1.2.3 Transformation of plasmid DNA into chemically-competent *H. influenzae* cells

The method used for making *H. influenzae* cells chemically competent was modified from Barcak *et al.* and Williams *et al.* (8, 97). At an OD_{600} of 0.30 – 0.33, cells in 10 ml of culture were washed with 10 ml of 25 mM cold $CaCl_2$ and collected by filtration. The cells were resuspended in 2 ml of 75 mM ice-cold $CaCl_2$, of which 1 ml of cells was pelleted again, and resuspended in 100 µl of 75 mM ice-cold $CaCl_2$. To the competent cells thus prepared, 1.0-2.0 µg of plasmid was added and the mixture was incubated on ice for 30 min. Heat-shock was performed at 37°C for 3 min, followed by addition of 1 ml of sBHI to the cells, which were then shaken at 37°C for 1.5 hours and plated on sBHI agar.

2.1.3 *E. coli* plasmid transformation

E. coli competent cells were prepared using the filtration method described by Williams *et al.* (97). Once grown in LB broth to an OD_{600} of ~0.375, 10 ml of the cells was collected by filtration, washed once with 10 ml of 100 mM ice-cold $CaCl_2$ and resuspended in 2 ml of 100 mM ice-cold $CaCl_2$. The cells were used immediately for transformation. Frozen stocks of competent cells were prepared by adding 0.5 ml of 80% glycerol to 2 ml of above cell suspension containing 100 mM ice-cold $CaCl_2$, dividing

into 150 µl aliquots and storing at –80°C. Transformation of *E. coli* was done by the standard method described by Sambrook *et al.* (77).

2.1.4 DNA manipulations

2.1.4.1 Isolation of plasmid DNA

Plasmid DNA was isolated and purified from *E. coli* or *H. influenzae* using Qiaprep spin miniprep kits (Qiagen) following the instructions of the manufacturer.

2.1.4.2 Isolation of chromosomal DNA

Chromosomal DNA of *H. influenzae* was extracted using the method described by Poje and Redfield (70). 1.5 ml of a fresh overnight culture was pelleted and resuspended in cell resuspension solution (50 mM Tris HCl, pH7.4, 50 mM EDTA). The cell suspension was then mixed with 50 µl of 10% SDS and incubated at 50°C for ~10 minutes for complete lysis of the cells. The chromosomal DNA was extracted twice with phenol/chloroform and precipitated with 2 volumes of 95% ethanol in the presence of 15 mM NaCl. The fibrous clump of DNA resulting from the precipitation was retrieved by winding it onto the sealed tip of a Pasteur pipette and was washed with 1.0 ml of 70% ethanol dribbling down the pipette. The DNA was then air-dried for one hour. The dry DNA was resuspended in 200 µl of dH_2O or TE buffer (10 mM Tris-HCL, pH7.4, 1 mM EDTA). A few hours were allowed for full dispersion of the DNA.

The method for extraction of *P. aeruginosa* PA01 chromosomal DNA was modified from the online protocol of Goldberg lab (34). From an overnight culture of *P. aeruginosa*

grown at 37°C in low salt LB broth (0.05% NaCl), 1ml of cells were pelleted and resuspended in 1ml TNE buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, pH 8.0). The cells were pelleted again and resuspended in 135 µl of TNE buffer. To lyse the cells, 135 µl of TNE with 2% Triton X-100 was added followed by addition of 30 µl freshly prepared 5 mg/ml lysozyme and incubation at 37°C for 30 minutes. The cell lysate was treated with 15 µl of 20mg/ml proteinase K at 65°C for 2 hours. Phenol-chloroform extraction was performed twice afterwards to purify the DNA. The subsequent procedures were the same as described above for *H. influenzae* DNA extraction.

2.1.4.3 Gel electrophoresis, gel extraction, restriction digestion and ligation

Agarose and polyacrylamide gel electrophoresis were performed as described by Sambrook *et al.* (77) and Ausubel *et al.* (5). DNA fragments were extracted and purified from agarose gels using QIAquick Gel Extraction Kits (Qiagen) according to the instructions of the manufacturer. DNA manipulations with restriction endonucleases and T4 DNA ligase were done according to the manufacturers' recommendations. The ligated products were transformed into *E. coli* DH5α, GM2163 (NEB) or Top 10 (Invitrogen) competent cells.

2.1.4.4 PCR amplifications

Amplification of DNA by PCR was done in a PTC-150 MiniCycler (MJ Research). *Taq* DNA polymerase was used for most PCR amplifications. *Pfu* polymerase was used if products longer than 3.5kb were to be amplified or if PCR products with no "A" overhang were required. For Inverse PCR of pPILB::Kan^R (a 6174 bp amplicon), the

Expand Long Template PCR System (Roche) was used. The PCR primers used in this study are listed in Table 2.3. Primers were designed using the online program NetPrimer of Premier Biosoft International (44). Simulation of PCR reactions were carried out using Amplify 1.2 (28). DNA sequencing was performed by the UBC NAPS Unit (Nucleic Acid Protein Service Unit).

For colony PCR, single colonies were picked up with 20-200 µl pipette tips and resuspended in water in PCR tubes. The cells were lysed at 95°C for 10 min and placed on ice afterwards for a few minutes. The remaining components were then added and PCR reaction was performed the same way as normal PCR.

Table 2.3 Primers used in this study.

Name	Sequence	Original usage	Source
PilBf PilBr	5′-GCTTGTGAGAATGCTAAACCAGA 5′-GGTCAGCAATCCTTTCTCAATATCT	Cloning of KW20 *pilB*	This study
pilCf pilCr	5′-TTCTTGGTTTGCCACGTTTG 5′-CAAGCTGGCGTACCGCTTAA	Real time PCR of *H. influenzae pilC*	This study
pilDf pilDr	5′-CACTTTTTTTGTTGAATAGAGGCAAA 5′-GCGATTTACGTTGAATTATTTCCA	Real time PCR of *H. influenzae pilD*	This study
LPICf LPICr	5′-AATGACTAAAAAACTCTTTTATTATCAAGC 5′-GTTG**TCTAGA**GGCAAAGTGCGGTTGAAATG *Xba*I	Cloning of *H. influenzae pilC.*	This study
VecF Vec2R	5′-CATTTTCCTTTTATTAAGCCTTTGTTGG 5′-AGGT**TCTAGA**GGTCGAATCGGCGTGTATCA *Xba*I	Inverse PCR of pSUPILB and pPILB	This study
Vec2F	5′-TTTA**TCTAGA**AATCGCCCATCTTGTGGAAGTC *Xba*I	Inverse PCR of pPILB::Kan^R	This study
piBF2 piBR	5′- CCAA**ACTAGT**GAACCTCAACAAGCACTTACC *Spe*I 5′-TCTT**ACTAGT**GCCCTCCCAGATGAAAAC *Spe*I	Cloning of *P. aeruginosa pilB* coding sequence and 200 bp upstream	This study
Vec3F Pro-10R	5′-CCTTT**TCTAGA**GCCTTTGTTGGCAACGACATTGTG *Xba*I 5′-AGGT**TCTAGA**GGTCGAATCGGCGTGTATCA *Xba*I	Inverse PCR of pSUPILB	This study

Table 2.3 Primers used in this study (continued).

Name	Sequence	Original usage	Source
piTF piTR	5′-AATGGATATTACCGAGCTGCTCGCCTTCA 5′-TCCT**ACTAGT**GAATCCTAGACGCAGTTCC *Spe*I	Cloning of *P. aeruginosa pilT* (Starts at ATG). The first "A" in piTF before the start codon ATG is the last residue of the *pil* promoter	This study
piTF2 piTR	5′-ATTGGGGAGTCCTATGGATATTACCGA 5′-TCCT**ACTAGT**GAATCCTAGACGCAGTTCC *Spe*I	Cloning of *P. aeruginosa pilT* (Starts at TTG). The first "A" in piTF2 before the start codon ATG is the last residue of the *pil* promoter	This study
K1 K2	5′-**GGATCC**GGGGGGGAAAGCCACGTTTGT *BamH*I 5′-AGCCGCCGTCCCGTCAAGT	Amplification of a 997 bp fragment from pWJC3 containing Kan^R cassette	(61)
PFRTPilA PRRTPilA	5′-AGTTCTTTGAGTTGGCTGCTTTCT 5′-TCAGCCTGTGAAACCATTGC	Real time PCR of *H. influenzae pilA.*	MN #304
SpBEf SpBr	5′-CTCTAGTGCCCTCCCAGATGAAAA 5′-CAGTCGTATCTCTGCTCGTCTCAA	Sequencing of pPAPB	This study
SpBf	5′-GGTAGAGTTCCTTCTGGTCCTCCTC	Sequencing of pPAPB	This study
SpBSf	5′-CGGAACTGCGTCTAGGATTCACTA	Sequencing of pPAPBT and pPAPBT2	This study
SpTSf	5′-TCCCAGTCACGACGTTGTAAAAC	Sequencing of pPAPBT and pPAPBT2	This study
PaBf PaBr	5′-GCTCTTCCGACCTGCACTTC 5′-AGCGCACCCGGTAGATCTT	Real time PCR of *P. aeruginosa pilB*	This study
PaTf PaTr	5′-CGGGACGTCTGAAACACGTT 5′-ATGGAGGAGCTTGGCATGG	Real time PCR of *P. aeruginosa pilT*	This study

2.1.5 DNA binding & uptake assays

DNA binding & uptake by competent *H. influenzae* cells was measured using *Bgl*II or *Hin*fI fragments of KW20 DNA end-labeled with α-^{33}P-dATP (Amersham). KW20 DNA (~ 6.8 ng) was treated with *Bgl*II or *Hin*fI in a 50 µl reaction, and 20 µl was used in a fill-in reaction with Klenow enzyme (Roche) in the presence of 6.25 mM dGTP/dTTP/dCTP and 16 pmol of α -^{33}P- dATP (40 µCi). The reaction was then chased with 0.625 mM dNTPs at 37°C for 15minutes. Unincorporated nucleotides were removed using Sephadex G25 mini columns (Amersham), yielding 450 µl of 8.4 ng/µl labeled DNA (1.45×10^4 cpm/ng).

2.1.5.1 Total DNA binding plus uptake

To measure the total DNA bound to the cell surface and taken up into the cells, ^{33}P labeled DNA (~ 100 ng) was added to 1 ml of MIV-induced competent cells. After incubation for 5-10 min at 37°C, the cells were washed twice with 1ml of MIV medium, pelleted, and resuspended in 100 µl of MIV. The cells and 100 µl of the supernatants of each wash were separately used for scintillation counting. The total amount of DNA binding plus uptake was determined from the radioactivity associated with the cells.

2.1.5.2 DNA binding and/or uptake

To measure DNA binding and uptake separately, ^{33}P labeled DNA (~ 100 ng) was added to 1 ml of MIV-induced competent cells. After incubation for 5-10 min at 37°C, the sample was then split into two 0.5 ml fractions. One 0.5 ml fraction was transferred to ice immediately to stop DNA uptake. The other fraction was incubated with 10 µl of 100

μg/ml DNase I for 15 min at 37°C. The cells were washed twice with 1ml of MIV medium, pelleted, and resuspended in 100 μl of MIV. Subsequently, the cells and 100 μl of the supernatants of each wash were subjected to scintillation counting. The total amount of DNA bound to the cell surface and taken up into the cells was determined from the radioactivity of the DNase I-free cell fraction, while the amount of DNA taken up into the cells was determined from the radioactivity of DNase I-treated cell fraction. The amount of DNA binding is obtained by subtracting the above two numbers.

Scintillation counting of the samples was performed in 1ml of liquid scintillation cocktail (Amersham) in a Beckman LS180 I liquid scintillation system.

2.1.6 Real time PCR

Real time quantitive PCR was performed using an ABI Prism 7000 Sequence Detection System. The primers used for the real time PCR of *H. influenzae pilA*, *pilC*, *pilD* and *P. aeruginosa pilB* and *pilT* in *H. influenzae* were designed using Primer Express software supplied by Applied Biosystems, and are listed in Table 2.3. Total mRNA was extracted from MIV-induced competent cells of *H. influenzae* using an RNeasy Mini Kit (Qiagen) and was treated with DNase I using a DNA-free kit (Ambion) to remove possible trace of DNA. Reverse transcriptase PCR was carried out in the PTC-150 MiniCycler (MJ Research) according to the protocol provided by the SYBR Green PCR Master Mix and RT-PCR protocol (Applied Biosystems) to get cDNA. Real time PCR was then performed using the cDNA as template in the ABI Prism 7000 Sequence Detection

System (Applied Biosystems). Data analysis was done using the software provided by the same company.

2.1.7 Nucleotide sequence analyses

Multiple sequence alignments were performed using NCBI BLAST programs (NCBI) and ClustalW (EMBL-EBI). Restriction maps of DNA were obtained using DNA Strider (24).

2.2 Plasmid and strain constructions

This section includes the overall strategy and the general procedures of plasmid and strain construction.

2.2.1 Construction of *H. influenzae* plasmids

2.2.1.1 pPILB and pPILB::Kan^R

The scheme for construction of pPILB is shown in Fig. 2.1. To generate pPILB, a fragment containing *H. influenzae* KW20 *pilB* and flanking sequences was amplified from MAP7 chromosomal DNA using *Taq* DNA polymerase and primers PilBf/PilBr. The 2326 bp PCR product was ligated to a pGEMT-easy vector (Promega), and transformed into GM2163 (*dam*$^-$, *dcm*$^-$) (NEB). This host was chosen because the restriction enzyme *BclI* used in the next step is blocked by Dam methylation. Transformants were plated on LB plates supplemented with Ampicillin, X-gal and IPTG.

White or light blue colonies were selected. Transformants carrying the desired pPILB construct were verified by restriction analysis using *Eco*RI, *Dra*I and *Sac*II.

For pPILB::Kan^R construction, a 1466 bp fragment bearing a Tn*903*-derived Kan^R cassette was obtained from pWJC3 by *BamH*I digestion and inserted at the *Bcl*I site of *pilB* on pPILB, resulting in the plasmid pPILB::Kan^R (Fig.2.2). Kan^R transformants carrying the right constructs were identified by restriction analysis with *BspH*I, *Pvu*I, *Nru* I, and *Ssp*I digestions.

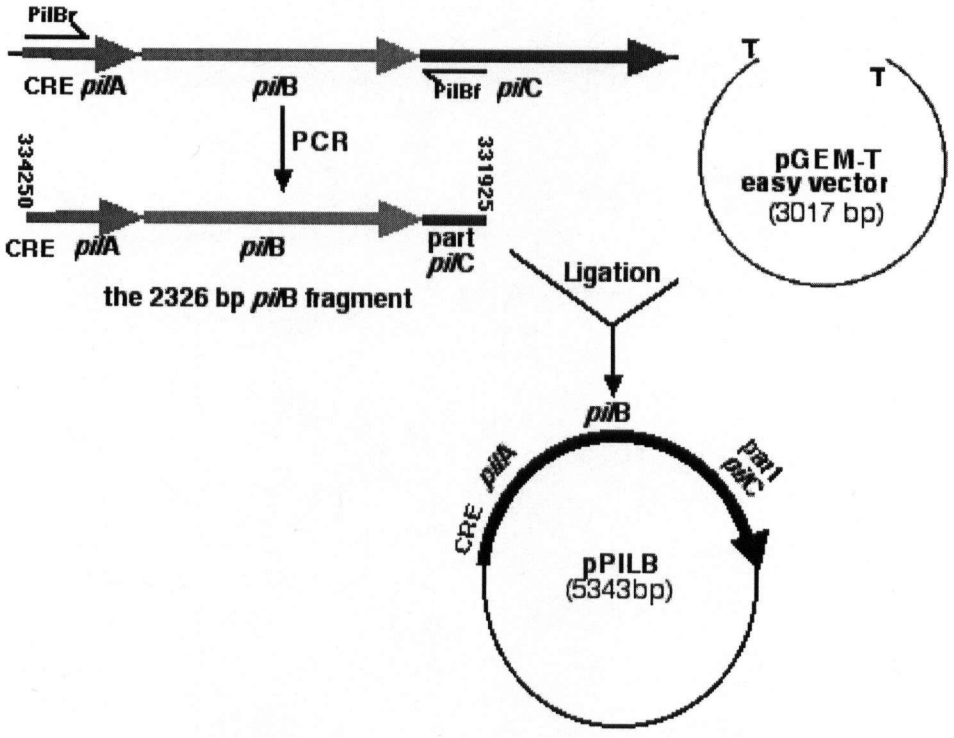

Fig. 2.1 Construction of pPILB. A fragment containing *pilB* and the flanking sequence was amplified from KW20 chromosomal DNA and inserted into the pGEM-T easy vector. The fragment includes the CRE promoter of the *pil* operon, *pil*A, *pilB* and about

330 bp of the *pilC* gene. The *BclI* site used for the later cassette insertion was at 1280bp, so that there are at least 1kb flanking region on either side for the future homologous recombination of the *pilB::Kan*^{R} fragment into the chromosome.

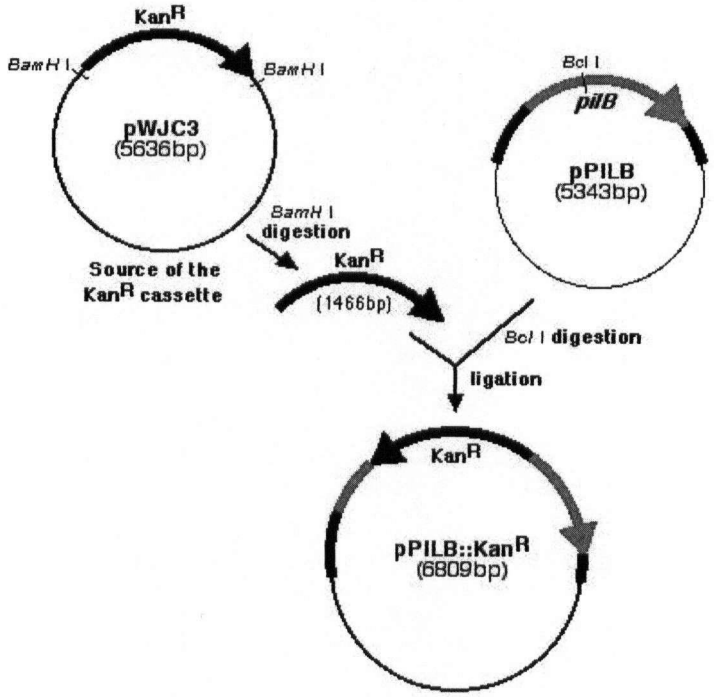

Fig. 2.2 Construction of pPILB::Kan^R. A 1466 bp fragment containing the Kan^R cassette was obtained with *BamHI* digestion of pWJC3 and inserted into the *BclI* site of *pilB* on pPILB. The orientation of the Kan^R cassette is opposite to that of *pilB*.

2.2.1.2 pPILC

The scheme for pPILC construction is shown in Fig. 2.3. A fragment containing *H. influenzae pilC* was amplified from KW20 chromosomal DNA and cloned into a vector containing a CRE promoter of the *H. influenzae pil* operon. The detailed procedures are described below.

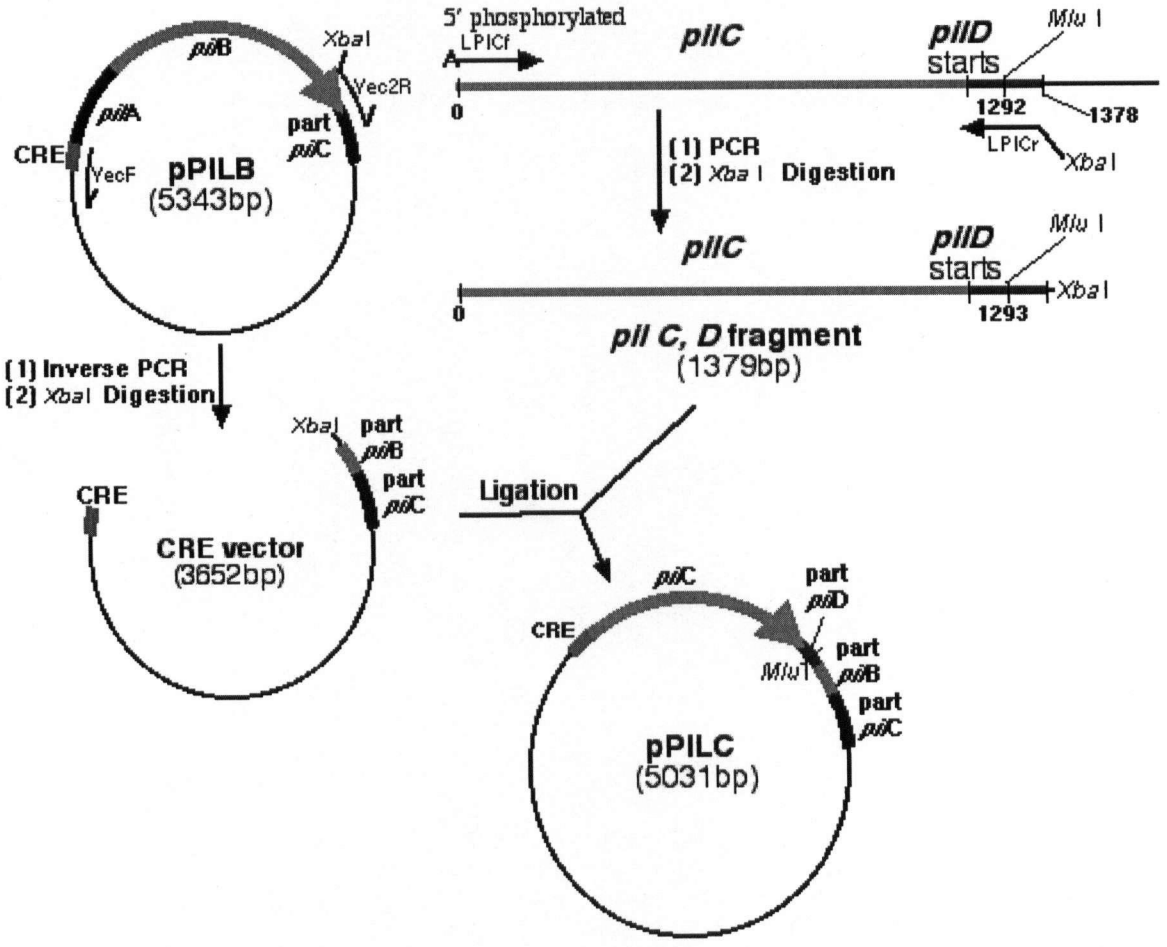

Fig. 2.3 Construction of pPILC. *H. influenzae pilC* was PCR amplified and inserted into a vector containing a CRE promoter of the *H. influenzae pil* operon, thus that *pilC* is under the control of a regulatory sequence of its own.

A 1384 bp fragment bearing the complete *pilC* gene and a small fraction of *pilD* was amplified by PCR using primer LPICf/LPICr. LPICf was 5′ phosphorylated and LPICr was engineered with an *Xba*I site. *Pfu* DNA polymerase was used in the PCR to get a blunt ended fragment.

To generate a pSU20-based shuttle vector containing the CRE promoter of the *H. influenzae pil* operon (simplified as "pSU-CRE vector" below), an inverse PCR of pPILB was performed using primers VecF/Vec2R. The Vec2R primer included an added *Xba*I site. *Pfu* DNA polymerase was used in the inverse PCR to get blunt-ended amplicons. The *Xba*I cleavage of the 3661 bp PCR product generated a pSU-CRE vector of 3652 bp that was ligated to the above *Xba*I digested *pilC* fragment, with the *pilC* start codon fused at the normal location of the *pilA* start codon. The ligation mixture was first transformed into Top10 competent cells (Invitrogen). Cam resistant transformants carrying the desired pPILC construct were verified by restriction analysis with *Eco*RI, *Hin*dIII, and *Ssp*I. Miniprep DNA from one of the colonies carrying the right construct was then transformed into GM2163 competent cells (*dam*⁻, *dcm*⁻) (NEB) to permit *Xba*I digestion in the downstream construction (Section 2.2.1.4). *Ssp*I digestion and *Xba*I/*Spe*I double digestion were used to confirm the restriction pattern of pPILC extracted from GM2163 cells. The sequence of pPILC around the 5′ ligation joint was examined by DNA sequencing using primer PilBr.

2.2.1.3 pSUPILB

To generate the plasmid pSUPILB, the insert of pPILB was released by *Eco*RI digestion and subcloned into *Eco*RI linearized pSU20 (Fig.2.4). The ligation mixture was

transformed into DH5α, plated on LB plates supplemented with Cam, ITPG and Xgal. White colonies bearing the desired pSUPILB construct were verified by restriction analysis with *Eco*RI, *Dra*I, *Hin*dIII, and by colony PCR using primers PilBf/PilBr. The sequence of pSUPILB in the CRE promoter region was examined by DNA sequencing using primer PilBr.

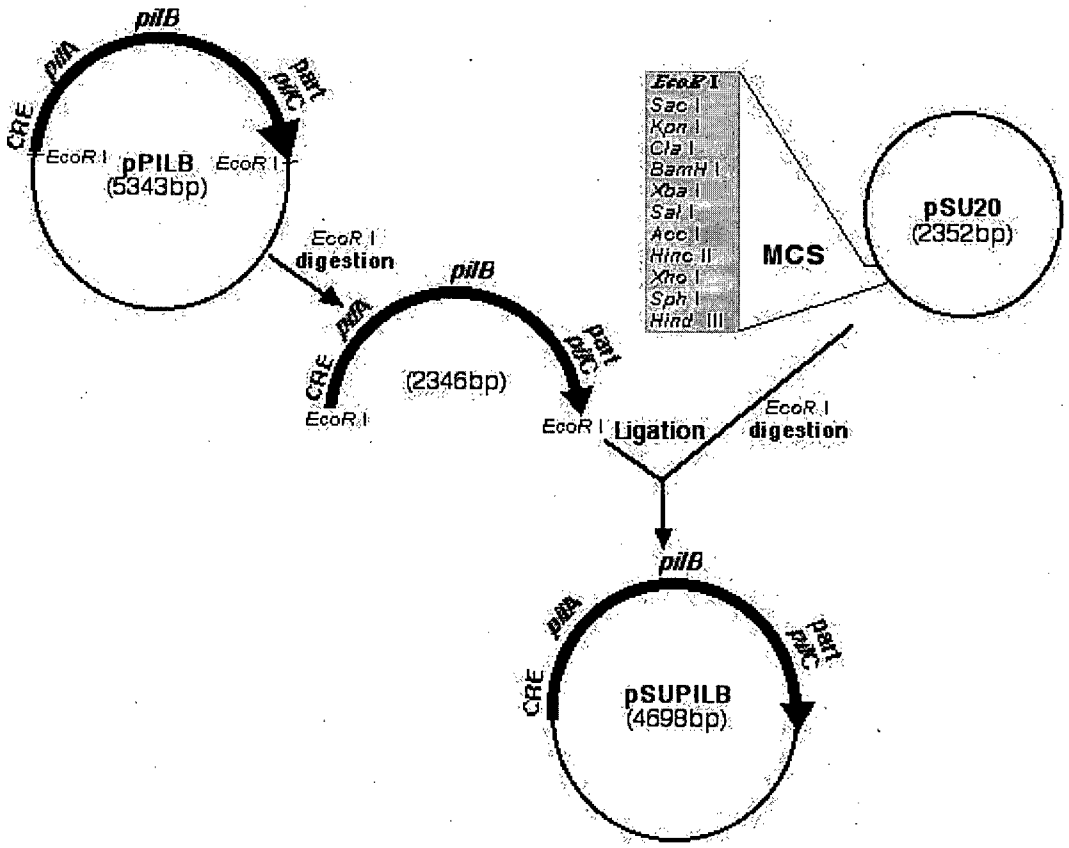

Fig. 2.4 Construction of pSUPILB.

2.2.1.4 pBKanCREC

To construct the plasmid pBKanCREC, a fragment containing the *pilC* gene under the control of the CRE promoter (*CRE/pilC* fragment) was subcloned into a vector containing a *H. influenzae pilB* knockout. The plasmid thus constructed contains a fragment bearing a CRE + a truncated *pilB* with a Kan^R insertion at the *Bcl*I + another CRE + *pilC* + part of *pilD* (the $\Delta pilB::Kan^R/CRE/pilC$ fragment) and can be used to generate a non-polar *pilB* knockout (Fig. 2.5). The detailed procedures for pBKanCREC construction are described below.

To obtain a vector containing a *H. influenzae pilB* knockout, inverse PCR was performed using plasmid pPILB::Kan^R and primers Vec2F/Vec2R, each with an *Xba*I site at the end. Expand Long Template PCR system" (Roche) was used to amplify the 6174 bp fragment. The PCR product was gel extracted to remove non-specific amplifications, followed by *Xba*I digestion to get the 6160 bp vector containing a *H. influenzae pilB* knockout. The 1537 bp *CRE/pilC* fragment was released from pPILC by *Spe*I/*Xba*I double digestions and ligated to the above vector . The ligation was transformed into Top10 *E. coli* competent cells (Invitrogen), selecting for Amp/Kan double resistance.

Transformants with the desired pBKanCREC constructs bearing the $\Delta pilB::Kan^R$/ *CRE/pilC* fragment were screened by restriction analysis using *Acc*I, *Eco*RI, *Hin*dIII, and *Sac*II/*Nde*I double digestion.

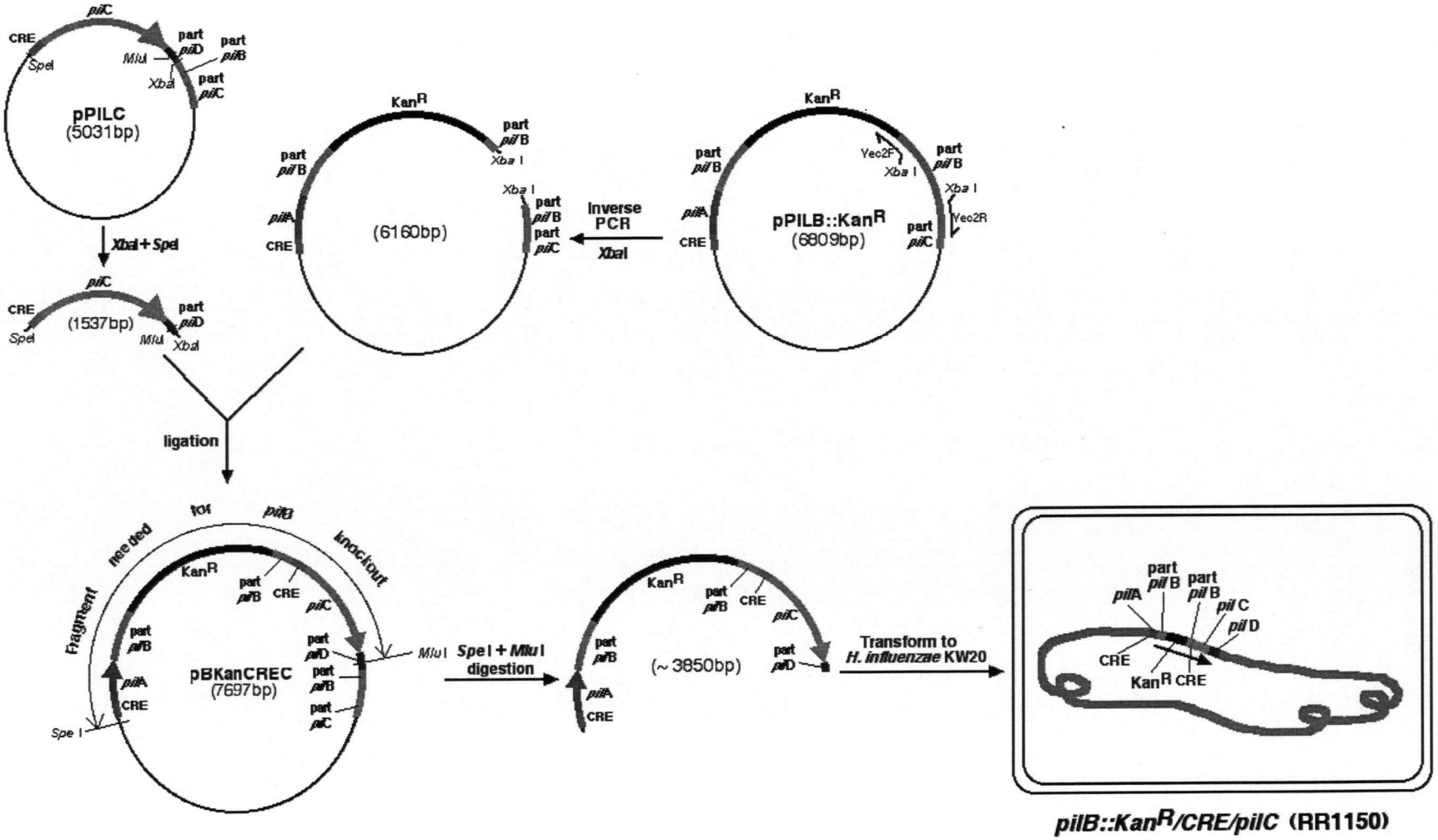

Fig. 2.5 Construction of pBKanCREC and the strain RR1150.

2.2.2 *H. influenzae* knockouts

2.2.2.1 RR1137 (*pilB::Kan*^{R})

To generate the *pilB* knockout strain RR1137, the insertional knockout of *pilB* constructed in pPILB::Kan^R was released from its vector by *Eco*RI digestion and transformed into KW20 by natural transformation, replacing the wild type *pilB* on the chromosome. To reduce the probability that one cell would take up multiple fragments of the *pilB* knockout, serially diluted DNA was used in the transformations. Colonies were chosen from the transformation in which the number of transformants was greatly reduced compared to the transformation using more DNA, suggesting that the amount of DNA used was low enough that no cells took up more than one fragment. Kan^R colonies were chosen from a transformation that used ~ 0.5 ng DNA and chromosomal DNA was extracted. Substitution of the wild type *pilB* with the knocked-out *pilB* was verified by PCR using chromosomal DNA and the primer pair PilBf/PilBr.

2.2.2.2 Construction of RR1150 (Δ*pilB::Kan*^{R}*/CRE/pilC*)

The overall strategy used to generate a non-polar *H. influenzae pilB* knockout is shown in Fig.2.5. The strategy was to place a regulatory sequence containing a duplicate copy of the *pil* CRE promoter immediately upstream of *pilC*, downstream of the insertionally inactivated *pilB*. To do this, the fragment containing the *pilB* knockout, *pilC* with a CRE promoter, and flanking sequences (Δ*pilB::Kan*^{R}*/CRE/pilC*) was released by *Mlu*I/*Spe*I double digestion of pBKanCREC and transformed into KW20 by natural transformation. For the same consideration as in Section 2.2.2.1, limited amounts and serial dilutions of

DNA (5 μl, 0.5 μl and 0.05 μl to 1 ml of competent cells) were used in the transformation. Colonies were chosen from the transformation with ~2.5 ng DNA, in which CFU were significantly reduced. PCR was used to screen for colonies carrying the recombined fragment in the chromosome, using primer pairs PilBf/PilBr, PilBf/PRRTPILA and PilBf/K2.

2.2.2.3 Construction of RR1136 (KW20 carrying pSUPILB), RR1138 (RR1137 complemented with pSUPILB) and RR1151 (RR1150 complemented with pSUPILB)

For construction of strain RR1136 (KW20 carrying pSUPILB), pSUPILB was introduced into KW20 by natural transformation. Since RR1137 (*pilB::Kan*^{R}) and RR1150 (Δ*pilB::Kan*^{R}*/CRE/pilC*) are defective in natural transformation, the strains RR1138 (RR1137 complemented with pSUPILB) and RR1151 (RR1150 complemented with pSUPILB), were obtained by introducing pSUPILB into RR1137 and RR1150 using chemical transformation, selecting for Cam/Kan double resistance. Presence of pSUPILB in the strains was verified by restriction analysis and colony PCR as described in the construction of pSUPILB.

2.2.3 Introduction of *P. aeruginosa* PA01 *pilB, pilT* to the *H. influenzae pilB* background

P. aeruginosa PA01 *pilB* and *pilT* were amplified from chromosomal DNA and placed under the control of the CRE promoter of *H. influenzae pil* operon. The cloning strategies are shown in Fig.2.6 and Fig.2.7 and Fig. 2.8. Details are given below.

2.2.3.1 Cloning of *P. aeruginosa pilT*

For construction of *P. aeruginosa pilT* plasmids pPAPT and pPAPT2, the two fragments containing *pilT* (starting at ATG_0 and GTG_{-12} respectively), were amplified from *P. aeruginosa* PA01 chromosomal DNA and cloned into a pSU20-based vector containing a CRE promoter of the *H. influenzae pil* operon. To do this, fragments containing *P. aeruginosa pilT* and *pilT*2 were PCR amplified using *Pfu* DNA polymerase and primers piTF/piTR or piTF2/piTR, respectively (Fig. 2.6). Both piTF and piTF2 were 5′ phosphorylated and a *Spe*I site was included in piTR. The amplified fragments start exactly at the first nucleotide of the start codon, and end at ~50 bp downstream of each ORF. The PCR products were digested with *Spe*I, resulting in 1096bp and 1108bp fragments. To get a pSU20-based vector containing a CRE promoter of the *H. influenzae pil* operon (simplified as pSU-CRE vector below), inverse PCR of pSUPILB was performed using *Pfu* DNA polymerase and the primer pair VecF/Vec2R. VecF was 5′ phosphorylated and Vec2R had a *Spe*I site at the 5′ end. The 3016 bp PCR product was purified by gel extraction, followed by *Xba*I digestion, resulting in a pSU-CRE-vector of 2481bp.

pPAPT and pPAPT2 were obtained by ligation of *Spe*I-digested *P. aeruginosa pilT* fragments into *Xba*I-digested pSU-CRE vector. The ligation mixture was transformed into DH5α and plated on LB plates selecting for Cam resistance. Restriction analyses were applied to screen for transformants carrying desired constructs. Restriction enzymes *Eco*RI, *Nco*I and *Xho*I were used in analysis of pPAPT candidates, and *Nco*I, *Pvu*II, *Xho*I were used first for analysis of pPAPT2 candidates. *Hin*fI digestion was

performed subsequently to confirm that pPAPT2 has the extra 12 bp not present in pPAPT. In addition, *Hin*dIII, a unique cutter of both constructs, was used as well to check the size of the two plasmids. Sequences of pPAPT and pPAPT2 around the ligation joint and the promoter were examined by DNA sequencing, using the primer pair PilBr/piTR.

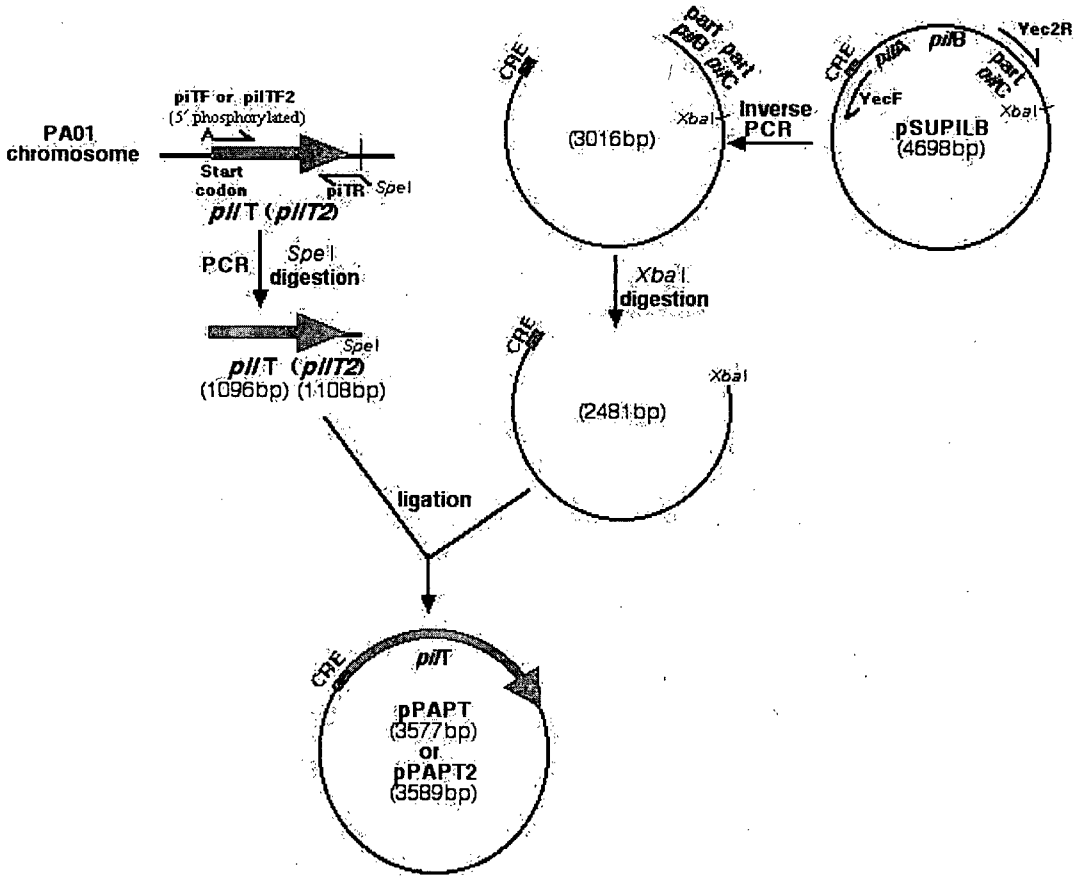

Fig. 2.6 Cloning of PA01 *pilT* and construction of pPAPT

2.2.3.2 Cloning of *P. aeruginosa pilB*

The overall strategy for *P. aeruginosa* PA01 *pilB* cloning is shown in Fig.2.7 and Fig. 2.8. *P. aeruginosa pilB* was cloned under the control of its own RBS (Ribosome Binding Site) and a CRE promoter of the *H. influenzae pil* operon. In the final *pilB* clone, the ligation joint of *P. aeruginosa pilB* and the *H. influenzae* CRE promoter was designed to be downstream of the transcription start and upstream of the RBS. This was to minimize the impact of short deletions at the ligation joint. To construct this, a fragment containing *P. aeruginosa* PA01 *pilB* and 200bp upstream was first amplified from *P. aeruginosa* PA01 chromosomal DNA and cloned into a pSU20-based vector containing a CRE promoter of the *H. influenzae pil* operon, giving the plasmid pPAPBpro. The RBS in the CRE promoter and most of the regulatory sequence of *P. aeruginosa pilB* except the RBS, were then removed by inverse PCR of pPAPBpro using the primer pair pilBF3 & Pro-10. The final clone of *P. aeruginosa pilB* was obtained by self-ligation of the PCR product, such that *P. aeruginosa pilB* uses *H. influenzae pilA*'s CRE promoter including the putative transcription start, and uses *P. aeruginosa pilB*'s own RBS. To ensure that the ligation point was not too closed to the transcription start and RBS, 6 bases downstream of *H. influenzae pilA*'s transcription start and 6 bases upstream of *P. aeruginosa pilB*'s RBS were included in the clone, so that in the final *pilB* clone, there should be 12 bases between the putative transcription start and RBS. The experimental procedures are described in more detail below.

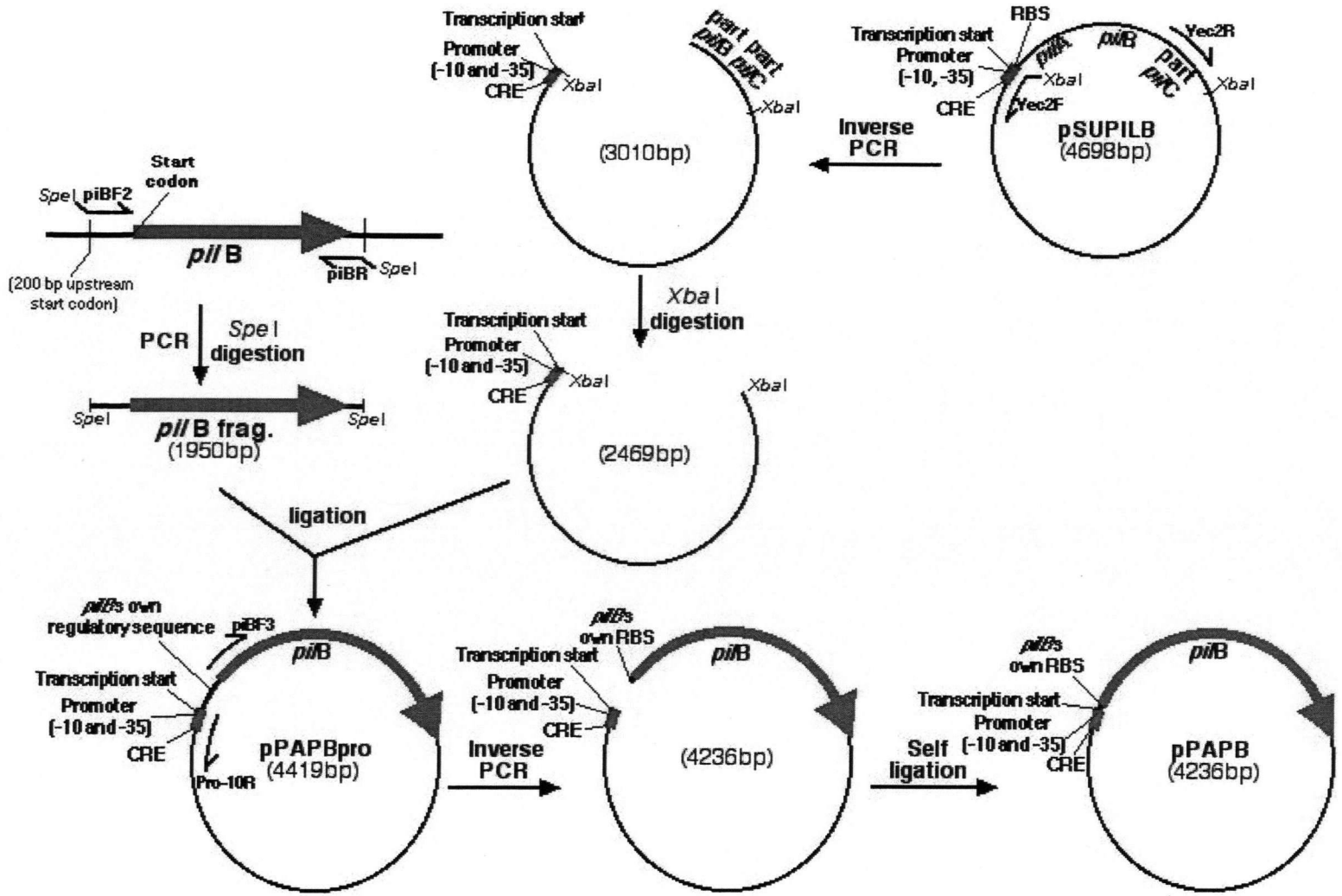

Fig. 2.7 Cloning of PA01 *pilB* and construction of pPAPB.

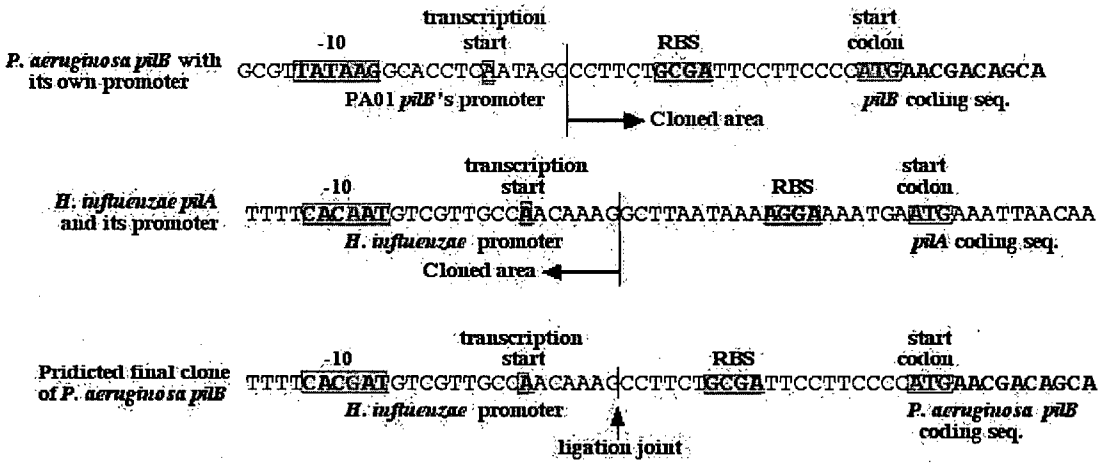

Fig. 2.8 The strategy for *P. aeruginosa* PA01 *pil*B cloning, showing the region and sequences around *H. influenzae pilA* and PA01 *pilB* chosen for the cloning, and the predicted sequence of pPAPB around the ligation point.

To construct the plasmid pPAPBpro, a fragment containing the *pilB* ORF and 200 bp upstream was amplified by PCR using the primer pair piBF2/piBR, each having a *Spe*I site at the 5′ end. The PCR product was digested with *Spe*I, which produced a 1950 bp fragment. A pSU20-based vector containing the CRE promoter (pSU-CRE vector) from *H. influenzae pil* operon was obtained by inverse PCR of pSUPILB using the primer pair Vec2F/Vec2R. An *Xba*I site was included at the 5′ end of Vec2F. *Xba*I digestion of the PCR product produced a 2469 bp pSU-CRE vector, which was ligated to the 1950 bp *pilB* fragment to get the plasmid pPAPBpro. The ligation mixture was transformed into DH5α, selecting for Cam resistance. Constructs carrying desired construct were screened with restriction analysis using *BamH*I, *Ban*I and *Pvu*II. Sequence of pPAPBpro around

the ligation joint and the promoter was examined by DNA sequencing, using primer PilBr.

pPAPB was obtained by removing the unwanted sequence in pPAPBpro, using inverse PCR and religation of the PCR product. *Pfu* DNA polymerase and the primer pair piB3F/Pro-10, both 5′ phosphorylated, were used in the PCR reaction. The PCR amplification removed most of *pilB*'s own regulatory sequence except its RBS (Ribosome Binding Site). Therefore the final *P. aeruginosa pilB* clone, which was obtained afterwards by self-ligation of the PCR product, should contain *pilB* with its own RBS and *H. influenzae pilA*'s CRE and promoter in the upstream region (Fig. 2.7). The predicted sequence at the joint of the self-ligation is shown in Fig. 2.8. Self-ligation of the PCR product was transformed into DH5α, selecting for Cam resistance. Restriction enzymes *BamH*I, *Ban*I and *Pvu*II were used to examine colonies bearing the desired construct. The sequences of *pilB* and the CRE promoter in pPAPB were examined by DNA sequencing, using primer pairs SpBf/PilBr and SpBEf/SpBr.

2.2.3.3 Construction of pPAPBT and pPAPBT2

The scheme for pPAPBT and pPAPBT2 is shown in Fig.2.9. Two fragments containing the *P. aeruginosa pilT* gene (starting at ATG_0 and GTG_{-12}, respectively) under the control of the CRE promoter were released from pPAPT and pPAPT2 by *Nhe*I/*Spe*I double digestion, and inserted into *Spe*I digested pPAPB. The ligation mixtures were transformed into DH5α and spread on LB plates, selecting for Cam resistance. The constructs were examined by restriction analysis with *BamH*I, *Nco*I and *Sac*II.

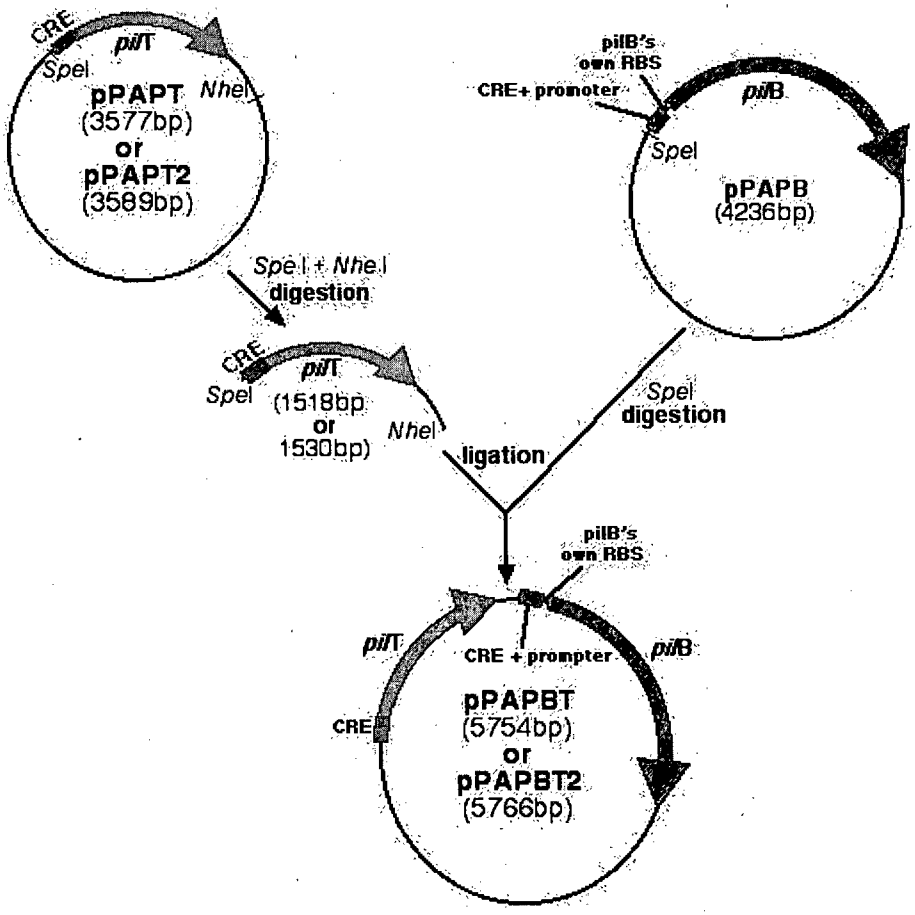

Fig. 2.9 Construction of pPAPBT and pPAPBT2.

2.2.3.4 Transforming the *P. aeruginosa* plasmids into *H. influenzae pilB*

Since *H. influenzae pilB* is deficient in natural transformation, the *P. aeruginosa* plasmids pPAPT, pPAPT2, pPAPB, pPAPBT and pPAPBT2 were first transformed into wild type *H. influenzae* KW20, chromosomal DNA of the non-polar *pilB* knockout was then introduced into the resultant strains by natural transformation (Fig.2.10). Details are given below.

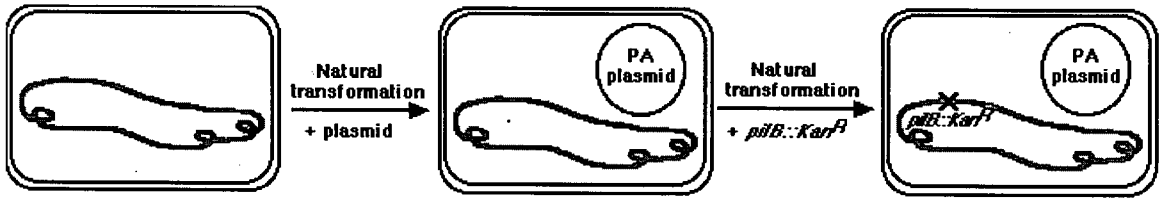

Fig. 2.10 Construction of *H. influenzae strains* containing both *pilB* knockout and *P. aeruginosa pilB*, *pilT* plasmids.

The 5 plasmids were first transformed into KW20, selecting for Cam resistance. Since the *pilB* plasmids (pPAPB, pPAPBT and pPAPBT2) contain multiple *Hin*dIII sites, they were treated with methylase *Hin*dIII (Takara) before transformation into KW20 (~ 0.6 units/μg) to protect them from digestion by *H. influenzae Hin*dIII. Presence of the *P. aeruginosa* plasmids was verified by restriction analyses. The resultant strains were then transformed with the chromosomal DNA of RR1150. Limited amount of DNA (100 ng, 10 ng and 0.1 ng to 1 ml of competent cells) was used in the transformation (See Section 2.2.2.1). Kan/Cam double resistant transformants were chosen from the 0.1 ng transformation. Chromosomal organization was examined by PCR using primer pairs PilBf/PRRTPILA and PilBf/K2. Sequences of the *P. aeruginosa* inserts in the resultant strains were verified by DNA sequencing (Fig. 2.11, Fig. 2.12, Fig. 2.13). The sequencing primers used were: for pPAPB, SpBEf, SpBr, SpBf and PilBr; for pPAPT, and pPAPT2, piTR and PilBr; and for pPAPBT and pPAPBT2, SpBsf, SpBr, SpBf, SpBsF, SpTsf and piTR.

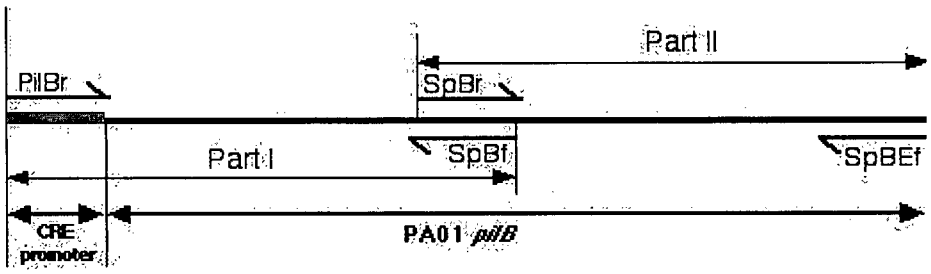

Fig. 2.11 Sequencing of *P. aeruginosa* PA01 *pilB* in pPAPB.

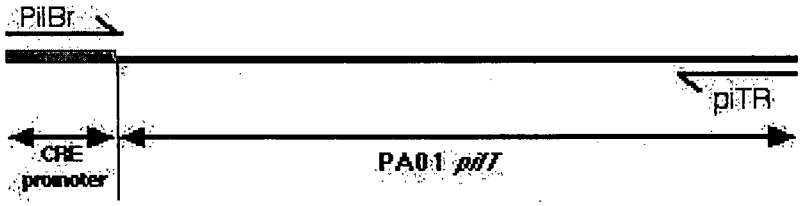

Fig. 2.12 Sequencing of *P. aeruginosa* PA01 *pilT* in pPAPT.

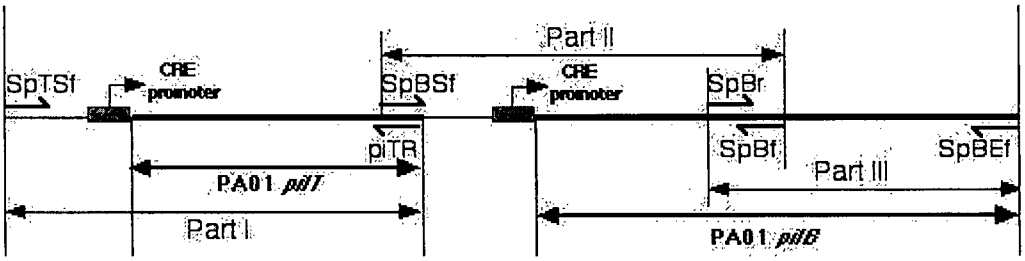

Fig. 2.13 Sequencing of *P. aeruginosa* PA01 *pilB and pilT in* pPAPBT and pPAPBT2. Since there are 2 CRE promoters in the plasmids, the primer PilBr that anneals to the CRE promoter cannot be used. Therefore SpTSf and SpBSf were designed.

Chapter 3: Results and discussion

3.1 Sequence analysis and functional prediction of *H. influenzae* PilB

H. influenzae pilB (gene HI0298) is 1395bp long and encodes a predicted protein of 464 amino acids. Sequence alignments showed that PilB is more similar to homologues of *P. aeruginosa* PilB (required for Tfp biogenesis), and less similar to PilT homologues (required for Tfp retraction). In a BLASTP with *H. influenzae* PilB, a number of Tfp assembly proteins (required for Tfp biogenesis) were obtained with high identities to PilB, including PilB (or HofB) in *Pasteurellaceae* Species, PilB of *Vibrio cholerae*, PilF of *N. gonorrhoeae* and *N. meningitides*, PilB of *Acinetobacter* sp. ADP1. Following the Tfp biogenesis proteins, twitching motility proteins (PilT homologues, required for Tfp retraction) were found with much lower E-values ($>10^{-30}$). Alignment scores of *H. influenzae* PilB homologues in *P. aeruginosa* and *N. gonorrhoeae* are shown in Table 3.1.

Table 3.1 Alignment scores of *H. influenzae* PilB homologues in *P. aeruginosa* and *N. gonorrhoeae*

	ClustalW score	BLAST E value	Identity to *H. influenzae* PilB
PaPilB	40	4e-89	38%
NgPilF	35	2e-73	41%
PaPilT	21	3e-30	38%
NgPilT	17	8e-27	31%

PilB and PilT homologues all belong to the PulE/GspE family (TrbB proteins). Members of this family are distinguished by conserved nucleotide binding and hydrolysis motifs (49). The nucleotide binding motifs of *H. influenzae* PilB are indicated in Fig. 3.1a. Fig. 3.1b shows the sequence alignment of the C-terminal of some PilB and PilT homologues. All the proteins share the three conserved domains related to nucleotide binding and ATP hydrolysis (Fig. 3.1a). In addition to the sharing of these domains, extended searching in the alignment also revealed some conserved regions shared by the PilT proteins but absent in the PilB proteins (also see (66)). This is consistent with the different functions known for PilB & PilT in *P. aeruginosa* and *N. gonorrhoeae* (4, 33). *H. influenzae* PilB shares high homology with other PilB proteins and does not contain the conserved regions unique to the PilT proteins. Therefore *H. influenzae* PilB is expected to function as an ATPase in the assembly of the Tfp-like apparatus for DNA uptake.

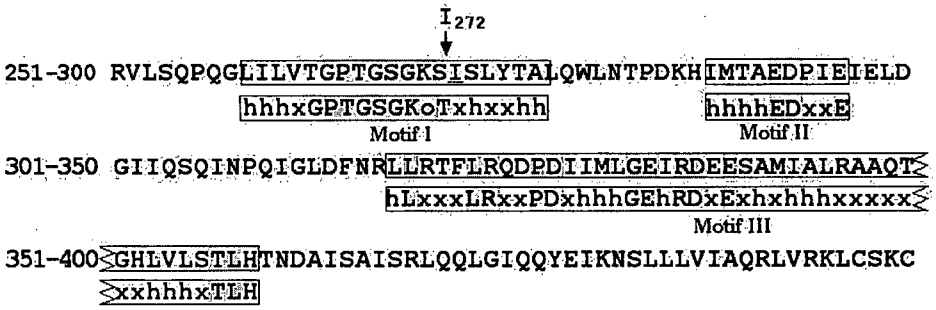

Fig. 3.1a Residues 251-400 of *H. influenzae* PilB showing the 3 nucleotide binding motifs predicted by Lessl and Lanka (49). h represents hydrophobic amino acids, o marks polar ones, and x any other amino acids. Highlighted capital letters represent residues that are strictly conserved in at least 12 of the 14 proteins analyzed by Lessl and Lanka. The sequence of PilB fits all the three motifs except residue Ile_{272} (underlined) that should be a Thr (the 13th residue) in Motif I.

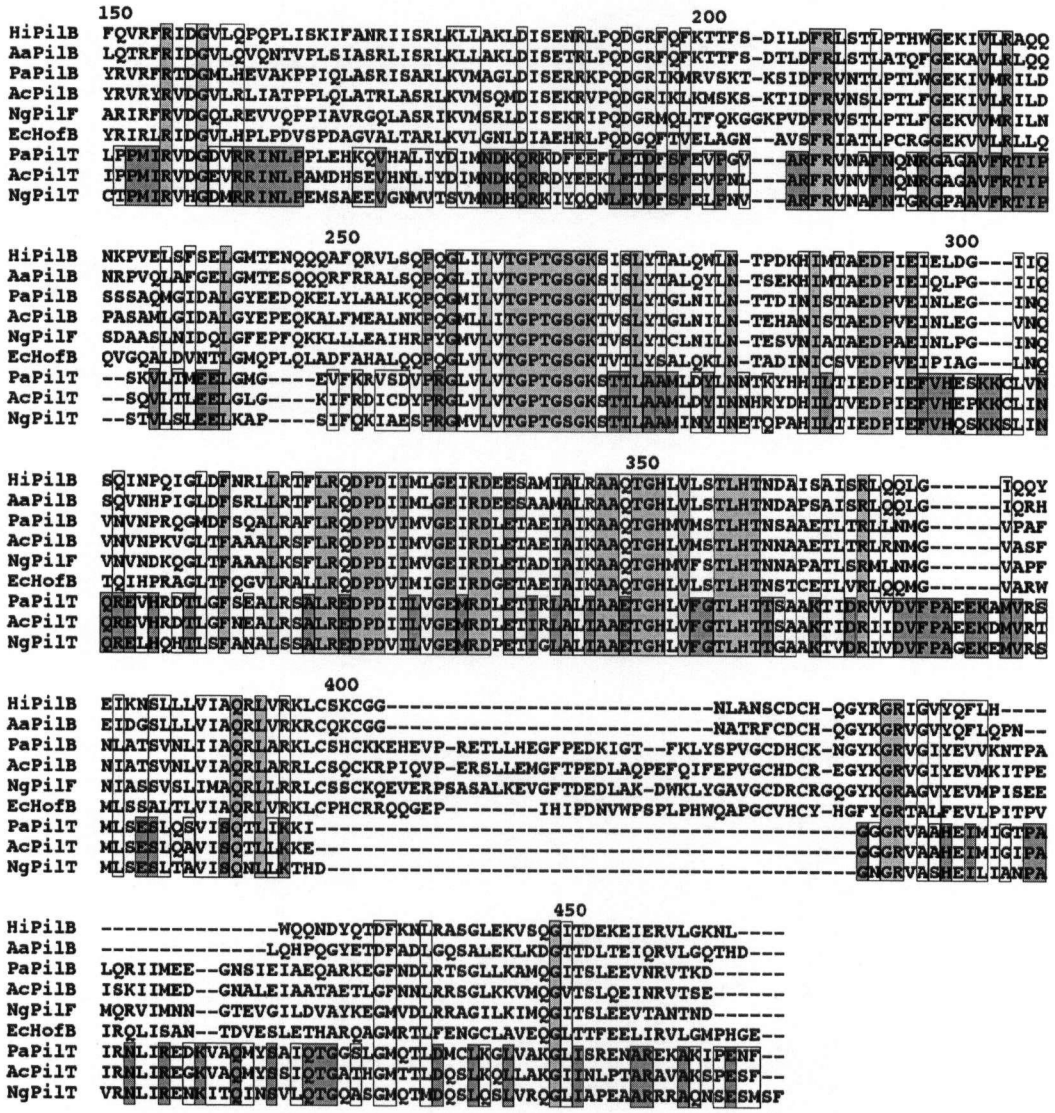

Fig. 3.1b Amino acid alignment of C-terminal segments of PA01 PilB and PilT homologues, accomplished by using ClustalW. The numbering is based on the *H. influenzae* PilB sequence. Conserved residues are enclosed in boxes, and identical residues shaded. The alignment begins in the regions corresponding to residue 150 of *H. influenzae* PilB where the sequences begin to align well. The boxes and shaded region within the last three sequences indicate the conserved regions unique to PilT family proteins. The following proteins are shown: HiPilB (*H. influenzae* PilB), AaPilB (*Actinobacillus actinomycetemcomitans* PilB), PaPilB (*P. aeruginosa* PilB), AcPilB (*Acinetobacter* sp. Strain BD413 PilB), NgPilF (*N. gonorrhoeae* PilF), EcHofB (*E. coli* HofB), PaPilT (*P. aeruginosa* PilT), AcPilT (*Acinetobacter* sp. Strain BD413 PilT), NgPilT (*N. gonorrhoeae* PilT).

BLAST searches with the well characterized nucleotide binding proteins PilB, PilT and PilU of *P. aeruginosa* and *N. gonorrhoeae* indicated that PilB is the only Tfp-related ATPase in *H. influenzae* (Section 1.6). Efficient DNA uptake by *H. influenzae* in the absence of a PilT homologue indicates that DNA uptake does not require a PilT-like ATPase. This has several possible explanations. First, PilB may function in both assembly and reaction of the Tfp-like apparatus. In this case, PilB may drive the two opposite processes (assembly/polymerization and retraction/depolymerization) under different conditions. The second explanation is that PilB only powers Tfp assembly. In this case, a non-VrbB/GspE-like protein could power the retraction of the Tfp-like machinery by either ATP hydrolysis or other process that can generate energy. Alternatively, *H. influenzae* might need no other proteins for Tfp retraction; that is, Tfp might automatically depolymerize and retract when the assembly protein PilB is not active. Thus the transforming DNA would be taken up during this process. The two alternative hypotheses (whether PilB is required for both Tfp assembly and retraction, or only required for Tfp assembly), can be tested by complementing a *H. influenzae pilB* knockout with *pilB/pilT* genes from a well-characterized Tfp system, which is the major approach of this study.

In this analysis I have assumed that pilus retraction is needed for DNA uptake by *H. influenzae*. The validity of this assumption is reconsidered in the discussion.

3.2 Construction of *H. influenzae pilB* knockouts

3.2.1 Overview

Two *H. influenzae pilB* knockouts, RR1137 and RR1150, were constructed in this study. As described below, inserting a Kan^R cassette at the *BclI* site of the *pilB* ORF generated the *pilB* knockout strain RR1137. However, complementation experiments and real time PCR showed that the *pilB* knockout in RR1137 is seriously polar to the downstream genes *pilC* and *pilD* (*i.e.* a mutation or knockout in one gene reduces the expression of the downstream genes) (more details below). This polarity was eliminated by placing an additional CRE regulatory sequence and a promoter before *pilC* in the *pilB::Kan*^{R} background, giving strain RR1150. The relevant genotypes of RR1137 and RR1150 are illustrated in Fig.3.2.

Construction of the necessary plasmids is described in detail in the Methods. The plasmids used for generation of RR1137 were pPILB and pPILB::Kan^R. The plasmids used for generation of RR1150 were pPILB::Kan^R, pPILC and pBKanCREC. pSUPILB was used to test polarity of the *pilB* mutations in both RR1137 and RR1150.

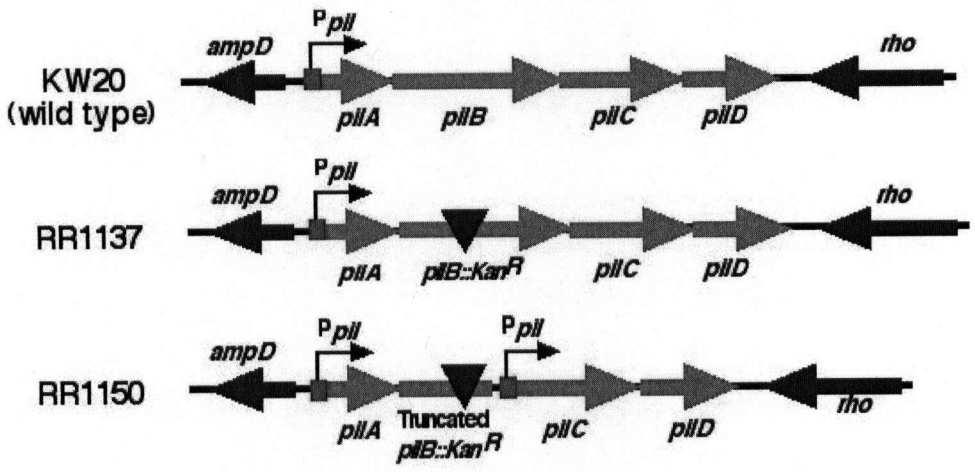

Fig. 3.2 Genetic organization of KW20, RR1137 and RR1150. P_{pil} = CRE promoter of the *pil* operon.

3.2.2 Construction of pPILB and pPILB::Kan^R

To investigate *H. influenzae pilB*'s function, a *pilB* knockout was generated using a Tn*903* Kan^R insertion. First, a plasmid carrying the *pilB* gene was constructed. A 2326 bp fragment encompassing *H. influenzae pilB* ORF and the flanking sequences was PCR amplified and cloned into a pGEM-T easy vector, resulting in pPILB.

To knock out *pilB*, a Kan^R cassette was inserted into the *pilB* gene on pPILB at the *Bcl*I site. The knockout plasmid was designated pPILB::Kan^R.

3.2.3 Construction of RR1137 (*pilB::Kan^R*)

The knockout was then introduced into *H. influenzae* KW20 by natural transformation, replacing the wild-type copy of *pilB*. Two colonies carrying the desired chromosomal organization were examined for exponential growth rate in sBHI and the ability to develop competence using a chromosomal DNA transformation assay. Wild type KW20 was used as the control. No discernable difference of growth rate was observed between the strains (data not shown). Since *H. influenzae pilB* codes for a nucleotide binding protein predicted to be required for the biogenesis and/or function of the Tfp-like machinery, the knockout of *pilB* was expected to give no transformation due to lack of a Tfp-like machinery. No transformants were obtained from either isolate, giving transformation frequencies less than 9.5×10^{-9}, which was 8.9×10^{5} lower than that of the wild type. One colony was frozen as strain RR1137.

The elimination of transformation could have resulted either from the disruption of *pilB* itself or from polarity, which significantly reduced expression of the downstream genes *pilC* and *pilD*, another two genes expected to be required for Tfp biogenesis (Section 1.5). In the later case, the lack of competence in RR1137 could not be attributed to the knockout of *pilB*. The polarity of the *pilB* knockout in RR1137 was examined below.

3.2.4 Complementation assays and real time PCR revealed severe polarity of RR1137 *pilB* knockout

To assess whether the *pilB* knockout in RR1137 was polar on the downstream genes, a plasmid expressing a wild type *pilB* was needed. Full complementation of the knockout by this plasmid would imply no polarity.

To generate a plasmid that can replicate in *H. influenzae* and contains a wild type *pilB*, the 2346 bp *Eco*RI fragment of pPILB containing *pilB* was subcloned into the *H. influenzae*/*E. coli* shuttle vector pSU20, giving pSUPILB. pSUPILB was then introduced into RR1137 by chemical transformation, as RR1137 is defective in natural transformation. The plasmid-carrying knockout strain RR1138 was then used in the transformational complementation assays. RR1137 and KW20 were included as negative and positive controls. Due to addition of Cam to keep pSUPILB, growth of RR1138 was slightly slower than that of RR1137 and KW20 (data not shown). Therefore another strain RR1136 (wild type strain containing pSUPILB) was included as a positive control in the second assay. On average of the 4 transformation assays, introduction of pSUPILB into RR1137 only restored transformation frequency to 1.75×10^{-6}, which is about 1800

fold lower than that of the wild type KW20 (Fig. 3.3). This suggests that the *pilB* knockout in RR1137 may be strongly polar on *pilC* and *pilD*.

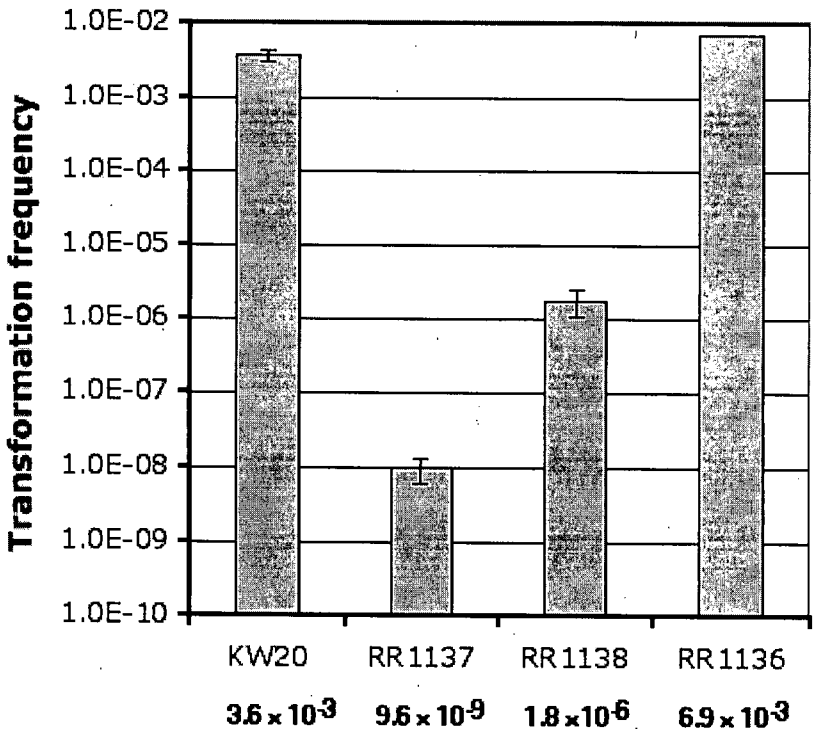

Fig. 3.3 Transformation tests of the *pilB* knockout in RR1137 complemented with wild type *pilB*.

To confirm that lack of complementation was due to polarity, real time PCR was used to measure the mRNA levels of *pilC* and *pilD*. Since the real time PCR primer pair for *pilA* was already available in the lab (MN#403), and pSUPILB contains *pilA* in addition to *pilB*, the *pilA* gene, instead of *pilB* was analyzed to examine the expression of the genes on pSUPILB. The gene *murG*, whose expression is largely constant (74), was used as an internal control. The result showed that the average expression levels of *pilC* and *pilD* in RR1137 were only 6.3% and 5.8%, respectively, of the same genes in the wild type KW20, indicating that the *pilB* knockout in RR1137 has serious polar effect on the downstream genes *pilC* and *pilD*. Thus I could not conclude that its competence defect is due to lack of PilB.

3.2.5 Construction of a non-polar *pilB* knockout RR1150

To circumvent the polar effect of the *pilB* insertional knockout on *pilC* and *pilD*, a competence inducible CRE promoter (see Section 1.3) was inserted in front of *pilC* (Fig. 2.5), downstream of the Kan^R insertion in *pilB*. The strain constructed using this strategy was designated RR1150.

To add an additional CRE promoter before *pilC*, a 1384 bp fragment containing *pilC* coding sequence was cloned into a pSU20 based vector containing a CRE promoter (Fig. 2.3) to get the plasmid pPILC. Subsequently, the *pilC* gene, together with the CRE promoter right before it, was subcloned into a vector containing a truncated *pilB* with a Tn*903* Kan^R insertion at the *Bcl*I site (Fig. 2.5). The resultant plasmid pBKan(CREC)5 thus constructed contains a fragment bearing a truncated *pilB* with a Kan^R + CRE

promoter + *pilC* (Fig. 3.4) . The fragment was then released by *Mlu*I/*Spe*I double digestion and introduced into *H. influenzae* KW20 by natural transformation, giving the strain RR1150.

3.3 Natural competence was eliminated in RR1150

Growth and natural competence of RR1150 were tested using the wild type KW20 as a control. Although the integrity of the *pilB* coding sequence was disrupted by the Kan^R insertion, RR1150 did not exhibit discernable difference in growth rate from that of the wild type (data not shown). Like RR1137, the transformation frequency was reduced to background level 5.6×10^{-9}, more than 10^5 fold lower than that of the wild type KW20 (9.0×10^{-3}).

3.4 RR1150 *pilB* knockout was complemented by wild type *pilB*

To confirm that insertion of a CRE promoter before *pilC* eliminated polarity, the *pilB* knockout in RR1150 was tested for complementation by the *pilB*-expressing plasmid pSUPILB. A strain RR1151 was constructed by transforming plasmid pSUPILB into RR1150. Transformation assays were performed using RR1150 ($\Delta pilB::Kan^R/CRE$ */pilC*), RR1151 (RR1150 complemented with pSUPILB), KW20, and RR1136 (KW20 carrying pSUPILB). The result showed that RR1151, with the wild type *pilB* introduced on the plasmid pSUPILB, had an average transformation frequency of 6.2×10^{-4}, which is only 6.6 fold lower than the average transformation frequency of 4.08×10^{-3} of KW20 (Fig. 3.5).

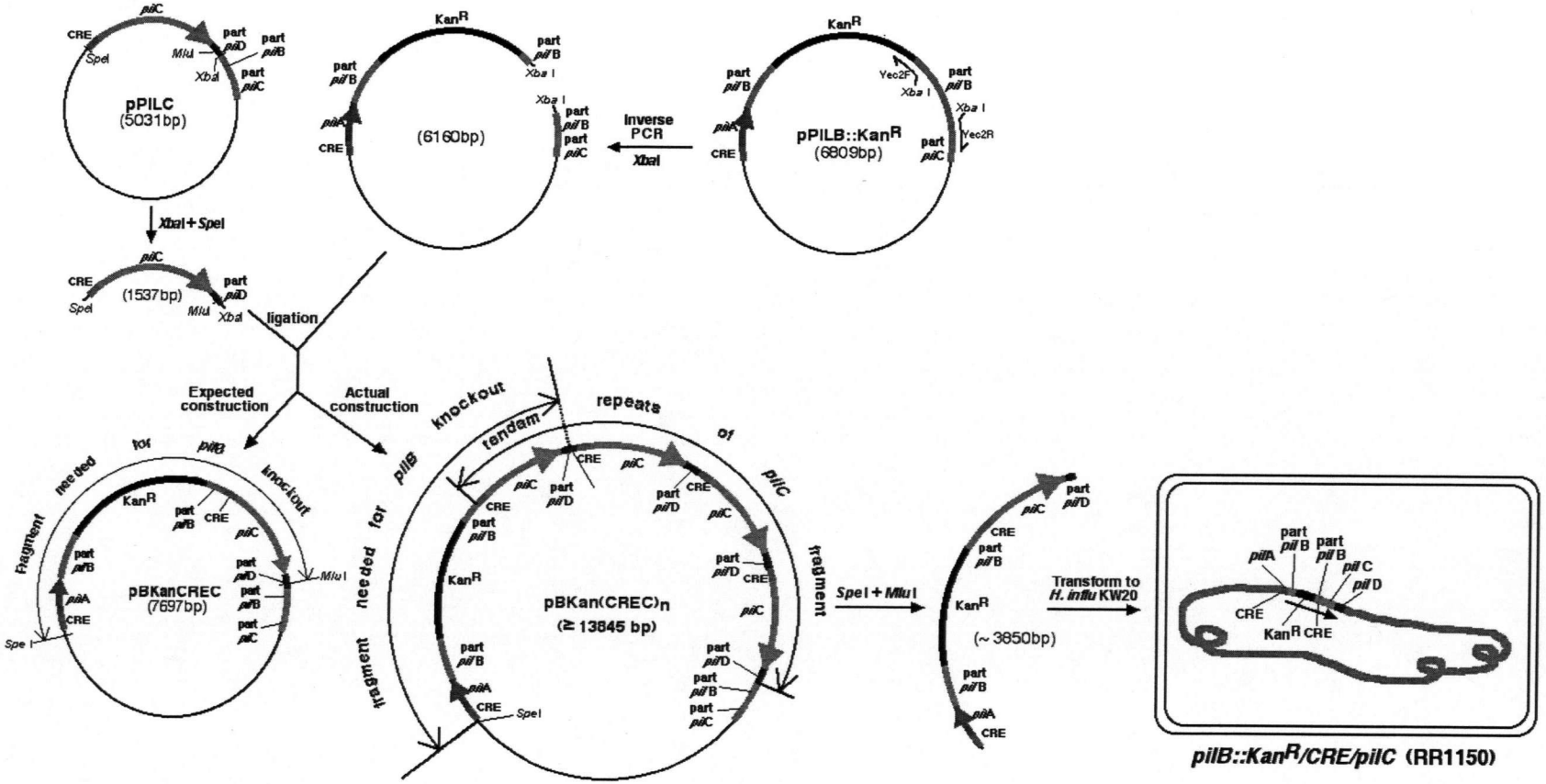

Fig. 3.4 Construction of RR1150. In construction of pBKanCREC, restriction analysis showed that a plasmid pBKan(CREC)$_n$ containing >5 tandem insertions of the 1537 bp *CREC/pilC* fragment was obtained instead of pBKanCREC. Since pBKan(CREC)$_n$ carries the final fragment for a non-polar *pilB* knockout construction, it was used to generate the strain RR1150.

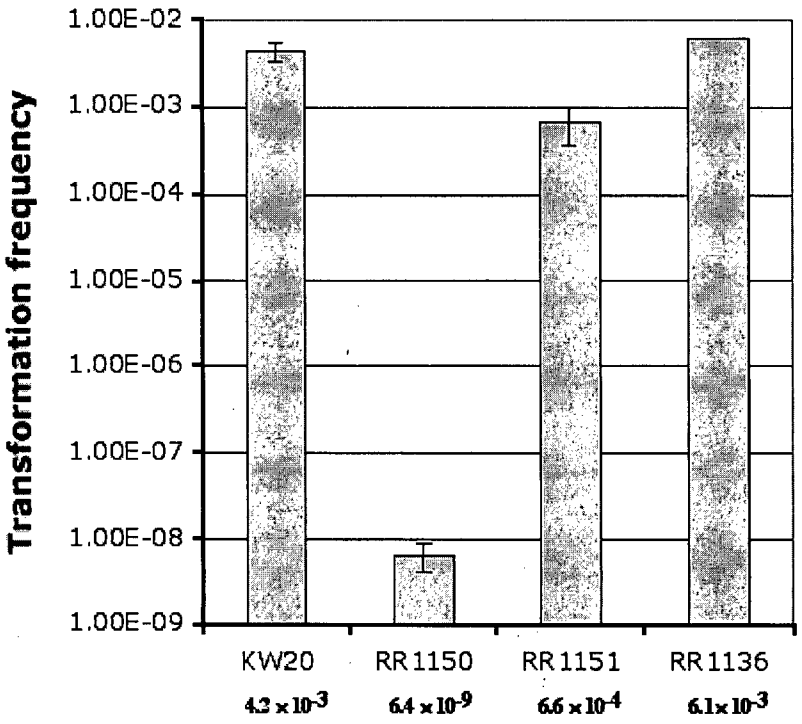

Fig. 3.5 Transformation tests of the *pilB* knockout in RR1150 complemented with wild type *pilB*.

Although the transformation frequency of the complemented *pilB* strain RR1151 is close to that of the wild type, it is still considerably lower than expected for a full complementation. To examine whether the regulatory sequence added next to the 5′ of *pilC* is correct, the region of CRE and the promoter in pPILC was sequenced using the primer PilBr. An A-to-G substitution was detected in the –10 area of the putative promoter (46), which could explain the low complementation. The substitution is also expected to be present in the CRE promoter of *pilC* in RR1150. This substitution may

have reduced the expression of the downstream gene *pilC* and *pilD,* which was supported by the later real time PCR that showed the expression of *pilC* in RR1150 was about 3.7 fold lower than that of KW20 (Table 3.2). Thus the incomplete complementation observed in RR1151, was still caused by polarity, although indirectly. Nevertheless, introduction of a wild type *pilB* into RR1150 has restored the transformation more than 10^{-5} fold, suggesting the *pilB* knockout in RR1150 is sufficient to account for the transformation deficiency of RR1150. This strain was used for later experiments and for construction of derivative strains.

Later sequencing analyses indicate that the A-to-G substitution found in the –10 segment of pPILC was originated from pPILB, the very first clone of *H. influenzae pilB* fragment (Fig. 3.6, Fig. 3.7). Therefore the pPILB-derived pSUPILB, used for complementation of RR1150, should have the substitution as well. This was confirmed later by DNA sequencing of pSUPILB.

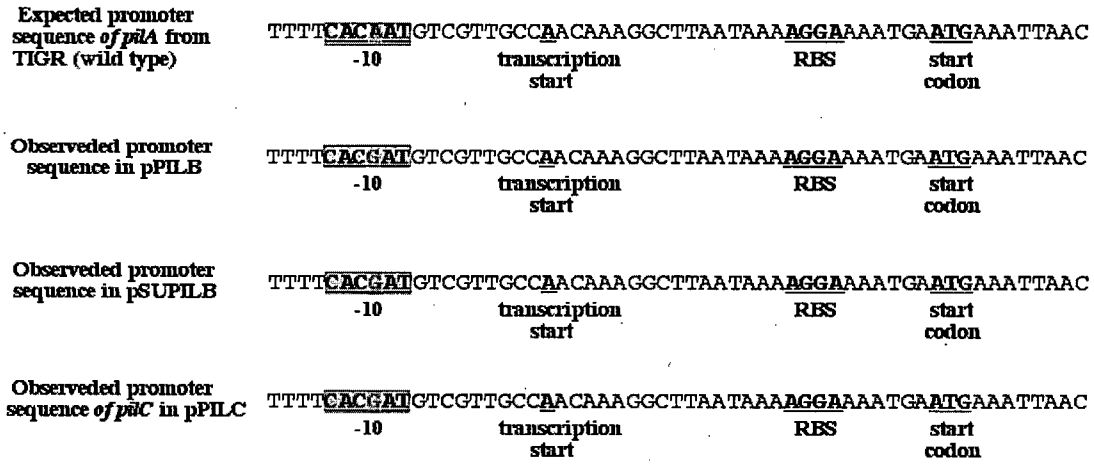

Fig. 3.6 The expected and observed sequences around the –10 region of *pil* promoter in the wild type and in pPILB, pSUPILB and pPILC, showing the A-to-G substitution.

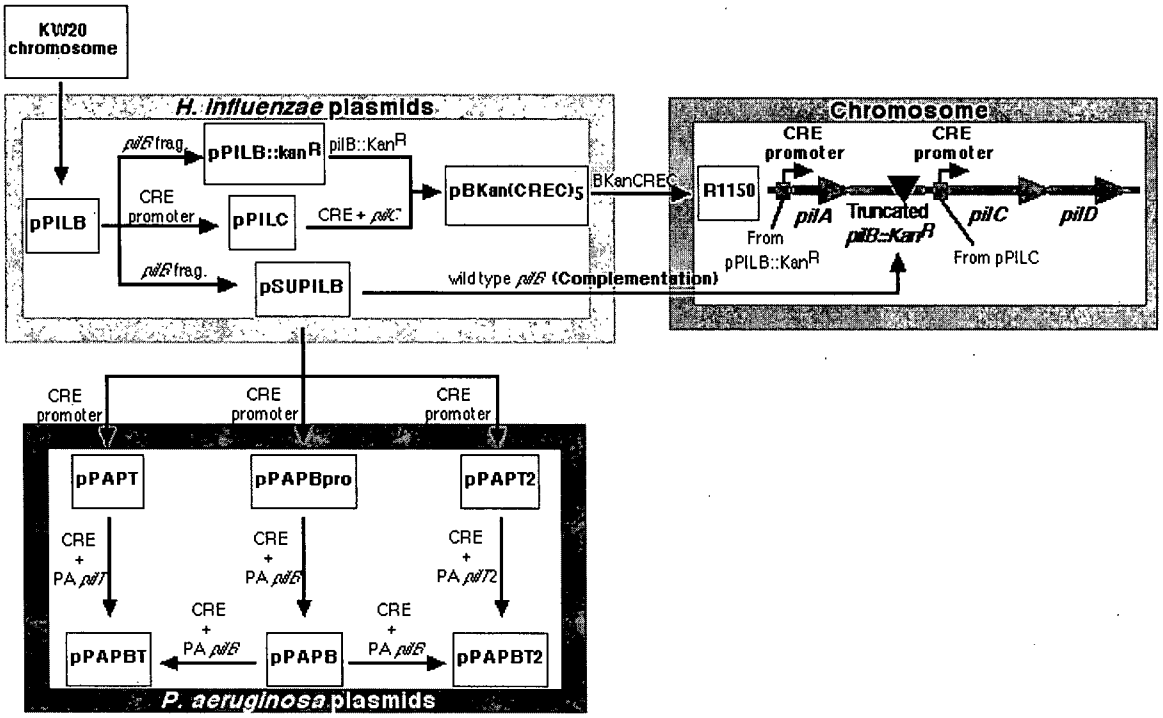

Fig. 3.7 Relationships between the plasmids of this study and the strain RR1150.

Table 3.2 Expression level of *pilC* in the non-polar *pilB* knockout compared to that of the wild type.

	Real Time PCR I	**Real Time PCR II**
***pilC* expression in competent KW20**	129	146
Average *pilC* expression in RR1150 complemented with Pa *pilB, T*	33	42.8
***pilC* expression in RR1150 relative to that of KW20**	Average 1 : 3.7	

Note:

(1) The quantities of the expression were normalized by the gene *murG*, the expression of which is constant.

(2) The *pilC* expression of the knockout came from the expression data of the *P. aeruginosa pilB, T* complemented RR1150.

(3) Pa = *P. aeruginosa*

3.5 Transformation defect of RR1150 is at the level of DNA uptake

As mentioned in the Introduction, natural competence includes steps in which DNA is bound, taken up, translocated across the outer and inner membranes and sometimes recombined with its homologous counterpart in the genome. A defect at any of these steps could be responsible for the transformation defect of RR1150. However, DNA binding and uptake would be expected to be normal if the defect occurs in the downstream steps such as translocation or recombination. As mentioned in Section 3.1, *H. influenzae* PilB was predicted as a Tfp-related ATPase responsible for assembly and/or retraction of the putative Tfp-like apparatus, which in other bacteria acts as DNA uptake machinery. RR1150 is expected to be deficient in DNA binding and uptake. To test this, DNA binding and uptake assays were performed for RR1150, using ^{33}P-end-labeled *Hin*fI fragments of KW20 DNA. MIV-induced KW20 and RR1151 (RR1150 complemented with pSUPILB) were used as positive controls for DNA binding and uptake, and non-competent KW20 cells (in exponential phase) were used as a negative control.

According to the current model, Type IV pili (or pseudopili) provide the structural basis for DNA binding, whereas retraction of Tfp drives DNA uptake. In *H. influenzae*, PilB is predicted to be required for assembly, or for both assembly and retraction of the Tfp-like machinery. In either case, knocking out *pilB* would result in a failure in Tfp assembly. Therefore no DNA binding & uptake is expected in RR1150. The assays of DNA binding plus uptake showed that the amount of DNA associated with RR1150 cells was ~

500 fold lower than that of the wild type KW20, and was not significantly different form that of the negative control (Fig. 3.8). This result indicates that RR1150 can neither bind nor take up DNA. The DNA binding and uptake of RR1151 (RR1150 complemented with pSUPILB) is ~7 fold lower than that of the wild type, but ~57 fold higher than that of RR1150. The defect in RR1150 suggests that the *pilB* knockout strain was unable to assemble a Tfp-like apparatus, thus leading to the strongly decreased DNA binding and uptake.

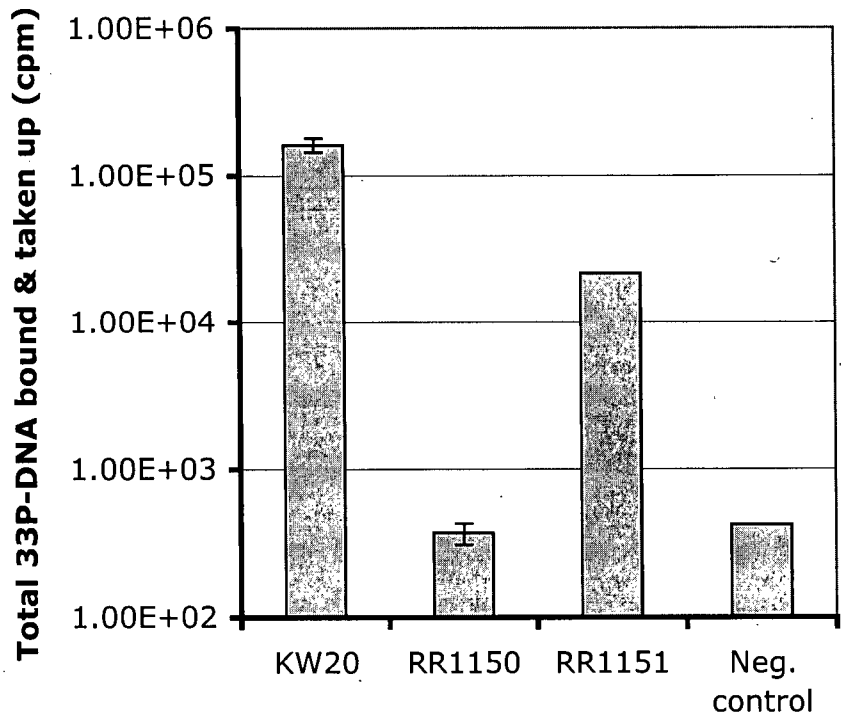

Fig. 3.8 DNA binding and uptake of RR1150. After incubation in MIV medium for 100 min, cells were incubated with α-^{33}P-dATP labeled KW20 DNA. DNase I was not added so that total cell-associated DNA (DNA bound or taken up) could be measured. Competent KW20 and RR1151 (RR1150 containing pSUPILB) were used as the positive controls, and non-competent KW20 (cells in log-phase) was used as the negative control.

3.6 Introduction of *P. aeruginosa pilB* and *pilT* into the *H. influenzae pilB* knockout

As mentioned above, PilB is the only *H. influenzae* Rd ATPase-like protein in the PilB, PilT and PilU family. If all the bacteria that have Tfp or Tfp-like machinery share similar mechanism in DNA binding and uptake, either *H. influenzae* PilB performs the functions of both Tfp biogenesis (required for DNA binding) and retraction (required for DNA uptake), or PilB only functions in Tfp assembly and another as-yet-uncharacterized protein is responsible for Tfp retraction.

3.6.1 Overview

Complementation of pili genes and type II secretion genes with foreign homologues has been reported (21, 30, 42, 68). *P. aeruginosa* PilB/PilT and *N. gonorrhoeae* PilF/PilT are the only well-characterized ATPases systems required for Tfp biogenesis/retraction. Both *P. aeruginosa* PilB and *N. gonorrhoeae* PilF share high similarity with *H. influenzae* PilB (identities 38% and 41%, BLAST E value 4e-89 and 2e-73) (see Table 3.1). Since *P. aeruginosa* PA01 PilB and PilT are a closer homologues of *H. influenzae* PilB than *N. gonorrhoeae* PilF and PilT, they were chosen for complementation of the *H. influenzae pilB* knockout RR1150 and for identification of the specific role of *H. influenzae* PilB in DNA uptake. If *H. influenzae* PilB is needed for both pseudopilus elongation & retraction, complementation of the *pilB* knockout would not be observed unless both *P. aeruginosa pilB* and *pilT* have been introduced (Table 3.3). If *P. aeruginosa* PilB can complement the assembly defect of *H. influenzae pilB,* then introduction of *P. aeruginosa pilB* might restore DNA binding but not uptake in RR1150.

On the other hand, if another *H. influenzae* protein is responsible for Tfp retraction, introduction of *P. aeruginosa pilB* would be able to restore both DNA binding and uptake in the *H. influenzae pilB* knockout. However, if *P. aeruginosa pilB* and *pilT* cannot interact with the *H. influenzae* Tfp genes, no complementation would be observed.

Table 3.3 Hypothesis about complementation of *H. influenzae pilB* with *P. aeruginosa* PA01 *pilB* & *pilT*

		Introduced *P. aeruginosa* gene	**Trans-formation**	**DNA binding**	**DNA uptake**
P. aeruginosa pilB and *pilT* able to complement	**Hypothesis I** *H. influenzae pilB* required for required for both assembly and retraction	-	-	-	-
		pilB	-	+	-
		pilT	-	-	-
		pilB + *pilT*	+	+	+
	Hypothesis II *H. influenzae pilB* required only for Tfp assembly	-	-	-	-
		pilB	+	+	+
		pilT	-	-	-
		pilB + *pilT*	+	+	+
P. aeruginosa pilB and *pilT* cannot interact with *H. influenzae* Tfp proteins		-	-	-	-
		pilB	-	-	-
		pilT	-	-	-
		pilB + *pilT*	-	-	-

P. aeruginosa PA01 *pilB* and *pilT* were cloned using similar strategies, placing each gene under the control of the CRE promoter of the *H. influenzae pil* operon (Section 2.2.3). However, two possible start codons are found in the 5′ of both *P. aeruginosa pilB* and *pilT* (Fig. 3.9) (87). Sequence alignment of PilB homologues indicated that ATG_0, rather than GTG_{117}, is the real start of translation of *pilB*, because the region from 0-117 bp is well conserved in homologues. Analysis for PilT, however, could not identify the true start codon of *pilT* from the two codons ATG_0 and GTG_{-12}, because only 4 amino acids were involved. The sequences upstream of both *pilT* putative start codons were also examined for ribosome binding sites. An obvious consensus sequence for a possible ribosome binding site could not be found. Consequently, two *pilT* plasmids were constructed, each using one of the candidate start codons.

Three types of *P. aeruginosa* plasmids were generated, containing *pilB* (pPAPB), *pilT* (pPAPT and pPAPT2), or both (pPAPBT and pPAPBT2), and were transformed into the *H. influenzae pilB* knockout RR1150. Plasmids containing *P. aeruginosa pilB* and *pilT* are listed in Table 3.4.

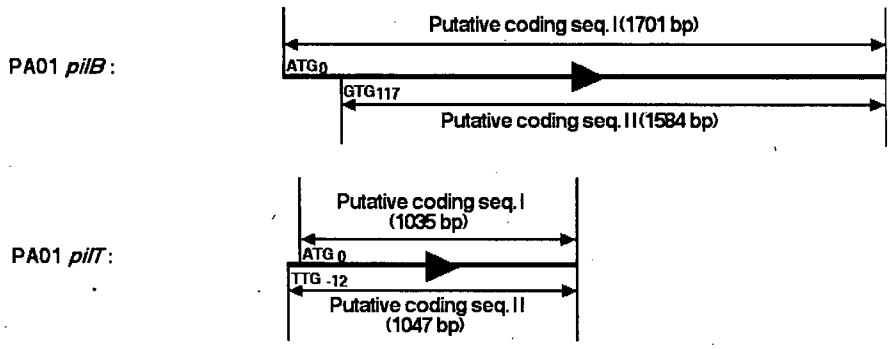

Fig. 3.9 Graphic presentation of the two putative coding sequences of *P. aeruginosa pilB* and *pilT*.

Table 3.4 Constructed *P. aeruginosa* plasmids.

No.	plasmids	*P. aeruginosa* gene	Start codon
1	pPAPB	*pilB*	
2	pPAPT	*pilT*	Starts at ATG_0
3	pPAPT2	*pilT*	Starts at TTG_{-12}
4	pPAPBT	*pilB* and *pilT*	*pilT* starts at ATG_0
5	pPAPBT2	*pilB* and *pilT*	*pilT* starts at TTG_{-12}

3.6.2 Cloning of *P. aeruginosa pilT*

3.6.2.1 Construction of pPAPT and pPAPT2

Fragments of 1100 bp (*pilT*, from start codon ATG_0) and 1112 bp (*pilT*2, from GTG_{-12}) containing either possible *P. aeruginosa pilT* coding sequences were amplified from chromosomal DNA (Fig.2.6) and cloned into a pSU20 based vector containing a CRE promoter of the *H. influenzae pil* operon, to get the plasmids pPAPT and pPAPT2.

As indicated by DNA sequencing, an A-to-G substitution in the putative –10 region of the promoter was found as well in both pPAPT and pPAPT2, as was found in the pPILC construct (Section 3.4) (Fig. 3.6, Fig. 3.7 and Fig. 3.10). As shown in the polarity examination of RR1150 (Section 3.4), the A-to-G substitution did not cause dramatic decrease in the expression of the downstream gene, because the transformation frequency of the complemented strain RR1151 (RR1150 complemented with pSUPILB) was only 6.6 fold lower than that of the wild type but was more than 10^5 fold higher than that of the *pilB* knockout. The pPAPT and pPAPT2 constructs were used afterwards for construction of pPAPBT (containing *P. aeruginosa pilB* and *pilT* that starts at ATG_0), pPAPBT2 (containing *P. aeruginosa pilB* and *pilT* that starts at GTG_{-12}) and for complementation of the *H. influenzae pilB* knockout.

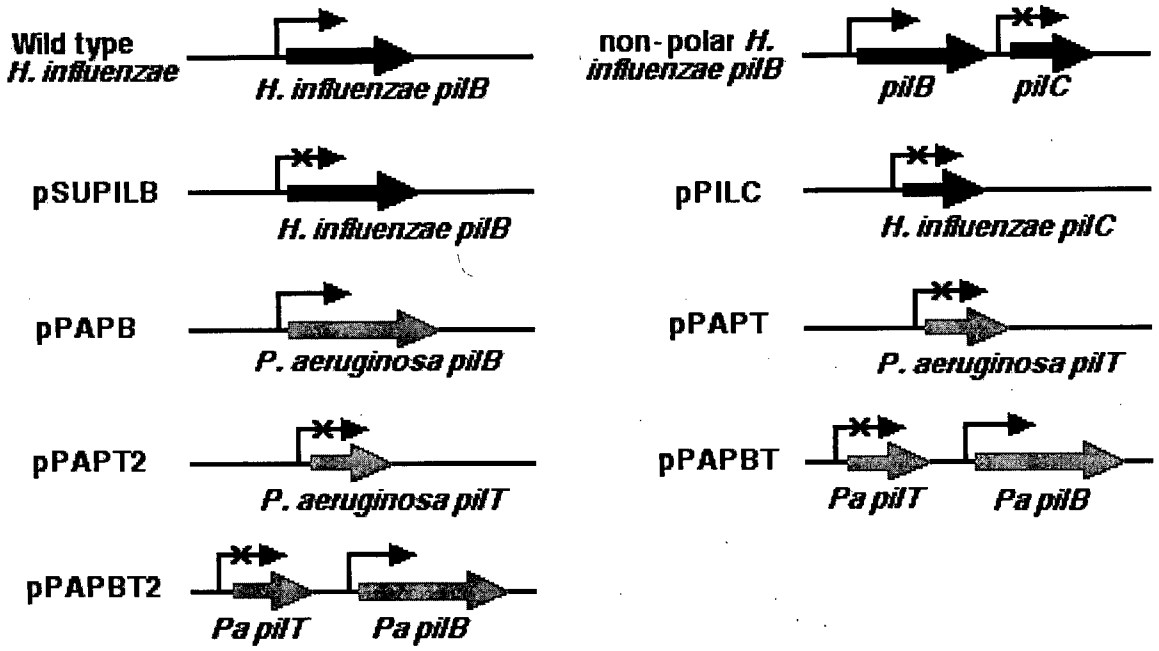

Fig. 3.10 The strains and plasmids with or without the A-G substitution in the putative –10 segment (indicated by ×). Pa = *P. aeruginosa.*

3.6.2.2 Construction of pPAPB

The strategy used to construct pPAPT and pPAPT2 was initially also used to construct the *P. aeruginosa pilB* plasmid pPAPB. However, sequencing of the constructs revealed that deletions occurred at the ligation joint of the end of the *pilA* promoter and start of *pilB*. The problem was not resolved by several modifications in experimental procedures. Therefore toxicity of *P. aeruginosa pilB* to the host cells DH5α was suspected. The original cloning strategy was modified by shifting the breakpoints in the promoter and in *pilB* (Fig. 2.7, Fig. 2.8). Instead of cloning only the coding sequence of *pilB*, a fragment extending 200 bp upstream was amplified from *P. aeruginosa* chromosomal DNA and

inserted into a pSU20 based vector containing a shortened version of *H. influenzae pilA* CRE promoter (simplified as pSU-CRE vector below). The unwanted *P. aeruginosa pilB* promoter was then removed, so that the final plasmid pPAPB contained the *P. aeruginosa pilB* gene and RBS under the *H. influenzae pilA* promoter.

The sequence of *pilB* and the upstream CRE promoter in pPAPB #6 was examined by DNA sequencing using primers SpBEf, SpBr, SpBf and PilBr (See Section 3.6.2.4 below). pPAPB #6 was used for heterologous complementation tests and for construction of pPAPBT, pPAPBT2, as described below.

3.6.2.3 Construction of pPAPBT and pPAPBT2

To get plasmids that express both *P. aeruginosa pilB* and *pilT*, the inserts of pPAPT and pPAPT2 were released with *Nhe*I/*Spe*I double digestion, and subcloned into *Spe*I-linearized pPAPB (Fig. 2.9). The resultant plasmids were designated pPAPBT and pPAPBT2, respectively.

3.6.2.4 Sequencing of the *P. aeruginosa* plasmids in *E. coli*

All five *P. aeruginosa* plasmids (pPAPT, pPAPT2, pPAPB, pPAPBT and pPAPBT2) were examined by DNA sequencing after they were constructed in DH5α. As in pSUPILB, the source of the CRE promoter, both pPAPT and pPAPT2 bears the same A-to-G substitution that tuned the original putative –10 from CAC**A**AT to CAC**G**AT. For unknown reason, however, the –10 area in pPAPB was restored to wild type CACAAT, although the CRE promoter originated from pSUPILB (Fig. 3.5, Fig. 3.6). 2 bases (CC) were also lost at the joint of the ligation in pPAPB (Fig. 3.11). However, as mentioned in

Section 2.2.3.2, the number of bases between the transcription start and RBS was determined arbitrarily in pPAPB, because the sequence in this area plays no significant role in the initiation of gene expression. The 2 bases deletion should not significantly affect the expression of *pilB*.

The other two plasmids pPAPBT and pPAPBT2 that are derived from pPAPB and pPAPT, consequently, both of the *pilT* promoters bear the substitution, whereas both of the *pilB* promoters are wild type (Fig. 3.9). Nevertheless, the rest of the sequences of all the 5 plasmids have no changes.

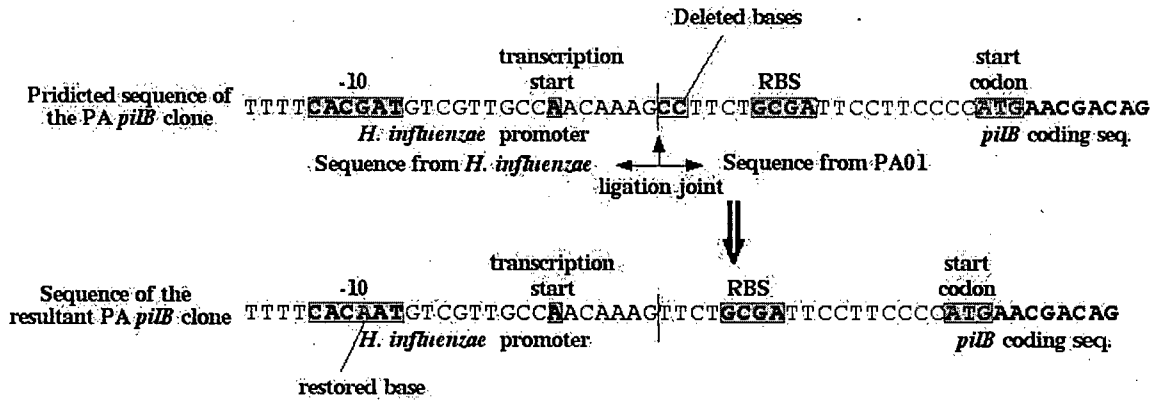

Fig. 3.11 The sequence of pPAPB #6 at the ligation joint, showing the CC 2 base deletion and the restored wild type –10 sequence.

3.6.3 Construction of *H. influenzae* strains bearing both the *pilB* knockout and the *P. aeruginosa* plasmids

The non-polar *H. influenzae pilB* knockout in RR1150 prevents transformation, so cells carrying both the *pilB* knockout and potentially complementing plasmids were constructed indirectly by first transforming the plasmids into the wild *H. influenzae* strain KW20 and then introducing the chromosomal *pilB* knockout by transformation (Fig. 2.10). The resultant strains are RR1156 (RR1150 complemented with pPAPT), RR1157 (RR1150 complemented with pPAPB), RR1158 (RR1150 complemented with pPAPBT), RR1159 (RR1150 complemented with pPAPT2) and RR1197 (RR1150 complemented with pPAPBT2).

Because of the severe interference caused by the *Hin*dIII restriction system in *H. influenzae*, great difficulties were experienced in the earlier attempts to transform the plasmids pPAPB, pPAPBT and pPAPBT2 into *H. influenzae* (data not shown), as these three plasmids contain a number of *Hin*dIII sites. To prevent the digestion of the plasmids by *Hin*dIII, they were treated with methylase *Hin*dIII before transformation into KW20.

3.6.4 Sequences of the *P. aeruginosa* plasmids in *H. influenzae pilB* have no mutations

All the 5 *P. aeruginosa* plasmids in the *H. influenzae pilB* background were re-sequenced. No changes were found compared to the plasmids from *E. coli* (Section 3.6.1.4).

3.6.5 The *P. aeruginosa* genes were strongly expressed in *H. influenzae pilB*

To examine the expression of the *P. aeruginosa pilB*, *pilT* genes in *H. influenzae*, real time PCR was performed for the complemented *H. influenzae pilB* knockouts. To induce CRE-regulated genes, exponentially growing cells were transferred into MIV and incubated as usually done for competence induction. The expression of *P. aeruginosa pilB* and *pilT* was measured by real time PCR of cDNA reverse transcribed from RNA samples of the above MIV cells. The result showed that both *pilB* and *pilT* were strongly expressed in *H. influenzae pilB* (Fig. 3.12 and Fig. 3.13).

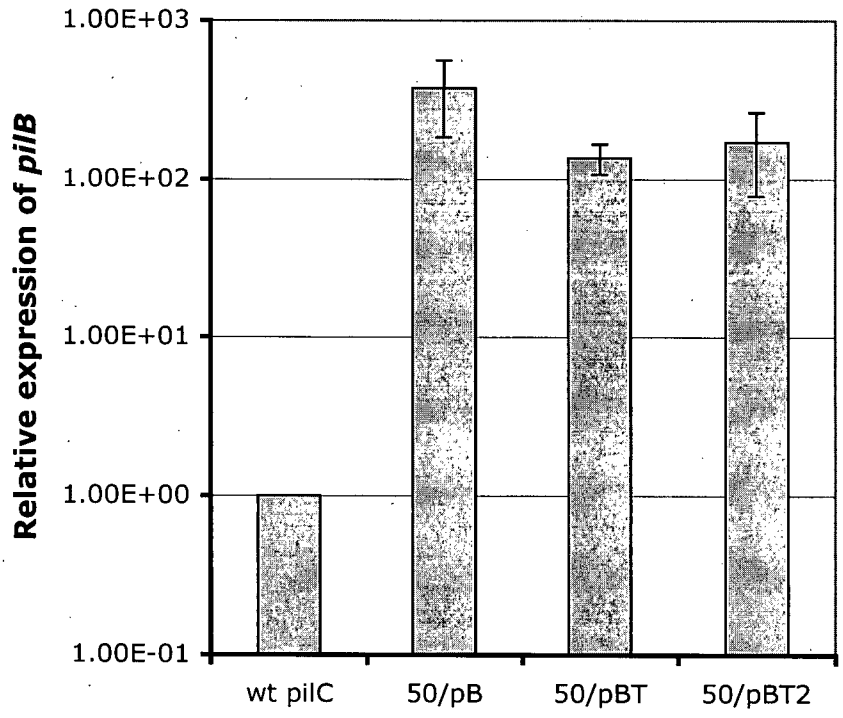

Fig. 3.12 Expression level of *P. aeruginosa pilB* in the *H. influenzae pilB* background relative to the expression of *H. influenzae pilC*, assuming the expression of *pilC* is 1. The expression level of *P. aeruginosa pilB* was compared to that of *H. influenzae pilC* in the same strain (*e.g.* the expression of *P. aeruginosa pilB* in RR1158 was compared to the *pilC* expression in RR1158 according to the difference in the cycle numbers). In order to know the expression of *P. aeruginosa pilB* relative to a wild type *H. influenzae* competence gene, the expression of *pilC* was normalized to the wild type level by subtracting 1.87 from its cycle number, because the expression of *pilC* in the *pilB* knockout is ~3.7 fold lower than that of KW20 (Section 3.4). Assuming ~ 2 fold/cycle difference of PCR amplification, the cycle number difference between RR1150 *pilC* and a wild type *pilC* expression was obtained by the formula $2^{dCt} = d_{exp}$ (dCt = cycle number difference, d_{exp} = difference in expression).

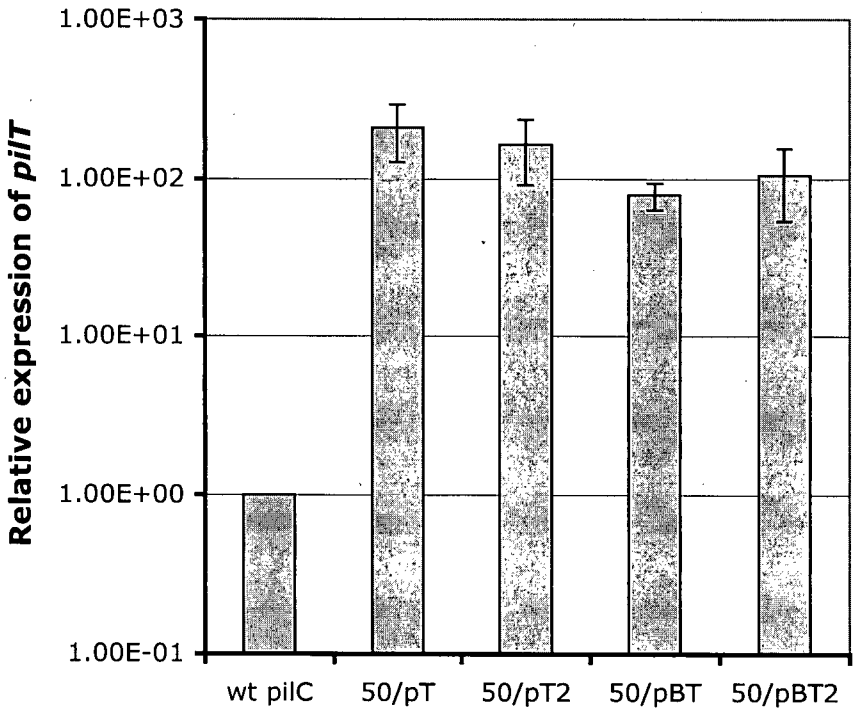

Fig. 3.13 Expression level of *P. aeruginosa pilT* in the *H. influenzae pilB* background relative to the expression of *H. influenzae pilC*, assuming the expression of *pilC* is 1. The expression level of *P. aeruginosa pilB* was compared to that of *H. influenzae pilC* in the same strain (*e.g.* the expression of *P. aeruginosa pilT* in RR1156 was compared to the *pilC* expression in RR1156 according to the difference in the cycle numbers). The expression of *pilC* has been normalized to the level of *pilC* in a wild type. See Fig. 3.11 for explanation.

3.7 The transformation defect of *H. influenzae pilB* could not be restored by *P. aeruginosa pilB* and *pilT*

If *P. aeruginosa pilB* and *pilT* are together functionally equivalent to *H. influenzae pilB,* and can interact with other *H. influenzae* Tfp genes, the defect of *pilB* should be restored effectively by introduction of *P. aeruginosa pilB, pilT* into *pilB*. Transformation assays were employed using the strains RR1156 (RR1150 complemented with pPAPT), RR1157 (RR1150 complemented with pPAPB), RR1158 (RR1150 complemented with pPAPBT), RR1159 (RR1150 complemented with pPAPT2) and RR1197 (RR1150 complemented with pPAPBT2). KW20, RR1150 ($\Delta pilB::Kan^R/CRE/pilC$) and RR1151 (RR1150 complemented with pSUPILB) were used as controls. No complementation was observed in any of the constructs (Fig. 3.14). In the several transformation tests performed, the non-polar *pilB* knockout complemented by the plasmid copy of wild type *H. influenzae pilB*, RR1151, had a transformation frequency approximately 6.8 fold lower than that of KW20, confirmed the previous result in polarity examination of RR1150 (Section 3. 2.4). The transformation frequencies of the *H. influenzae pilB* knockout complemented with *P. aeruginosa pilB* and *pilT*, however, were all below 8.5×10^{-9}, which are not significantly different from that of the uncomplemented strain RR1150 ($\leq 8.1 \times 10^{-9}$).

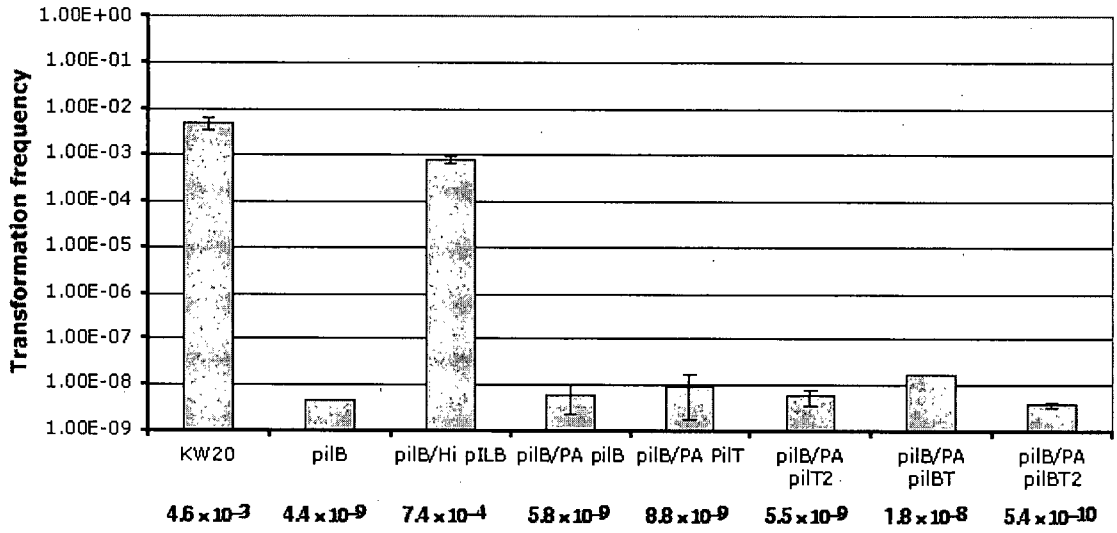

Fig. 3.14 Transformation assays of the *P. aeruginosa pilB, pilT* complemented *H. influenzae pilB*. The numbers below the figure are the average transformation frequencies.

3.8 The defect of DNA binding and uptake of *H. influenzae pilB* could not be complemented by *P. aeruginosa pilB* and *pilT*

If introduction of *P. aeruginosa* genes only restores the Tfp biogenesis function of *H. influenzae pilB*, but not the retraction of the Tfp-like machinery, then the cells containing the above constructs might only bind DNA but not take it up. To examine this, DNA binding experiments were performed using ^{33}P-dATP end-labeled *Hin*fI fragments of KW20 DNA. None of the five complemented *H. influenzae pilB* strains had detectable DNA binding (Fig. 3.15). The radioactivities associated with the cells of the five strains and RR1150 were at similar levels, which all were more than 10^2 fold lower than that associated with KW20 cells.

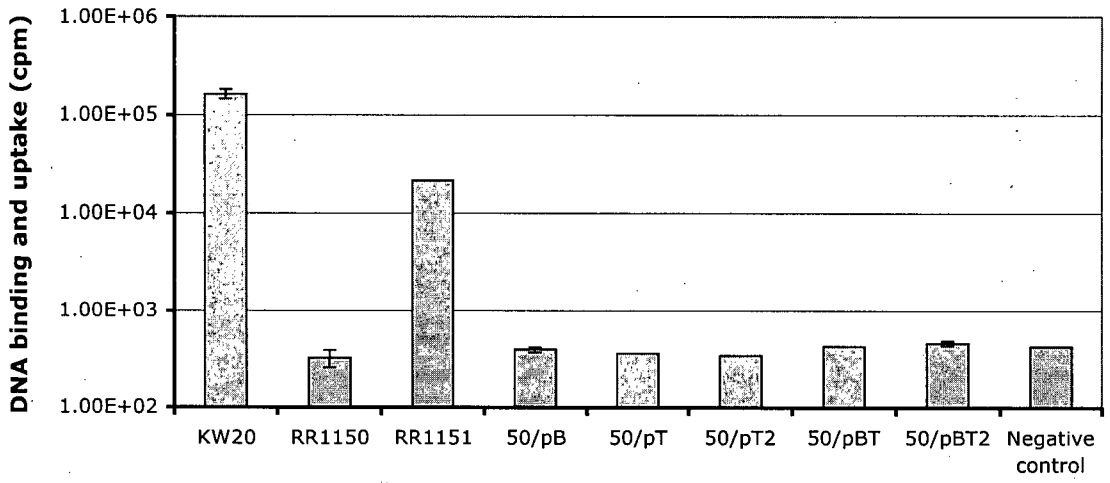

Fig. 3.15 DNA binding assays of the *P. aeruginosa pilB, pilT* complemented *H. influenzae pilB.*

3.9 Discussion

The *pil* operon is required for natural transformation in *H. influenzae* Rd (23). To investigate the specific mechanism of Tfp mediated natural competence, the second gene of this operon, *pilB,* was chosen as the focus of this study. Sequence analysis predicts PilB to be a GspE family ATPase responsible for Tfp biogenesis (49). However, the absence of a PilT homologue in *H. influenzae* led to several speculations about PilB's function (Section 3.1).

Inactivation of *H. influenzae pilB* with a non-polar Kan^R insertion, eliminated natural competence. The defect was because the mutant was unable to bind and take up DNA. When the *pilB* knockout was complemented with the homologous genes *pilB* and *pilT*

from *P. aeruginosa*, neither transformation nor DNA binding and uptake were restored, although the *P. aeruginosa* genes were strongly expressed.

P. aeruginosa and *N. gonorrhoeae* are the only species where the roles of PilB & PilT are well established. *P. aeruginosa* PilB and PilT are closer homologues of *H. influenzae* PilB than *N. gonorrhoeae* PilF and PilT (Table 3.1, Table 3.5). Therefore they were chosen for complementation of *H. influenzae pilB*.

Complementation with a heterologous protein has been commonly used for investigation of a protein's function. The probability of producing a functional hybrid machinery depends on the phylogenetic distance between the organisms and the extent of the similarity between the proteins involved. In Type II secretion systems (T2ss), most components have exchangeable examples except GspC (a component unique to T2ss) and GspD (a PilQ homologue) (30). In Tfp systems, there are several reported cases of complementation by heterologous proteins, including pilin, peptidase and GspE family ATPases (21, 42, 68, 80). A cosmid clone bearing *Pseudomonas syringae* pv. *Tomato* DC3000 DNA was able to complement a mutation of the *pilB* gene in *P. aeruginosa* (75). In *Aeromonas hydrophila,* the gene *tapB* was identified to be a homologue of *P. aeruginosa pilB* by its ability to complement a *pilB* mutation in *P. aeruginosa* (68), although A. *hydrophila* is not more closely related to *P. aeruginosa* than *H. influenzae* (Table 3.5). Another Tfp protein that can substitute the homologue in other species is peptidase (PilD homologues), probably because its function is solely enzymatic and PilD does not need to interact with other proteins (21, 30, 68, 75). The most permissive T2ss

and Tfp components are pilins and pseudopilins, which can often be exchanged, more or less efficiently (30, 42, 80).

Table 3.5 Taxonomic relationships between *Haemophilus influenzae, Pseudomonas aeruginosa, Nesseria gonorrhoeae* and *Aeromonas hydrophila* (adapted from NCBI).

Bacteria	**Class**	**Order**
Neisseria gonorrhoeae	Betaproteobacteria	Neisseriales
Aeromonas hydrophila	Gammaproteobacteria	Aeromonadales
Haemophilus influenzae	Gammaproteobacteria	Pasteurellales
Pseudomonas aeruginosa	Gammaproteobacteria	Pseudomonadales

However, complementation does not often occur, despite the conservation of the Tfp systems and Type II secretion systems (30, 78), suggesting that there is specificity within the machinery itself. The situation of GspE like ATPases appears to be especially complicated. In addition to the nucleotide binding motifs in the C-termini that are thought to perform the enzymatic function, the N-termini may interact with other proteins, thus determining species specificity (30). Sandkvist showed that T2ss *V. cholerae* EpsE (GspE homologue) is associated with and stabilized by the cytoplasmic membrane via interaction with EpsL (79). In *P. aeruginosa,* localization of PilB to both poles of the cells requires PilC, suggesting that PilB and PilC interact (15). Therefore, in addition to the phylogenetic distance of the two organism involved, the functional interactions between the introduced protein and other members of the Tfp machinery in the organism are also important constraints on heterologous complementation.

In *P. aeruginosa,* although the *pilB* mutation was complemented by *Aeromonas hydrophila tapB,* the two proteins PilB and TapB are substantially more similar than the *H. influenzae* & *P. aeruginosa* PilBs. The failure of *P. aeruginosa* PilB and PilT to complement the *H. influenzae pilB* knockout, therefore, is likely to be at least partly because the *P. aeruginosa* homologues could not interact with *H. influenzae* PilC to form a functional hybrid Tfp machinery.

However, the result of a recent work and our sequence analyses suggest that the reason of the non-complementation of PilB in this study may also be because the difference in the Tfp energetics of *H. influenzae* and *P. aeruginosa.* Another *H. influenzae* strain has been

recently shown to express functional Tfp and twitching motility (7), the first report of type IV pili in *H. influenzae*. It was also shown that the Type IV pili in this strain are required for twitching motility, which in other Tfp systems requires pilus retraction. However, a PilT protein was not found in this *H. influenzae*. This raised the question of which protein would drive the retraction required for twitching motility. Since a PilT is absent in *H. influenzae* Rd as well, I was interested to see whether there is a PilT in *A. pleuropneumoniae* and *P. multocida*, the other two Pasteurellaceae species that possess type IV pili (76, 102). Similarly, a PilT was not found.

PilT proteins are required for Tfp retraction and Tfp mediated functions (*e.g.* adhesion, cell motility) in Gram negative bacteria including *P. aeruginosa, P. stutzeri, N. gonorrhoeae, Myxococcus xanthus, Aquifex aeolicus, Synechocystis sp.* PCC 6803 and *Ralstonia solanacearum* (9, 37, 50, 94, 98, 101). The absence of a PilT homologue in Pasteurellaceae implies the presence of an as-yet-uncharacterized protein to provide the force for Tfp retraction, and a difference between energetics of Tfp in Pasteurellaceae species and other Gram negative bacteria.

On the other hand, however, the Pasteurellaceae species may not need a protein to drive Tfp retraction. In *P. stutzeri*, an insertion in the *pilT* gene resulted in a hyperpiliated mutant, which was able to bind DNA but could not take it up (37). This transformation defect was suppressed by replacement of the six C-terminal amino acids in the pilin subunit PilAI with six histidine residues. It was hypothesized in the study that the *pilT*-independent transformation can promoted by mutant PilA protein either as single molecules or as minimal pilin assembly structures in the periplasm which may resemble

depolymerized pili, and that these cause the outer membrane pores to open for DNA entry. Both of these two explanations have problems. Studies have shown that DNA binding is a function of intact pili (91). Therefore the DNA uptake machinery has to be a Tfp-like assembly that is at least long enough to penetrate to the outside of the outer membrane to reach DNA, although fimbriae may not be seen on the cell surface. The current model postulates that the assembled Tfp fimbriae are translocated across the outer membrane through the secretin pore of PilQ (16). Since the predicted structure of Type IV pilus does not have space for a DNA channel in the center of the pilus (47), transforming DNA is thought to be transformed through the PilQ secretin pore (6, 26). DNA would not be allowed to come in until the secretin pore become unoccupied by pilus disassembly. Therefore disassembly of the Tfp-like machinery into the periplasm would be required for transport of DNA. Consequently, a more plausible explanation of the observation in the above study would be that the mutation in PilAI altered the association between the pilin subunits such that the pilus assembly was shorter and less stable at the base than a regular pilus and it spontaneously depolymerized when PilB was not active, thus a PilT protein was not needed.

Similarly, the absence of a PilT-like ATPase in Pasteurellaceae species, therefore, may also be because a PilT protein is not needed. Sequence analysis showed some considerable differences between the prepilin protein PilA of nontypeable *H. influenzae* and other Type IV pilins (7). The leader peptides of nontypeable *H. influenzae* prepilins are longer than that of *P. aeruginosa and N. gonorrhoeae* prepilins, whereas the length of the mature pilins in nontypeable *H. influenzae* are shorter than that in *P. aeruginosa and*

N. gonorrhoeae. The sequence features of *H. influenzae* pilins are also found in the pilins of *A. pleuropneumoniae* and *P. multocida* (data not shown). The Tfp fibers of the Pasteurellaceae species may be able to disassemble from the base spontaneously from the base when PilB is not active, therefore a PilT protein would not be required and the *pilT* gene might have been lost during evolution.

In summary, the absence of a PilT protein in Pasteurellaceae may be either because an-as-yet uncharacterized protein drives Tfp retraction, or a PilT is not required for pilus disassembly in Pasteurellaceae. To test which of the above hypotheses is right, experiments can first be done in either of the two *H. influenzae* strains, to look for the putative protein that the first hypothesis predicts to drive the Tfp retraction in Pasteurellaceae species. According to the first hypothesis, this protein should be unique to Pasteurellaceae species. Moreover, if Pasteurellaceae Tfp are disassembled at the base, the protein that drives retraction may not be in the outer membrane and would be either in the periplasm, or intrinsic or peripheral to the inner membrane. This as-yet-uncharacterized protein could be an ATPase that does not belong to the GspE family, or could use another energy source, such as proton motive force. Bremer, *et al.* showed that proton motive force is needed for DNA uptake by *H. influenzae* (11). Comparison analysis of the genomes of *H. influenzae* and *P. aeruginosa* can be performed to look for genes present in Pasteurellaceae but not in *P. aeruginosa.* Sequence analysis including BLAST search and reserved domain analysis could be used to predict location and function of the proteins. Subsequently, High-throughput gene mutagenesis can be used. A gene whose knockout strain can bind DNA but cannot take it up in *H. influenzae* Rd, or

a gene whose knockout can express Tfp but is deficient in twitching motility in the other *H. influenzae* strain that possess visible Tfp, would be the one responsible for Tfp retraction. As a result, if such a protein cannot be found, it would suggest that a protein may be unnecessary in Pasteurellaceae. However, more convincing evidence would be required to confirm it.

The lack of another GspE family protein and of visible Tfp in *H. influenzae* Rd, makes it difficult to test whether *H. influenzae* PilB function both in the assembly and retraction of the Tfp-like machinery or only function in assembly. Due to the failure of complementation by *P. aeruginosa* homologues, *H. influenzae* PilB's specific function in DNA uptake remains unidentified. However, sequence analysis indicates that *H. influenzae* PilB is more related to PilB proteins than to PilT proteins (67, 69). These suggest that *H. influenzae* PilB only functions in the assembly of the Tfp-like DNA uptake machinery, although more convincing evidence is not available yet. Furthermore, the existence of functional type IV pili in some Pasteurellaceae in the absence of a PilT homologue suggests that a GspE family ATPase may not be required for retraction of the type IV pili in this family (7, 76, 102).

In future experiments, a *H. influenzae* strain that can express visible pili would be useful to identify PilB's specific function in Tfp assembly/retraction. It has been shown that the majority of cells of *H. influenzae* 86-028NP express Tfp only when growing under defined nutrient conditions at an alkaline pH (pH8.5 to 9.0) (7). *H. influenzae* Rd might also do so under similar conditions. On the other hand, the hypertransformable *Sxy*

mutant strain might be able to express visible Tfp as well, because pilin genes are hyperinduced in this strain (73, 74). If either of the above can express visible Type IV pili, electron microscopy and/or motility assays using agar slabs can be used to examine Tfp expression in the above strains and in the *pilB* knockout RR1150 (7). The presence of visible Tfp in the wild type *H. influenzae* or in the Sxy mutant, and the absence of Tfp in RR1150 would indicate that PilB is responsible for Tfp assembly.

References

1. **Aas, F. E., C. Lovold, and M. Koomey.** 2002. An inhibitor of DNA binding and uptake events dictates the proficiency of genetic transformation in *Neisseria gonorrhoeae*: mechanism of action and links to Type IV pilus expression. Mol Microbiol **46:**1441-50.
2. **Aas, F. E., M. Wolfgang, S. Frye, S. Dunham, C. Lovold, and M. Koomey.** 2002. Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. Mol Microbiol **46:**749-60.
3. **Alexander, H. E., and G. Leidy.** 1951. Determination of inherited traits of *H. influenzae* by deoxyribonucleic acid fractions isolated from type-specific cells. J Exp Med **93:**345-59.
4. **Alm, R. A., and J. S. Mattick.** 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa.* Gene **192:**89-98.
5. **Ausubel, F. M., R. Brent, R. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. John Wiley & Sons, Inc.
6. **Averhoff, B., and A. Friedrich.** 2003. Type IV pili-related natural transformation systems: DNA transport in mesophilic and thermophilic bacteria. Arch Microbiol **180:**385-93.
7. **Bakaletz, L. O., B. D. Baker, J. A. Jurcisek, A. Harrison, L. A. Novotny, J. E. Bookwalter, R. Mungur, and R. S. Munson, Jr.** 2005. Demonstration of Type IV pilus expression and a twitching phenotype by *Haemophilus influenzae.* Infect Immun **73:**1635-43.
8. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae.* Methods Enzymol **204:**321-42.
9. **Bhaya, D., N. R. Bianco, D. Bryant, and A. Grossman.** 2000. Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp. PCC6803.
10. **Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock, and J. Tommassen.** 1998. Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa.*
11. **Bremer, W., J. Kooistra, K. J. Hellingwerf, and W. N. Konings.** 1984. Role of the electrochemical proton gradient in genetic transformation of *Haemophilus influenzae.* J Bacteriol **157:**868-73.
12. **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nat Rev Microbiol **2:**241-9.
13. **Chen, I., and E. C. Gotschlich.** 2001. ComE, a competence protein from *Neisseria gonorrhoeae* with DNA-binding activity. J Bacteriol **183:**3160-8.
14. **Chen, W. J., L. Gross, K. E. Joho, and W. T. McAllister.** 1992. A modified kanamycin-resistance cassette to facilitate two-codon insertion mutagenesis. Gene **111:**143-4.
15. **Chiang, P., M. Habash, and L. L. Burrows.** 2005. Disparate subcellular localization patterns of *Pseudomonas aeruginosa* Type IV pilus ATPases involved in twitching motility. J Bacteriol **187:**829-39.

16. **Collins, R. F., L. Davidsen, J. P. Derrick, R. C. Ford, and T. Tonjum.** 2001. Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure.
17. **Comolli, J. C., A. R. Hauser, L. Waite, C. B. Whitchurch, J. S. Mattick, and J. N. Engel.** 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect Immun **67:**3625-30.
18. **Craig, L., M. E. Pique, and J. A. Tainer.** 2004. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol **2:**363-78.
19. **Craig, L., R. K. Taylor, M. E. Pique, B. D. Adair, A. S. Arvai, M. Singh, S. J. Lloyd, D. S. Shin, E. D. Getzoff, M. Yeager, K. T. Forest, and J. A. Tainer.** 2003. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Mol Cell **11:**1139-50.
20. **Danner, D. B., R. A. Deich, K. L. Sisco, and H. O. Smith.** 1980. An eleven-base-pair sequence determines the specificity of DNA uptake in *Haemophilus* transformation. Gene **11:**311-8.
21. **de Groot, A., I. Heijnen, H. de Cock, A. Filloux, and J. Tommassen.** 1994. Characterization of type IV pilus genes in plant growth-promoting *Pseudomonas putida* WCS358. J Bacteriol **176:**642-50.
22. **De Reuse, H., and M. K. Taha.** 1997. RegF, an SspA homologue, regulates the expression of the *Neisseria gonorrhoeae pilE* gene. Res Microbiol **148:**289-303.
23. **Dougherty, B. A., and H. O. Smith.** 1999. Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. Microbiology **145 (Pt 2):**401-9.
24. **Douglas, S. E.** 1995. DNA Strider. An inexpensive sequence analysis package for the Macintosh. Mol Biotechnol **3:**37-45.
25. **Drake, S. L., and M. Koomey.** 1995. The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*.
26. **Dubnau, D.** 1999. DNA uptake in bacteria. Annu Rev Microbiol **53:**217-44.
27. **Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling.** 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence.
28. **Engels, W. R.** 1993. Contributing software to the internet: the Amplify program. Trends Biochem Sci **18:**448-50.
29. **Fernandez, L. A., and J. Berenguer.** 2000. Secretion and assembly of regular surface structures in Gram-negative bacteria. FEMS Microbiol Rev **24:**21-44.
30. **Filloux, A.** 2004. The underlying mechanisms of type II protein secretion. Biochim Biophys Acta **1694:**163-79.
31. **Finkel, S. E., and R. Kolter.** 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J Bacteriol **183:**6288-93.
32. **Freitag, N. E., H. S. Seifert, and M. Koomey.** 1995. Characterization of the *pilF-pilD* pilus-assembly locus of *Neisseria gonorrhoeae*. Mol Microbiol **16:**575-86.
33. **Fussenegger, M., T. Rudel, R. Barten, R. Ryll, and T. F. Meyer.** 1997. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae*--a review. Gene **192:**125-34.

34. **Goldberg, G. B.** 1995, posting date. Goldberg lab standard protocols. Cayne, M. [Online.]
35. **Goodgal, S. H.** 1982. DNA uptake in *Haemophilus* transformation. Annu Rev Genet **16:**169-92.
36. **Graupner, S., and W. Wackernagel.** 2001. *Pseudomonas stutzeri* has two closely related *pilA* genes (Type IV pilus structural protein) with opposite influences on natural genetic transformation. J Bacteriol **183:**2359-66.
37. **Graupner, S., N. Weger, M. Sohni, and W. Wackernagel.** 2001. Requirement of novel competence genes *pilT* and *pilU* of *Pseudomonas stutzeri* for natural transformation and suppression of *pilT* deficiency by a hexahistidine tag on the type IV pilus protein *PilAI*. J Bacteriol **183:**4694-701.
38. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557-80.
39. **Herdendorf, T. J., D. R. McCaslin, and K. T. Forest.** 2002. *Aquifex aeolicus* PilT, homologue of a surface motility protein, is a thermostable oligomeric NTPase. J Bacteriol **184:**6465-71.
40. **Herriott, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J Bacteriol **101:**517-24.
41. **Hobbs, M., and J. S. Mattick.** 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol Microbiol **10:**233-43.
42. **Hoyne, P. A., R. Haas, T. F. Meyer, J. K. Davies, and T. C. Elleman.** 1992. Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. J Bacteriol **174:**7321-7.
43. **Inamine, G. S., and D. Dubnau.** 1995. ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. J Bacteriol **177:**3045-51.
44. **International, P. B.** 1994, posting date. NetPrimer. [Online.]
45. **Kahn, M. E., and H. O. Smith.** 1984. Transformation in *Haemophilus*: a problem in membrane biology. J Membr Biol **81:**89-103.
46. **Karudapuram, S., and G. J. Barcak.** 1997. The *Haemophilus influenzae dprABC* genes constitute a competence-inducible operon that requires the product of the *tfoX* (*sxy*) gene for transcriptional activation. J Bacteriol **179:**4815-20.
47. **Keizer, D. W., C. M. Slupsky, M. Kalisiak, A. P. Campbell, M. P. Crump, P. A. Sastry, B. Hazes, R. T. Irvin, and B. D. Sykes.** 2001. Structure of a pilin monomer from *Pseudomonas aeruginosa*: implications for the assembly of pili. J Biol Chem **276:**24186-93.
48. **Koga, T., K. Ishimoto, and S. Lory.** 1993. Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili.
49. **Lessl, M., and E. Lanka.** 1994. Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell **77:**321-4.
50. **Liu, H., Y. Kang, S. Genin, M. A. Schell, and T. P. Denny.** 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system.

51. **Long, C. D., D. M. Tobiason, M. P. Lazio, K. A. Kline, and H. S. Seifert.** 2003. Low-level pilin expression allows for substantial DNA transformation competence in *Neisseria gonorrhoeae*. Infect Immun **71:**6279-91.
52. **Lory, S., and M. S. Strom.** 1997. Structure-function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa*--a review. Gene **192:**117-21.
53. **Macfadyen, L. P.** 2000. Regulation of competence development in *Haemophilus influenzae*. J Theor Biol **207:**349-59.
54. **Maier, B., L. Potter, M. So, C. D. Long, H. S. Seifert, and M. P. Sheetz.** 2002. Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci U S A **99:**16012-7.
55. **Martin, P. R., M. Hobbs, P. D. Free, Y. Jeske, and J. S. Mattick.** 1993. Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*.
56. **Martin, P. R., A. A. Watson, T. F. McCaul, and J. S. Mattick.** 1995. Characterization of a five-gene cluster required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*.
57. **Martinez, E., B. Bartolome, and F. de la Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* alpha reporter gene of pUC8/9 and pUC18/19 plasmids. Gene **68:**159-62.
58. **Mattick, J. S.** 2002. Type IV pili and twitching motility. Annu Rev Microbiol **56:**289-314.
59. **Mattick, J. S., M. M. Bills, B. J. Anderson, B. Dalrymple, M. R. Mott, and J. R. Egerton.** 1987. Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*.
60. **Merz, A. J., M. So, and M. P. Sheetz.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98-102.
61. **Molnar, S.** 2004. Identification and characterization of DNA receptor candidates in *Haemophilus influenzae*. Thesis for Master of Science. The University of British Columbia, Vancouver.
62. **Nudleman, E., and D. Kaiser.** 2004. Pulling together with type IV pili. J Mol Microbiol Biotechnol **7:**52-62.
63. **Nunn, D.** 1999. Bacterial type II protein export and pilus biogenesis: more than just homologies? Trends Cell Biol **9:**402-8.
64. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili.
65. **Parge, H. E., K. T. Forest, M. J. Hickey, D. A. Christensen, E. D. Getzoff, and J. A. Tainer.** 1995. Structure of the fibre-forming protein pilin at 2.6 A resolution. Nature **378:**32-8.
66. **Park, H. S., M. Wolfgang, and M. Koomey.** 2002. Modification of type IV pilus-associated epithelial cell adherence and multicellular behavior by the PilU protein of *Neisseria gonorrhoeae*. Infect Immun **70:**3891-903.
67. **Peabody, C. R., Y. J. Chung, M. R. Yen, D. Vidal-Ingigliardi, A. P. Pugsley, and M. H. Saier, Jr.** 2003. Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. Microbiology **149:**3051-72.
68. **Pepe, C. M., M. W. Eklund, and M. S. Strom.** 1996. Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterization of a type IV leader peptidase/N-

methyltransferase required for extracellular protein secretion. Mol Microbiol **19:**857-69.

69. **Planet, P. J., S. C. Kachlany, R. DeSalle, and D. H. Figurski.** 2001. Phylogeny of genes for secretion NTPases: identification of the widespread tadA subfamily and development of a diagnostic key for gene classification.
70. **Poje, G., and R. J. Redfield.** 2003. General methods for culturing *Haemophilus influenzae*. *In* M. A. Herbert, D. W. Hood, and E. R. Moxon (ed.), *Haemophilus influenzae* protocols. Human Press, Totowa, New Jersey.
71. **Poje, G., and R. J. Redfield.** 2003. Transformation of *Haemophilus influenzae*. *In* M. A. Herbert, D. W. Hood, and E. R. Moxon (ed.), *Haemophilus influenzae* protocols. Human Press, Totowa, New Jersey.
72. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol Rev **57:**50-108.
73. **Redfield, R. J.** 1991. *sxy*-1, a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence.
74. **Redfield, R. J., A. D. Cameron, Q. Qian, J. Hinds, T. R. Ali, J. S. Kroll, and P. R. Langford.** 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J Mol Biol **347:**735-47.
75. **Roine, E., D. M. Raineri, M. Romantschuk, M. Wilson, and D. N. Nunn.** 1998. Characterization of type IV pilus genes in *Pseudomonas syringae* pv. tomato DC3000. Mol Plant Microbe Interact **11:**1048-56.
76. **Ruffolo, C. G., J. M. Tennent, W. P. Michalski, and B. Adler.** 1997. Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. Infect Immun **65:**339-43.
77. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning - A Laboratory Manual, Second Edition ed. Cold Spring Harbour Laboratory Press, New York.
78. **Sandkvist, M.** 2001. Biology of type II secretion. Mol Microbiol **40:**271-83.
79. **Sandkvist, M., M. Bagdasarian, S. P. Howard, and V. J. DiRita.** 1995. Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*.
80. **Sauvonnet, N., P. Gounon, and A. P. Pugsley.** 2000. PpdD type IV pilin of *Escherichia coli* K-12 can be assembled into pili in *Pseudomonas aeruginosa*. J Bacteriol **182:**848-54.
81. **Sauvonnet, N., G. Vignon, A. P. Pugsley, and P. Gounon.** 2000. Pilus formation and protein secretion by the same machinery in *Escherichia coli*. Embo J **19:**2221-8.
82. **Smeets, L. C., and J. G. Kusters.** 2002. Natural transformation in *Helicobacter pylori*: DNA transport in an unexpected way. Trends Microbiol **10:**159-62; discussion 162.
83. **Strom, M. S., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili.
84. **Strom, M. S., D. Nunn, and S. Lory.** 1991. Multiple roles of the pilus biogenesis protein PilD: involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. J Bacteriol **173:**1175-80.

85. **Strom, M. S., D. N. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc Natl Acad Sci U S A **90:**2404-8.
86. **Stuy, J. H.** 1979. Plasmid transfer in *Haemophilus influenzae*. J Bacteriol **139:**520-9.
87. **TIGR** 1999-2004, posting date. http://www.tigr.org/. [Online.]
88. **Tomb, J. F., H. el-Hajj, and H. O. Smith.** 1991. Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd.
89. **Tonjum, T., N. E. Freitag, E. Namork, and M. Koomey.** 1995. Identification and characterization of *pilG*, a highly conserved pilus-assembly gene in pathogenic *Neisseria*. Mol Microbiol **16:**451-64.
90. **Turner, L. R., J. C. Lara, D. N. Nunn, and S. Lory.** 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J Bacteriol **175:**4962-9.
91. **van Schaik, E. J., C. L. Giltner, G. F. Audette, D. W. Keizer, D. L. Bautista, C. M. Slupsky, B. D. Sykes, and R. T. Irvin.** 2005. DNA binding: a novel function of *Pseudomonas aeruginosa* type IV pili. J Bacteriol **187:**1455-64.
92. **Wang, Y., S. D. Goodman, R. J. Redfield, and C. Chen.** 2002. Natural transformation and DNA uptake signal sequences in *Actinobacillus actinomycetemcomitans*. J Bacteriol **184:**3442-9.
93. **Watson, A. A., R. A. Alm, and J. S. Mattick.** 1996. Identification of a gene, *pilF*, required for type 4 fimbrial biogenesis and twitching motility in *Pseudomonas aeruginosa*.
94. **Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick.** 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria.
95. **Wiesner, R. S., D. R. Hendrixson, and V. J. DiRita.** 2003. Natural transformation of *Campylobacter jejuni* requires components of a type II secretion system. J Bacteriol **185:**5408-18.
96. **Wilcox, K. W., and H. O. Smith.** 1975. Isolation and characterization of mutants of *H. influenzae* deficient in an adenosine 5'-triphosphate-dependent deoxyribonuclease activity. J Bacteriol **122:**443-53.
97. **Williams, P., W. L. Hung, and R. J. Redfield.** 1996. Cell transfer by filtration: evaluation of protocols for transformational competence. FEMS Microbiol Lett **137:**183-7.
98. **Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert, and M. Koomey.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. Mol Microbiol **29:**321-30.
99. **Wolfgang, M., J. P. van Putten, S. F. Hayes, and M. Koomey.** 1999. The *comP* locus of *Neisseria gonorrhoeae* encodes a type IV prepilin that is dispensable for pilus biogenesis but essential for natural transformation. Mol Microbiol **31:**1345-57.

100. **Wu, S. S., and D. Kaiser.** 1995. Genetic and functional evidence that Type IV pili are required for social gliding motility in Myxococcus xanthus.
101. **Wu, S. S., J. Wu, and D. Kaiser.** 1997. The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced.
102. **Zhang, Y., J. M. Tennent, A. Ingham, G. Beddome, C. Prideaux, and W. P. Michalski.** 2000. Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae*. FEMS Microbiol Lett **189:**15-8.