MOLECULAR BIOLOGY OF ANTIBIOTIC RESISTANCE ELEMENTS IN HUMAN *HAEMOPHILUS* SPP.

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ABSTRACT

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Antibiotic resistance in *Haemophilus influenzae* emerged in the 1970s and rapidly rose in prevalence worldwide. The principal source of resistance was a large conjugative resistance element often referred to as a plasmid. The origins of this resistance element that integrates and conjugates have not been determined. The main aim of this thesis was to investigate the origin of this integrative and conjugative element (ICE) through comparative analysis of whole sequenced elements. Two *Haemophilus* resistance ICEs, ICEHin1056, from the UK, and ICEHin299, from Greece, were completely sequenced. Two further ICEs, ICEHin2866 from the USA and ICEHpa8F from a UK *Haemophilus parainfluenzae* were compared. These ICEs consisted of highly homologous sequences that were predicted to form functional modules. The properties of these predicted modules accounted for replication, conjugation and integrative and excisive recombination with tRNA\textsubscript{leu}. As the sequence diversity of resistance associated genes common to these ICEs were highly conserved compared to core ICE sequences they probably have been recently acquired by these ICEs suggesting a relatively recent origin. Further comparative analysis of these ICEs revealed a common evolutionary origin with genomic islands (GIs) found among bacteria belonging to β- and γ-Proteobacteria. These GIs share coherently organised core genes indicating that they have originated from a distant common ancestor. This finding contradicts the hypothesis that GIs were derived from independently evolving modules. Furthermore, the preservation of a coherent core gene structure between distantly related GIs suggests these core genes acting together confer a particular fitness advantage, which has contributed to the dissemination of adaptive genes such as antibiotic resistance genes. The methods used to compare these genomic islands could in the future be used to classify all genomic islands.
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**Note of declaration**

I, Zaini Mohd Zain hereby, declare that the work carried out in this thesis was solely carried out by myself. The sequences of *H. influenzae* 2866 were kindly provided by Dr. Arnold Smith from Seattle Biomedical Research Institute, USA. Dr. Thomas Inzana from Virginia Tech, Blacksburg, USA, provided the genome sequences of *H. somnus* 2336, and the sequences of ICE*Hp* gave by Dr. Lucielle Mansfield from NDCLS, Oxford University.

"This thesis is dedicated to Rahmat and Afiqah"
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LIST OF ABBREVIATIONS

A adenine
AA/aa amino acid
Ala alanine
amp r ampicillin resistant
asn asparagines
BBB blood-brain barrier
BHI brain heart infusion
bp base pair
C cytosine
°C degrees in Celsius
cat chloramphenicol acetyltransferase
chlor r chloramphenicol resistant
CIAP calf intestine alkaline phosphatase
CO₂ carbon dioxide
CSF cerebral spinal fluid
CsCl caesium chloride
DNA deoxyribonucleic acid
DNTP deoxynucleotide triphosphate
dsDNA double-stranded DNA
EDTA ethylene diamine tetra-acetic acid
EM electron microscopy
EMBL European Molecular Biology Laboratory
EtBr ethidium bromide
G guanine
g gram
g gravitational force
GC guanine and cystine
Gi genomic island
HEPES N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HTH helix-turn-helix
ICE integrative and conjugative element
IHF integration host factor
IPTG isopropylthio-β-D-galactosidase
IS insertion sequence
kb kilobases
kDa kilodalton
L litre
LB Luria broth
Leu leucine
LPS lipopolysaccharide
Lys lysine
Mda megadalton
MgCl₂ magnesium chloride
MH Muller Hinton
MIC minimal inhibitory concentration
µg microgram
µl microlitre
ml millilitre
MLEE  multi-locus enzyme electrophoresis
MLST  multi-locus sequence typing
NAD  nicotinamide adenine dinucleotide
NaOAc  sodium acetate
NCBI  National Centre for Biotechnology Information
OMP  outer membrane protein
ORF  open reading frame
oriT  origin of transfer
oriV  origin of replication
PAI  pathogenicity island
PBS  phosphate buffer saline
PBP  penicillin binding protein
PCR  polymerase chain reaction
PEG  polyethylene-glycol 8000
Phe  phenylalanine
PI  isoelectric point
Pro  proline
PRP  polynribosyl ribitol phosphate
UK  United Kingdom
USA  United States of America
UV  ultraviolet
R plasmid  resistant plasmid
RAPD  random amplified polymorphic DNA fingerprinting
REA  restriction endonuclease analysis
RFLP  restriction fragment length polymorphism
rif’  rifampicin resistant
RNA  ribonucleic acid
rpm  revolution per minute
s  seconds
Ser  serine
strep’  streptomycin resistant
T  thymine
TE  tris-EDTA
TER  Terminator/stop codon
ter  site for termination of replication
tet’  tetracycline resistant
TMHMM  transmembrane helix motif
Tn  transposons
TSB  tryptose soy broth
W  watt
X-Gal  5-Bromo-4-chloro-3-indolyl-β-D-galactosidase
CHAPTER ONE

HAEMOPHILUS INFLUENZAE AND ANTIBIOTIC RESISTANCE

1.1. Introduction

The genus *Haemophilus* includes commensal species with a wide range of pathogenic activities. They are small, non-motile Gram-negative bacteria in the family *Pasteurellaceae* (Barrow and Feltham, 1993; Holt *et al*., 1994). The family also include *Pasteurella* and *Actinobacillus*, two other genera of bacteria that are commensals of animals.

Species in the genus of *Haemophilus* are host-specific. In humans, the *Haemophilus* spp. constitute up to 10% of the normal bacterial flora of the upper respiratory tract and are also occasionally found as normal flora of the conjunctivae and genital tract (Turk, 1982; Turk, 1984). The species of *Haemophilus* that are of clinical importance in humans are *Haemophilus influenzae*, the causative agent for meningitis and septicaemia; *Haemophilus aegypticus* causing conjunctivitis and Brazilian purpuric fever; and *Haemophilus ducreyi* which is the major cause of human genital ulcer disease (chancroid) in many developing countries. Other species of *Haemophilus* rarely infecting humans include *Haemophilus aphrophilus*, *Haemophilus haemolyticus*, *Haemophilus parainfluenzae*, *Haemophilus paraphrophilus*, and *Haemophilus segnis*. *H. parainfluenzae* is an abundant commensal species that is relatively non-pathogenic, but is rarely described as causing pneumonia or bacterial endocarditis. *H. aphrophilus* is a member of the normal flora of the mouth and occasionally causes bacterial endocarditis.

In animals, species of *Haemophilus* are host-specific; some examples include *Haemophilus somnus*, *Haemophilus parasuis* and *Haemophilus paragallinarum*. *H. somnus* infects the respiratory tract of cattle causing pneumonia, but infection in the
genital tract of a pregnant cow may cause abortion (Miller et al., 1983). *H. parasuis* and *H. paragallinarum* causes respiratory tract infection in swine and poultry, respectively.

1.2. Historical background

*H. influenzae* is the major human pathogen in the genus of *Haemophilus*. It was first characterised by Robert Koch in 1883 in patients with conjunctivitis and was identified by Pfeiffer nine years later (Killian et al., 1981). The organism was named accordingly because it was mistakenly thought to be the cause of the disease influenza during the influenza pandemic of 1890. The name ‘Haemophilus’ was given due to the fact that haemin is required for growth.

1.3. Characteristics of *Haemophilus* spp.

*Haemophilus* spp. are facultative anaerobes, non-motile, and do not form spores. The average size of the bacterium is approximately, 0.3-0.5 μm by 0.5-1.0 μm and the morphology varies from coccobacilli to pleomorphic depending upon cultural environment. As the culture ages, filamentous forms usually develop. Although *H. influenzae* stains poorly with Gram’s stain, it is Gram’s negative.

Haemophilus (meaning “loves heme”) requires a precursor of heme in order to grow. *H. influenzae* prefers a complex medium and requires preformed growth factors that are present in blood. Specifically, it requires X factor, which is supplied by iron containing pigments, and V factor, usually in the form of nicotinamide adenine dinucleotide (NAD) (Barrow and Feltham, 1993). In the laboratory, it is usually grown on chocolate blood agar and grows best at 35-37°C under slight CO₂ tension (5% CO₂). Heme is required for the growth of *H. influenzae* type B but non-typeable *H. influenzae*
NTHI strain grows in medium containing free iron and protoporphyrin IX in place of heme (Sanders et al., 1994).

Most strains of Haemophilus spp. are usually non-capsulated. Capsule is found in some types of H. influenzae, although most strains of H. influenzae are devoid of capsules. On colourless medium such as Columbia agar and Levinthal agar, iridescence can be observed in colonies of capsulated H. influenzae (Pittman, 1930).

1.4. H. influenzae genome

The H. influenzae Rd strain KW20 chromosome was the first complete genome sequence of a free-living organism (Fleischmann et al., 1995). This strain was also the source of the first DNA restriction enzyme, HindIII. The circular Rd chromosome is 1,830,137 bp long with an approximately 38% G+C nucleotide content. The sequence has 1,743 open reading frames (ORFs) of which, 63 contain frameshifts or stop codon when compared to homologues from other species and therefore, may be pseudogenes (Fig. 1.1).
Fig. 1.1. A circular representation of *H. influenzae* Rd chromosome. Outer circle: the location of each predicted coding region containing a database match (colour coded by functional grouping) as well as selected global features of the genome including a unique *NotI* restriction endonuclease site (designated as nucleotide 1). Second circle: GC content bias (red =>42%, blue =>40%, green =>36%, black =>34%). Central zone: the purple l-bars within the circle indicate the large-insert (lambda phage) clones used as a framework to map the sequence contig relative to each other. Third circle: RNA genes (ribosomal RNAs, tRNAs). Fourth circle: simple repeats the green arrows show the putative origin of replication (Fleischmann *et al.*, 1995).

**1.5.  Virulence factors**

*H. influenzae* does not produce any demonstrable exotoxins (van der Zwan *et al.*, 1978). Apparently, each step in the pathogenesis of *H. influenzae* infection depends on the expression of a combination of several specific virulence determinants. These determinants include lipopolysaccharide (LPS), pili, outer-membrane proteins (OMPs), IgA1 proteases and capsule.
1.5.1. Lipopolysaccharide

Lipopolysaccharide (LPS) is a major suprastructure of glycoprotein on Gram-negative bacteria, which contributes greatly to the structural integrity of the bacteria, and protects them from host immune defences and a major virulence determinant. A feature of *H. influenzae* LPS is that surface exposed epitopes of the oligosaccharide are subject to high-frequency on-off switching of express (phase variation). This heterogeneity may be an advantage to the bacteria allowing them to better confront different host compartments and microenvironments and to survive the host immune response.

*H. influenzae* LPS consists of two covalently linked regions, lipid A and core oligosaccharides (Richards *et al.*, 2001). The toxicity of LPS was shown to be attributed mainly to the activity of the lipid A portion. Since the lipid A portion is embedded in the outer membrane, its endotoxicity is exerted mostly when the organism is lysed. To activate host cells, the LPS binds a plasma LPS-binding protein (LBP) to form a LPS-LBP complex, which then binds the host cell receptor, CD14 (Lazou Ahren *et al.*, 2001; Wang *et al.*, 2002). CD14 then interacts with Toll-like receptor proteins culminating in the transduction of a cytoplasmic signal (Chow *et al.*, 1999; Medzhitov *et al.*, 1997). Through the activation of a complex cascade of events, the production of cytokines is triggered. The activity of cytokines and complements may lead to septic shock.

The oligosaccharide portion of LPS extends outward from the bacterial surface. Between strains of *H. influenzae*, the oligosaccharide displays significantly different patterns of sugars. The oligosaccharide can influence interactions of the bacteria with host cells and components of the host immune system (Richards *et al.*, 2001).
1.5.2. OMPs

Strains of *H. influenzae* express between 10 and 20 OMPs (Murphy and Apicella, 1987). Among all, the porin protein P2 is the most abundant in the outer membrane of *H. influenzae* type b (Murphy and Bartos, 1988). Other major OMPs are P5 and P6 proteins (Munson and Granoff, 1985). The P5, which has a 50% identity and 65% similarity to the OmpA porin of *Escherichia coli* (Munson et al., 1993), is thought to be involved in the invasion of the mucosal epithelium (Chanyangam et al., 1991), while the P6 has been showed to possess immunogenic properties and constitutes a promising vaccine candidate antigen (Murphy et al., 1992; Nelson et al., 1988).

1.5.3. Pili

Many *H. influenzae* isolates express pili (fimbriae), which mediate adherence to epithelial cells and facilitate colonisation (Guerina et al., 1982; Pichichero et al., 1982). The expression of pili in *H. influenzae* is regulated by phase variable, spontaneously switching from piliated to non-piliated and vice-versa (Connor and Loeb, 1983; Guerina et al., 1982; Pichichero et al., 1982). Generally, strains isolated from invasive infections do not express pili, while those colonising the nasopharynx more commonly do (Mhlanga-Mutangadura et al., 1998).

1.5.4. Immunoglobulin A1

*H. influenzae* and a number of other mucosal pathogens, such as *Neisseria meningitis*, *Neisseria gonorrhoeae*, and *Streptococcus pneumoniae* constitutively secretes immunoglobulin A1 (IgA1) protease (Reinholdt and Kilian, 1997), an enzyme that cleaves and inactivates human IgA1 to facilitate colonisation (Plaut, 1978).
The most important virulence factor in pathogenic *H. influenzae* is the capsule (Roberts *et al.*, 1981). Capsulated *H. influenzae* isolates are classified according to their polysaccharide capsule (Pittman, 1931). There are a total of six structurally and antigenically distinct capsular types (serotypes) designated a through f (typeable strains). Strains that lack capsule and do not agglutinate with antisera against the capsular serotypes are considered as non-capsulated (non-typeable or NTHI).

Amongst all the serotypes, serotype b is the most virulent. The polysaccharide capsule of serotype b is made up of polyribosyl ribitol phosphate (PRP) known to be the major virulence factor of *H. influenzae* (Moxon and Maskell, 1992). The capsule of *H. influenzae* has anti-phagocytic properties. This is especially prominent with the type b capsule.

The classification method of serotyping of *H. influenzae* was primarily based on serological techniques. One disadvantage of this technique is that cross-reactions between the serotypes may occur. Molecular methods using polymerase chain reaction (PCR) have been developed as a confirmative method to determine capsular type (Jordens *et al.*, 1993). The PCR method for typing the strains of *H. influenzae* are now being adopted for differentiating strains of capsulated (a-f) from non-capsulated and non-capsulated variants of capsulated strains (deletion mutation) of *H. influenzae* (Falla *et al.*, 1994; Jordens *et al.*, 1993).

All encapsulated (typeable) strains possess the cap genetic locus, a segment of approximately 18 kb comprised of a region that shows serotype specificity flanked by regions sharing homology among all capsular types (Kroll, 1992). Most types a, c-f strains possess one copy of the cap locus. In contrast, most type b clinical isolates possess two copies in tandem. The cap locus, either as the tandem duplication of most type be
strains or the single copy of strains of other capsular types, is flanked by IS-like sequences in most strains, suggesting that the region was required by a primordial strain through transposition (Kroll et al., 1991).

One of the conserved regions of the *cap* locus contains the *bex* genes, which are responsible for exporting the capsular polysaccharide (Kroll et al., 1990). The tandem duplication of the *cap* locus in type b strains is characterised by a mutation in the *bexA* gene of one copy. This duplication results in an unstable gene region, with relatively high potential for recombination events. During recombination of the *cap* locus, mutants may be formed that possess a single copy of the *cap* locus containing the *bexA* mutation, which results in non-encapsulated mutants. On the other hand, such a recombination event may generate mutants possessing additional (n) copies of the *cap* locus with n-1 copies of *bexA*. Such mutants are hyper-encapsulated and show increase pathogenicity in the infant rat model (Kroll and Moxon, 1988).

### 1.6. Pathogenesis

*H. influenzae* typically colonises human respiratory mucosal surfaces and occasionally the female genital tract (Gilsdorf et al., 1997). From the nasopharynx, organisms are transmitted from one individual to another by airborne droplets or by direct contact with secretions (Farley and Stephens, 1992). Invasion by *H. influenzae* begins with colonisation of the upper respiratory mucosa that is mediated by pili (fimbriae) (St.-Geme-III, 1994). Inability to express fimbriae results in impaired ability of *H. influenzae* to colonise the nasopharynx (Weber et al., 1991). In addition, presence of fimbriae is associated with a capacity to agglutinate human erythrocytes (van Alphen et al., 1988). The fimbrial gene cluster, is found on an 8-kb fragment of *H. influenzae* type b, but is missing in Rd strain (van Ham et al., 1994).
Fimbriated *H. influenzae* type b isolates have also been found to show efficient adhesion to mammalian extracellular matrices (ECMs) (Virkola et al., 1996), which may promote the invasion of *H. influenzae* type b strains into the circulation. The encapsulated organisms penetrate the epithelium of the nasopharynx and gain access to the blood by passing through the respiratory epithelia and vascular endothelia (Rubin and Moxon, 1983). Specific interactions are believed to occur between *H. influenzae* and the blood-brain barrier (BBB). The BBB is a single layer of unique endothelial cells, which is largely responsible for the maintenance of biochemical homeostasis within the central nervous system (Betz and Goldstein, 1986). These endothelial cells exhibit continuous tight junctions and a marked paucity of pinocytosis (Patrick et al., 1992). It is believed that the *H. influenzae* cells adhere to the BBB and then translocate across or between the cellular tight junctions, to enter the cerebral spinal fluid (CSF). Damage to the BBB enhances *H. influenzae* entry into the CSF. Once in the CSF, the population of *H. influenzae* may continue to expand and infect the meninges of the brain, causing meningitis.

The invasive type b *H. influenzae* is able to resist phagocytosis by polymorphonuclear leukocytes because of the presence of polyribosyl ribitol phosphate (PRP) capsule. The capsule is an important factor enabling its survival in the blood, and the resultant bacteraemia (Noel et al., 1992). Bacteraemia seems to precede the entry of the organisms in the CSF and meningitis (Moxon et al., 1977; Moxon and Murphy, 1978).

The less invasive non-typeable strains may induce an inflammatory response at mucosal locations, such as the middle ear, the sinuses, the conjunctiva, or the lower respiratory tract, which is thought to contribute to disease at these sites. Following
colonisation there may be local invasion of the respiratory tract by contiguous spread (St.-Geme-III, 1994).

1.7. Genetic variation between strains

Besides different capsular types, there are other classification systems that could be used to subtype *H. influenzae* strains. Typing of strains provides epidemiological information about the spread of individual clones of the organisms, which helps to identify hyper-virulent strains or distinguish between recurrent and repeated infections (Edmonson *et al.*, 1982; Kostman *et al.*, 1993). In addition, characterising bacterial populations with subtyping techniques may also aid in phylogenetic or evolutionary studies (Aparicio *et al.*, 1996). Methods frequently used for subtyping include: biotyping, whole-cell protein profiling, outer membrane protein (OMP) subtyping, multi-locus enzyme electrophoresis (MLEE) or multi-locus sequencing typing (MLST).

In the case of biotyping, *H. influenzae* strains are classified according to a series of biochemical reactions such as ornithine decarboxylase activity, indole production and urease activity. The biotypes are designated as I through VI (Killian, 1976). This classification has been superseded by superior methods.

Diversity of *H. influenzae* isolates can be studied by looking at their whole cell protein profiles. Using protein profiling, it was found that *H. influenzae* type b belongs to a subset of single clone, whereas non-typeable strains were mostly heterogeneous (Musser *et al.*, 1985; Paterson *et al.*, 1987). This technique has now been replaced by DNA sequence based methods.

Methods utilising the whole genome provide useful information for epidemiological studies (Jordens *et al.*, 1993; Jordens, 1998; Mitsuda *et al.*, 1999). These include random amplified polymorphic DNA fingerprinting, RAPD (Jordens *et al.*, 1993),
restriction fragment length polymorphism, RFLP (van Belkum et al., 1994), restriction endonuclease analysis, REA (Bruce and Jordens, 1991; Omikunle et al., 2002), ribotyping (Leaves and Jordens, 1994) and PCR-ribotyping (Smith-Vaughan et al., 1995).

Multi-locus genotyping method is based on characterising unique combinations of virulence genes or alleles of virulence genes (Musser et al., 1986; Musser, 1996). This method has shown that most *H. influenzae* type b diseases are caused by nine related clones that have no close genetic relationships to strains of other serotype (Musser et al., 1990).

The most recently developed typing scheme is multi-locus sequencing typing (MLST). This scheme provides an unambiguous characterisation of both encapsulated and non-capsulated isolates of *H. influenzae*. This typing was developed based from the principles of multi-locus enzyme electrophoresis (MLEE), but assigns alleles at multiple housekeeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products (Maiden et al., 1998; Spratt, 1999). Although both MLEE and MLST are capable of differentiating between capsulated strains from non-capsulated strains, MLST is able to show related lineages between encapsulated strains of *H. influenzae* (Meats et al., 2003). Through this scheme, it was found that recombination appeared to have more impact on the divergence of non-capsulated isolates compared to encapsulated isolates. This could be due to their increased ability to be transformed and/or increased opportunities for non-capsulated isolates to meet other *H. influenzae* within the nasopharynx (Meats et al., 2003).

### 1.8. Clinical manifestations of *H. influenzae* infection

Diseases caused by *H. influenzae* infection can be classified as invasive infections and non-invasive infections (Table 1.1). The invasive infections are usually associated with
serotype b, whereas the non-invasive are usually caused by non-typeable (NTHI) *H. influenzae*.

**Table 1.1. Important infections caused by *H. influenzae***

<table>
<thead>
<tr>
<th>Invasive infection</th>
<th>Non-invasive infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteraemia</td>
<td>Conjunctivitis</td>
</tr>
<tr>
<td>Epiglottitis</td>
<td>Otitis media</td>
</tr>
<tr>
<td>Meningitis</td>
<td>Respiratory disease (chronic bronchitis)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td></td>
</tr>
</tbody>
</table>

*a90% caused by *H. influenzae* type b, b mostly caused by untypeable strains*

### 1.8.1. Serotype b infections

Amongst all the encapsulated strains of *H. influenzae*, serotype b is found to be the most virulent and most invasive. Studies confirmed that *H. influenzae* type b strain was responsible for approximately 85% of all invasive *H. influenzae* disease in children (Booy *et al.*, 1993; Wenger *et al.*, 1992). It was the leading cause of bacterial meningitis and other systemic diseases in infants and young children under the age of 5 years (Turk, 1984; Uduman *et al.*, 2000).

Epidemiological data of 3,931 patients from 21 countries worldwide reported that meningitis was the leading disease manifestation of *H. influenzae* type b (Fig. 1.2) (Peltola, 2000). Other diseases caused by type b *H. influenzae* include epiglottitis, cellulitis, osteomyelitis, and arthritis in older children and also adults (Turk, 1984).

25
Fig. 1.2. Worldwide spectrum of *H. influenzae* type b disease (non-bacteraemia pneumonia excluded). Data collected in the 1970s to 1990s from 3,931 patients in 21 studies from various parts of the world (Peltola, 2000).

### 1.8.2. Infections caused by non-type b serotypes

The other *H. influenzae* serotypes, a, c, d, e and f, have been shown to be associated with invasive disease at low frequency (Musser *et al.*, 1990). Non-type b strains rarely cause serious diseases in children (Heath and McVernon, 2002). Amongst all serotypes, the serotype f is regarded to be the most common cause of the non-type b invasive infections (Gonzalez Lopez *et al.*, 2000; Urwin *et al.*, 1996). Over a period of six years from 1989 to 1994, the incidence of invasive *H. influenzae* due to serotype f rose from 1% to 17% with overall mortality of 30% among adults and 21% among children (Urwin *et al.*, 1996).

### 1.8.3. Infections associated with non-typeable strains

Non-capsulated or non-typeable *H. influenzae* strains are normally commensal organism in the human nasopharynx and occupy this niche as their natural habitat (Howard *et al.*, 1988; Lerman *et al.*, 1979). Their principle association is with otitis media in children. They are found in the middle ear fluid of over a third of cases with otitis media undergoing tympanocentesis. They may also cause acute or chronic sinusitis in patients of all ages, and occasionally, causes meningitis in adults (Makela, 1988). Another disease with which NTHI are associated is chronic bronchitis. They are commonly isolated from
the bronchial secretions of such cases. The bacteria can persist in lower respiratory tract of these patients for months and appear to escape immunological defence because the major outer membrane protein changes during persistence (Groeneveld et al., 1988).

1.9. Control and prevention of *H. influenzae* infection

1.9.1 Host defences

Exposure to the organism induces the host to produce antibodies against the capsule and antigens. These antibodies are both bactericidal and promote phagocytosis and are highly effective in preventing infection. During the first six months of life, infants are protected against *H. influenzae* infection due to maternal IgG antibodies. However, children between six-months and five years are most susceptible to *H. influenzae* type b infection. Healthy children above the age of 5 years are rarely infected. The presumed reason for this age distribution is the acquisition of humoral immunity to *H. influenzae* type b with increasing age. This protection is most likely acquired as a result of sub-clinical infection or asymptomatic colonisation. It has been reported that, in addition to acquired humoral immunity involving bactericidal antibody, innate immunity mediated by C-reactive protein together with complement is sufficient to kill *H. influenzae* (Weiser et al., 1998).

1.9.2 Immunisation

Outbreaks of serious infection due to *H. influenzae* type b can be prevented by vaccination or prophylactic therapy. In 1985, the first *H. influenzae* type b vaccine was prepared from purified PRP capsular material of the bacteria (Anderson et al., 1977). This PRP vaccine consists of type b capsular polysaccharide that elicits a strong primary antibody response. This vaccine, however, was poorly immunogenic in young children
and did not provide long-term immunity. It was not efficacious in children under 18 months of age (Peltola et al., 1977).

In 1991, a polysaccharide-diptheria toxoid (PRP-T) conjugate *H. influenzae* type b vaccine was developed. This vaccine consists of PRP conjugated to a tetanus toxoid protein, which induces persistent antibody level in children and proved to be more effective than the earlier vaccine (Eskola et al., 1987).

Routine immunisation was introduced in the UK in 1992. With the implementation of the vaccination programme, the incidence of invasive disease declined dramatically and remained at very low rates until 1998 (Heath and McVernon, 2002). Despite the 93% immunisation uptake rate, the *H. influenzae* type b continues to be a public-health threat in the UK (Garner and Weston, 2003; Pushparajah et al., 2003). In 1999, *H. influenzae* type b disease had started to increase again in children, with the greatest number of cases occurring in vaccinated children (Fig. 1.3) (http://www.hpa.org.uk/infections) (Ramsay et al., 2003). The reduction in vaccine effectiveness partly coincided with the introduction to the UK of a new vaccine (combined Hib and acellular pertussis) (McVernon et al., 2003). Factors such as vaccine uptake rate in immigrant populations, effectiveness of conjugate vaccines, effect on individual immunity and immunological memory, carriage rates, and herd immunity could have contributed to the immunisation failure (Heath and McVernon, 2002).
Fig. 1.3. Prevalence of *H. influenzae* type b reported to Communicable Disease Surveillance Centre (CDSC) for England and Wales, 1990-2002.

1.10. **Antibiotic resistance in *Haemophilus* spp.**

1.10.1. **Changing trends in antimicrobial susceptibility**

In many countries, over-prescription of antibiotics and increasing use of disinfectants for routine hygiene and heavy use of antibiotics for veterinary medication and/or growth promotion in agriculture, aquaculture and animal husbandry have been shown to contribute to the selection pressure for antibiotic resistance bacteria (Guillemot, 1999; Okeke and Edelman, 2001; Watson *et al.*, 1999).

It is assumed that as a result of increased exposure to antibiotic for treatment of respiratory infections (mainly otitis media), *H. influenzae* is exhibiting increased antibiotic resistance (Fallon, 1982; Gessner, 2002; Philpott-Howard and Williams, 1982; Williams and Moosdeen, 1986). Resistance to chloramphenicol and ampicillin amongst *H. influenzae* isolates has risen since the 1970’s to the extent that neither antibiotic can be recommended as a first-line therapeutic agent for meningitis (de Groot *et al.*, 1991; Jorgensen, 1992). Fortunately, *H. influenzae* is almost uniformly susceptible to third
generation cephalosporins, but there is always a possibility that it will acquire broader antibiotic resistance (Cohen, 1992).

1.10.2. Prevalence of antibiotic resistant H. influenzae

The increasing prevalence of antibiotic resistance in *H. influenzae* is recognised worldwide. The first report of ampicillin resistant in *H. influenzae* was in 1972 (Mathies, 1972). Between 1977 and 1981, the prevalence of ampicillin resistance increased from 1.6% to 6.6% (Philpott-Howard and Williams, 1982). However, over the past decade, the level of resistance to ampicillin has not changed significantly (PHLS, 2002). Up to 2000, ampicillin was the most common antibiotic to which *H. influenzae* type b is resistant (Table 1.2).

Subsequent reports showed emergence of multi-drug resistant *H. influenzae* isolates. Clinical isolates *H. influenzae* resistant to a variety of antimicrobial agents such as ampicillin, chloramphenicol, tetracycline and cotrimoxazole has been reported in many countries (Baquero, 1996; Jorgensen, 1992).

<table>
<thead>
<tr>
<th>Year</th>
<th>n</th>
<th>Resistant (%)</th>
<th>β-lactamase +ve (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amp (%)</td>
<td>Chlor (%)</td>
</tr>
<tr>
<td>1992</td>
<td>610</td>
<td>118</td>
<td>19%</td>
</tr>
<tr>
<td>1993</td>
<td>252</td>
<td>51</td>
<td>20%</td>
</tr>
<tr>
<td>1994</td>
<td>84</td>
<td>25</td>
<td>30%</td>
</tr>
<tr>
<td>1995</td>
<td>77</td>
<td>10</td>
<td>13%</td>
</tr>
<tr>
<td>1995</td>
<td>62</td>
<td>14</td>
<td>23%</td>
</tr>
<tr>
<td>1997</td>
<td>69</td>
<td>13</td>
<td>19%</td>
</tr>
<tr>
<td>1998</td>
<td>50</td>
<td>19</td>
<td>38%</td>
</tr>
<tr>
<td>1999</td>
<td>68</td>
<td>14</td>
<td>21%</td>
</tr>
<tr>
<td>2000</td>
<td>113</td>
<td>22</td>
<td>19%</td>
</tr>
</tbody>
</table>

*a*ampicillin, *b*chloramphenicol, *trimethoprim. Source: Haemophilus Reference Unit
1.10.3. **Medical impact of antibiotic resistance**

Antibiotic resistance is increasingly regarded as a major problem complicating the management of *H. influenzae* infections. Infections caused by resistant microbes respond poorly to treatment resulting in an increase in morbidity and even mortality of patients. Antibiotic resistance also results in an increase in the cost of medical care (e.g. resulting from prolonged hospitalisations and the use of more expensive antibiotics).

1.11. **Detection of antibiotic resistance**

Antibiotic resistance in bacteria is most commonly detected by standard laboratory investigations to establish the best choice of treatment. Some of the methods used for detection of antibiotic resistance include: minimal inhibition concentration (MIC) (Barry, 1986); E-Strip test (AB Biodisk, Solna, Sweden); nitrocefin chromogenic cephalosporin test for detection of β-lactamase (Boughton, 1982); iodometric detection (Catlin, 1975), penicillinase paper strip (Jorgensen et al., 1977); chloramphenicol CAT assay; and acidometric method (Scheifele et al., 1976). Besides these methods, PCR detection of β-lactamase provides an important supplement or confirmation to phenotypic methods (Tenover et al., 1994).

1.12. **Mechanisms of antibiotic resistance**

Generally, antibiotic resistance mechanisms in bacteria can be subdivided as follows:

a. Loss or modification of site of action (e.g. point mutation)

b. Efflux pump (e.g. tetA)

c. Enzymatic inactivation of antibiotics (e.g. β-lactamase).

In multi-drug resistance *Haemophilus* spp. strains, more than one of the mechanisms could be present in a single bacterium.
1.13. Ampicillin resistance in *H. influenzae*

Ampicillin resistance in *H. influenzae* is could be due to either one of these mechanisms: plasmid-mediated production of TEM β-lactamase, altered penicillin-binding proteins, and/or diminished permeability (de Groot *et al.*, 1991). The predominant mechanism for *H. influenzae* resistance to ampicillin in clinical isolates is clearly due to β-lactamase production (Gutmann *et al.*, 1988; Heffron *et al.*, 1975).

1.13.1. Types of β-lactamases in *H. influenzae*

β-lactam antibiotics include penicillin, ampicillin and cephalosporins. The enzyme β-lactamase produced by *H. influenzae* hydrolyses penicillin antibiotics by cleaving one bond of the four-membered ring of the β-lactams. The open-ringed structure of the penicillin is incorporated into the cell wall but sufficiently different from the normal pentapeptide that transpeptidase does not recognise it as a substrate. The cleavage product of penicillin by β-lactamase is different enough that it is not used as a substrate in cell wall synthesis. Hence, the cell is not weaken by the antibiotic thus allowing normal growth (Quintiliani and Courvalin, 1995).

Two types of β-lactamases have been identified in *H. influenzae*; TEM-1 β-lactamase and ROB-1 β-lactamase (Gutmann *et al.*, 1988). ROB-1 β-lactamase produces a plasmid-determined enzyme with broad-spectrum activity like that of TEM-1 but with an isoelectric point (pI) of 8.1 (quite different from the TEM-1 pI of 5.4) (Rubin *et al.*, 1981). These two β-lactamases share limited amino acid sequence homology and have similar substrate profiles and relative rates of ampicillin hydrolysis (Bush, 1989). Ampicillin resistant *H. influenzae* generally produce either one of the two β-lactamases, but rare isolates produce both β-lactamases (Daum *et al.*, 1988; Scriver *et al.*, 1994).
Although both of the genes code for ampicillin-resistance, TEM-1 and ROB-1 enzymes are thought to be unrelated.

1.13.2. TEM β-lactamase

There are 43 naturally occurring TEM β-lactamase derivatives (Bush and Jacoby, 1997). The TEM-1 β-lactamase is the most commonly found in *H. influenzae* (Elwell *et al.*, 1975). TEM-1 β-lactamase is encoded by the *bla* gene. The structural gene for the β-lactamase was considered to reside on a plasmid genome (Heffron *et al.*, 1975; Laufs and Kaulfers, 1977). A plasmid of *H. influenzae* strain G32 (RSF007) was found to share about 48% with the TEM-type β-lactamase transposon, *TnA* (Elwell, 1994).

1.13.3. ROB β-lactamase

Most of the ampicillin-resistant *H. influenzae* isolated in the USA were found to possess ROB-1 enzymes (Daum *et al.*, 1988). ROB-1 enzymes were believed to have originated from *Actinobacillus pleuropneumoniae*, a pathogen that is frequently isolated from pigs (Juteau *et al.*, 1991; Livrelli *et al.*, 1988; Medeiros *et al.*, 1986). The ROB-1 enzyme is most commonly encoded by small non-conjugative R plasmids prevalent in *Enterobacteriaceae* (Brunton *et al.*, 1986; Medeiros and O’Brien, 1975). Plasmids with ROB-1 enzymes in *Pasteurella haemolytica* isolated from cattle and sheep is believed to have originated from *H. influenzae* (Azad *et al.*, 1992).

1.14. Tetracycline resistance in *H. influenzae*

Tetracycline resistance in *Haemophilus* spp. was initially reported in 1975 (Van *et al.*, 1975). Since then, numerous *Haemophilus* spp. with this resistance have been isolated (Kaulfers *et al.*, 1978; Powell *et al.*, 1992). Presently, tetracycline resistant *H. influenzae*
is commonly isolated. In UK, it ranks second (2.7%) as the most frequent antibiotic resistance after ampicillin (7.8%) (Powell et al., 1987). In several of these resistant strains, it was found that the resistance appeared to reside on transposons, \( \text{TnD} \) (now recognised as \( \text{Tn10} \)) on a resident plasmid (Elwell et al., 1977, Kaulfers et al., 1978).

Resistance to tetracycline often occurs as a result of energy-dependent pumping of the antibiotic from the cell so that the levels of antibiotics are decreased and the ribosome is not inhibited (Speer et al., 1992). Antibiotic efflux is mediated by the Tet membrane proteins, which use an antiport mechanism of transport involving the exchange of a proton for a tetracycline-cation complex (McMurry et al., 1980).

1.15. Chloramphenicol resistance in \textit{H. influenzae}

Resistance to chloramphenicol was first reported in 1972 (Barrett et al., 1972). Since then, increasing numbers of \textit{H. influenzae} type b are resistant to chloramphenicol have been reported (Kinmonth et al., 1978; Simasathien et al., 1980; Uchiyama et al., 1980). In England and Wales, 1% of the \textit{H. influenzae} type b isolated in 2000 was resistant to chloramphenicol (Table 1.2).

Most chloramphenicol resistance in \textit{H. influenzae} is associated with chloramphenicol acetyltransferase production which enzymatically inactive the antibiotics (Powell and Livermore, 1988; Roberts et al., 1980). Although several investigators believed that chloramphenicol acetyltransferase, \( \text{cat} \) gene was plasmid-borne (Roberts et al., 1980; Smith and Kelsey, 1989; van Klingerren et al., 1977), others believed that the resistance genes were integrated into the chromosome (Mendelman et al., 1984, Powell et al., 1988, Stuy, 1980b).
1.16. Resistance to other antimicrobials

1.16.1. Kanamycin resistance

To date, *H. influenzae* isolates resistant to kanamycin alone have not been reported. All the kanamycin resistant *H. influenzae* were found in association with ampicillin-chloramphenicol or ampicillin-chloramphenicol-tetracycline resistance. Resistance to these antibiotics was encoded by a 37 to 44-MDa conjugative plasmid (Levy et al., 1993).

1.16.2. Trimethoprim resistance

*H. influenzae* resistant to trimethoprim-sulfamethoxazole have also been reported to occur worldwide (Sahm et al., 2000). Resistance to trimethoprim was shown to be due to both permeability changes and to a mutation in the chromosomal dihydrofolate reductase gene (de Groot et al., 1988).

1.16.3. Multi-drug resistance

Bacteria can develop resistance to many antibiotics. Multi-resistance may develop by chromosomally located resistance determinants or mutations in a resident gene; however, it may also develop through the acquisition of resistance genes or an array of resistance genes by horizontal transfer (Rowe-Magnus and Mazel, 1999).

In multi-resistant *H. influenzae* isolates, especially those resistant to both tetracycline and chloramphenicol, resistance epitopes were found to be encoded on a single plasmid and could frequently transfer as a single unit (Mendelman et al., 1984; Roberts et al., 1980; van Klingeren et al., 1977).
1.17. **Mobile genetic elements**

Bacteria acquire and transfer genetic information as an important mechanism to survive in hostile environments including those containing antibiotics. The acquisition of resistance genes generally involves transfer on mobile elements such as transposons, integrons and plasmids (Gomez-Lus, 1998; Jahn et al., 1979; van Klingeren et al., 1977). These genetic mobile elements are not only able to transfer genetic information to and from within the chromosome, but they are also able to exchange information with other self-replicating genetic elements within the cell (plasmids and phages) (Dionisio et al., 2002). This exchange of information can also occur between strains and species and involves more than just resistance genes (Levy et al., 1993). These elements play important roles in the bacterial evolution, providing mechanisms to generate diversity and for the dissemination of DNA among other bacteria (Gomez-Lus, 1998; Ochman et al., 2000).

1.18. **Transposon mediated resistance**

Transposons, or ‘jumping genes’ are known to be responsible for the dissemination of genes encoding resistance to antibiotics and heavy metals and genes that degrade chemicals (Osborn and Boltner, 2002). Transposons are characterised by the presence of short terminal sequences (10-50 bp) at their two ends. These usually form part of flanking repeat sequences known as insertion elements (IS). The size of IS elements usually range between 800 to 1800 bp. Generally, IS elements do not encode any function other than a transposase (Tnp) gene (Chandler and Mahillon, 2002). Transposase recognises inverted tandem repeats (ITRs), and uses this for transposition. They can transposase/integrate into numerous non-homologous sequences of DNA, and are capable of inserting at multiple sites in a target DNA. They can also move from one plasmid to another, and in both directions between plasmids and chromosomes in a manner that does not require the
recombination functions of the bacteria. Many transposition events cumulatively result in resistance plasmids (Gomez-Lus, 1998).

Transposons can either be composite mobile elements or non-composite mobile elements. Composite transposons contain other gene(s) for survival and adaptation (e.g. antibiotic resistance genes) that is (are) flanked by IS elements. The IS elements provides transposase actively, although one of the copies is often inactive. An example of composite transposons is Tn10 (Chalmers et al., 2000). The non-composite transposons, such as Tn3 do not have insertion sequences at their extremities but only consist of the inverted repeats necessary for recognition by the transposase. All the activities required for transposition, including the transposase and resolvase, are encoded by sequences in the element (West and Tang, 2000).

11.8.1. Tn10

Tn10 is a Class I, 9,147-kb composite transposon, consisting of a central element of about 6,700 bp of non-repeated sequences that specifies resistance to tetracycline. Both ends of the transposon have inverted repeats of 1,329-bp representing the insertion sequence, IS10 (Fig. 1.4) (Chalmers et al., 2000; Lawley et al., 2000). The IS10-right is a fully functional insertion that can transpose as an individual unit but the IS10-left is inactive because it specifies a non-functional transposase protein (Foster et al., 1981; Kleckner, 1989).

To date, there are only two genes involved in expressing resistance. They are tetA and tetR. The tetA is a membrane-bound efflux pump and tetR functions as its repressor (Hillen and Berens, 1994). The Tn10 carries other additional structural genes, tetC and tetD of unknown functions (Kleckner, 1989). The tetD gene product is membrane-associated and inducible by tetracycline, however, no role for tetC is known.
An example of a transposon commonly found in antibiotic resistance bacteria is the ampicillin resistance transposon, Tn3. It is 4,957 kb in size and contains two genes for transposition and one for ampicillin resistance, TEM-1 β-lactamase gene (Roy, 1999). Transposon Tn3, is a non-composite transposon that does not have insertion sequences at either extremity, but it has the inverted repeats necessary for recognition by transposase (Fig. 1.5). The inverted repeats are generally 38-bp long, with an outer sequence starting with GGGG and terminating internally with TAAG (Chandler and Mahillon, 2002; Takeya et al., 1979). In this thesis, the features of Tn3 and Tn10 present on mobile elements of H. influenzae will be considered further in the results chapters.

Fig. 1.4. Transposon Tn10. IS10-left and IS10-right flank the tetR, tetA, tetC, and tetD genes. The short arrows at the termini of the IS10 elements indicate nearly perfect inverted repeat sequences. Open arrows within the tet genes indicate the direction of transcription; the solid arrow within IS10-Right indicates transcription of the transposase gene; the broken arrow within IS10-left indicates transcription of the defective transposase genes of this element. This figure was adapted from Mobile DNA (Kleckner, 1989).

1.18.2. Tn3

Fig. 1.5. Genetic organisation of Tn3 family transposons. tnpA gene is the transposase and tnpR gene is the repressor and resolvase. The crosshatched box is the res site.
1.19. **Conjugative and non-conjugative plasmids**

Plasmids are autonomously replicating extra-chromosomal DNA elements, distinct from the normal bacterial genome and nonessential for cell survival under non-selective conditions (Hardy, 1986). Plasmids play important roles in bacterial survival and diversity. In order to continue their existence and adapt to new environments, a plasmid will have to acquire the ability to survive and propagate, which includes replication, partitioning, post-segregational killing and conjugative transfer as shown in Fig. 1.6 (Thomas, 2000).

In general, bacterial plasmid replicate independently of the host chromosome, although usually they rely on some host-encoded factors for their replication. Plasmid partition systems, one of the maintenance mechanisms, are normally plasmid encoded, although host cellular functions may be required (Austin, 1988; Williams and Thomas, 1992). The mechanism of partition apparently involves the recognition of pairs of plasmid molecules and membrane proteins followed by an active process of separation of the members of the pair to the opposite halves of the dividing bacterial cell (Firshein and Kim, 1997; Thomas, 2000). Unlike in plasmid, the regions of origin of the daughter nucleoids were anchored to the cell membrane at a central position of the cell (Draper and Gober, 2002).

Conjugative plasmid refers to a self-transmissible plasmid that carries genes that can initiate conjugation with another bacterium. Examples of conjugative plasmids include, F plasmid and RSF1010 (Zechner et al., 2000), a 50-kb pRE25, multi-resistance plasmid from Enterococcus faecalis RE25 (Schwarz et al., 2001) and a 32-kb pF3028, isolated from H. aegyptius (Kroll et al., 2002). These plasmids carry genes for replication, conjugation (tra genes) and also genes for mobilization of smaller plasmids. They do not have genes for integration into host chromosome. In contrast, pBtoxis, a toxin-coding
megaplasmid (127-kb) of *Bacillus thuringiensis* subsp. *israelensis* has genes for replication but does not have *tra* genes (Berry *et al.*, 2002). This is one example of a plasmid that is non-conjugative.

Conjugative plasmids should not be confused with conjugative elements because conjugative elements are not able to replicate autonomously even though they can integrate into the host’s chromosome (Salyers *et al.*, 1995). One example is CTnDOT, a conjugative transposon from *Bacteriodes sp.*, which self-transfers, integrate and excise using recombinase genes not related to known conjugation plasmids (Bonheyo *et al.*, 2001; Cheng *et al.*, 2000). Another such element is the SXT element found in *Vibrio cholerae*. SXT is different from CTnDOT because of their unrelated conjugative transfer genes. In contrast to conjugative plasmids, the genome of SXT did not contain genes for autonomous replication or segregation, but its genes for the chromosomal recombination resembles those of plasmids (Beaber *et al.*, 2002; Hochhut and Waldor, 1999).

A group of elements that excise and integrate like prophages, and transfer by conjugation like plasmids, has been proposed and is the integrative and conjugative elements (ICEs) (Burrus *et al.*, 2002a). These elements are characterised by their ability to excise by site-specific recombination, transfer by conjugation and recombine into their host’s chromosome at a specific site. An example is a 35-kb ICEStI, an element of *Streptococcus thermophilus*, which encodes integrative and conjugative functions (Burrus *et al.*, 2002b). Since nomenclature for ICEs has only recently been suggested, some ICEs were initially described as plasmids or transposons and many such elements have not been renamed.
1.20. Resistance plasmids (elements) in *H. influenzae*

The sudden emergence of ampicillin resistance mediated by β-lactamase in *H. influenzae* was encoded by large conjugative plasmids (Elwell *et al.*, 1975; Elwell *et al.*, 1977). Ampicillin-sensitive Haemophilus strains, by contrast, did not contain plasmid DNA (Elwell *et al.*, 1975; Roberts and Smith, 1980). Similar sized plasmids were also found in tetracycline resistant *H. influenzae* (Jahn *et al.*, 1979; Marshall *et al.*, 1984).

Molecular studies performed on resistant strains of *H. influenzae* showed that antibiotic resistance is usually encoded by genes located on one of two types of plasmids (de Graaff *et al.*, 1976). Large plasmids (>45 kb) were responsible for carrying antibiotic resistance genes in *H. influenzae* (Elwell, 1994). Some large plasmids also encoded genes for multi-drug resistance. Besides β-lactamase that encoded ampicillin resistance, they
carried genes for resistance to tetracycline and chloramphenicol (Brightman et al., 1990; Brunton et al., 1986; Campos et al., 1989; de Groot et al., 1991; Dimopoulou et al., 1992; Mendelman et al., 1984).

Generally, small (<10 kb) plasmids in H. influenzae only code for TEM β-lactamase gene (Brunton et al., 1986). Laufs et al (1979) found that the structural gene for the β-lactamase was located on a 4.4-kb plasmid (Laufs et al., 1979), while another 4.4-kb plasmid was found to harbour ROB-1 β-lactamase (Rubin et al., 1981).

Cryptic plasmids have been found in some Haemophilus strains. The function of these plasmids is still unknown. Some researchers believe that resistance plasmids in H. influenzae were originally cryptic plasmids that have acquired resistance genes from H. parainfluenzae (Laufs et al., 1981).

1.21. Transfer of plasmids

Horizontal transfer of resistance genes is a successful mechanism for the transmission and dissemination of antibiotic resistance among bacteria including pathogens. Three major mechanisms, transformation, transduction and conjugation have been recognised (Davison, 1999).

1.21.1. Transfer by transformation

Transformation involves the direct uptake of exogenous DNA. H. influenzae has an efficient natural competence system whereby the cells are able to take up DNA from the environment without the direct interaction of the donor and recipient cells due to the presence of uptake sequences (Danner et al., 1980; Goodgal and Mitchell, 1990; Smith et al., 1999). Transformation was not thought to be not the mechanism for transfer of
antibiotic resistance determinants, because the *H. influenzae* was Rec independent and was resistant to DNAs (Dimopoulou *et al.*, 1992; Saunders and Sykes, 1977).

### 1.21.2. Transfer by conjugation

Conjugative transfer is the process whereby a DNA molecule is transferred from a donor to a recipient bacterium via a specialised protein complex (Zechner *et al.*, 2000). This process usually requires close physical contact between donor and recipient bacteria, which is usually mediated by plasmids and transposons.

The transfer of antibiotic resistance genes in *Haemophilus* spp. is through conjugation of large plasmids (elements) that are chromosomally integrated (Farrar and O'Dell, 1974; Laufs and Kaulfers, 1977; Stuy, 1980b). Thorne and Farrar (1975) showed that *H. influenzae* transferred an ampicillin resistance determinant to a recipient cell through the process of conjugation (Thorne and Farrar, 1975). Transfer of resistance plasmids through conjugation requires cell-to-cell contact but does not occur in suspension (Stuy, 1980b). Dimopoulou *et al.* (1992) confirmed that transfer of resistance determinants between *H. influenzae* strains occurred by conjugation rather than by transformation (Dimopoulou *et al.*, 1992). Apart from ampicillin, chloramphenicol, tetracycline and kanamycin resistance, genes encoding resistance to other antibiotics (e.g. trimethoprim) could not be transferred by conjugation (Dimopoulou *et al.*, 1992; Elwell *et al.*, 1977; van Klingerden *et al.*, 1977).

Transfer of plasmids between different strains of *H. influenzae* and *H. parainfluenzae* by conjugation have been also reported. Beta-lactamase specifying strains of *H. parainfluenzae* can conjugally transfer their plasmids to *H. influenzae* recipients (Scheifele and Fussell, 1981a; Scheifele *et al.*, 1982). As a result of this observation, Scheifele and Fussell (1986) postulated that *H. parainfluenzae* could play a role as a
vector in the spread of resistance genes to *H. influenzae* (Scheifele and Fussell, 1986). Leaves et al. (2000) found that the sequences detectable by PCR for β-lac\(^+\) amp\(^{r}\) in *H. parainfluenzae* were similar to a family of large plasmids in *H. influenzae* and is consistent with the idea that *H. influenzae* plasmids with β-lac\(^+\) amp\(^{r}\) have originated from the population of *H. parainfluenzae* (Leaves et al., 2000).

Conjugative plasmid transfer between strains of the same serotype was efficient, but was less efficient when transfer was between different serotypes. According to Stuy (1980), large resistance plasmids transfer less efficiently (~10\(^{-5}\)) between *H. influenzae* strains, but transfer between different serotypes are even more inefficiently (Stuy, 1979, 1980b). He reported that conjugation efficiency was unaffected by whether the strains are rec\(A^{-}\) or rec\(A^{+}\). Presence of host capsule and the types of restriction enzymes in the host also do not affect conjugation efficiency (Stuy, 1979).

### 1.22.3. Transfer by transduction

Transduction is the process of DNA transfer mediated by bacteriophage. Fragments of bacterial chromosome are incorporated into phage heads and are transduced into new host cell. The transfer of antibiotic resistance genes by transduction in *Haemophilus* spp. has not been shown. Furthermore, the presence of an efficient conjugative system for transferring resistance genes makes transduction an improbable means of explaining antibiotic resistance gene transfer (Saunders and Sykes, 1977).

### 1.23. Restriction and modification system

Regardless of how DNA enters a recipient cell, once inside the cell, the foreign DNA may either survives (i.e. replicate and/or recombine with the recipient DNA) or, it may be degraded by restriction endonuclease enzymes. If the DNA survives, it is due to the
modification activity of the donor cell accomplished by sequence specific methylase enzymes. In any cell with a restriction-modification system, both the restriction and modification enzymes have the same sequence specificity (Lewin, 1990).

In *H. influenzae* strain Rd, methylase II and methylase III were found to protect *H. parainfluenzae* transforming DNA from inactivation by *H. influenzae* endonuclease enzymes (Roszczyk and Goodgal, 1975) which suggests that DNA of *H. parainfluenzae* could be transferred into *H. influenzae* without being cleaved by *Hind* II and *Hind* III.

### 1.24. Site-specific recombination of *H. influenzae* plasmids

Plasmids in *H. influenzae* are found integrated in the host’s chromosome (Murphey-Corb *et al.*, 1984; Stuy, 1980a). Therefore, usual plasmid isolation procedures are not able to detect extra-chromosomal DNA from clinical isolates. However, following conjugative transfer, integrated plasmids in clinical isolates could be readily detected as closed circular plasmid in recipient (Dimopoulou *et al.*, 1992; Overturf *et al.*, 1987).

Dimopoulou *et al.* have found common sequence (66-bases) structures on the plasmid/element (*attP*) and on host chromosome (*attB*), indicative of site-specific integration (Dimopoulou *et al.*, 2002), see Fig. 1.7 (Leaves *et al.*, 2000). The *attP* of the closed circular element was the point of re-circularisation. Once integrated on the chromosome, the repeat sequences were located at the left (*attL*) and right (*attR*) junctions of the insert. These 66-base sequences were also identical to the 3’ of tRNA\textsubscript{leu}, which suggest that the plasmid was integrated on the chromosome at the tRNA\textsubscript{leu}. These *att* sites are shared *attP* of HP1 bacteriophage of *H. influenzae* (Dimopoulou *et al.*, 2002). The conservation of *att* sites between the bacterium, plasmid and bacteriophage suggests that they possibly recombine site-specifically by similar mechanisms, which require an
integrase (*int*). DNA binding protein (IHF) contributes to recombination by promoting the binding of *int* to the *att* sites (Astumian *et al.*, 1989; Craig, 1988).

![Diagram of plasmid and chromosomal PCR primer attachment sites.](image)

**Fig. 1.7.** Plasmid and chromosomal PCR primer attachment sites. The orientation at their respective sites on the chromosomes, closed circular (excise) plasmid and plasmid recombined (integrated) with the chromosome; *attB*, chromosomal attachment site; *attP*, plasmid attachment site; *attL*, left plasmid chromosomal junction; *attR*, right plasmid chromosomal junction (taken from Leaves *et al.* (2000) (Leaves *et al.*, 2000).

### 1.25. Excisive recombination of plasmid

Excisive recombination of an integrated plasmid is likely to involve recombination between *attL* and *attR* to generate intact *attP* and *attB* sites. The excisive site-specific recombination of the temperate bacteriophage, HP1, has been studied (Craig, 1988). Excisive recombination requires *int*, IHF and the Cox protein (Esposito and Scocca, 1994, 1997a). The Cox protein is similar to the Xis protein of *E. coli* phage P2, inhibits the integrative recombination of *attP* and *attB* *in vitro*, and activates the excisive recombination between *attL* and *attR* (Esposito and Scocca, 1994).

### 1.26. Origin of resistance genes and conjugative plasmids

Several investigators suggested that large conjugative plasmids/elements encoding antibiotic resistance arose as a result of the transposition of resistance genes onto closely related cryptic plasmids/elements that had a common evolutionary origin (Elwell *et al.*, 1997).
1975; Laufs et al., 1981; Saunders and Sykes, 1977). Laufs et al. (1981) speculated that the multi-resistance plasmids of *H. influenzae* that have emerged in different parts of the world were of multi-clonal evolutionary origin (Laufs et al., 1981).

The genetic relatedness of multi-resistance plasmids (ampicillin-chloramphenicol-tetracycline-kanamycin) of *H. influenzae* isolated in Belgium and Spain was investigated (Levy et al., 1993). Homoduplex analysis showed that they had similar molecular organisation and identical structures, indicating that plasmids in epidemiologically unrelated strains shared a high proportion of shared core sequences. Ampicillin resistant plasmids isolated in Alaska, though different from ampicillin resistant plasmids in the United States, have nevertheless been reported to share significant DNA homology (Mendelman et al., 1985). These observations led to a suggestion that a common genetic element was responsible for the dissemination of this type of resistance in Alaska.

1.27. **Role of plasmids in gene transfer and bacterial evolution**

Mobile elements, particularly plasmids, have been shown to play important roles in horizontal (or lateral) gene transfer and bacterial evolution. When bacterial genes are transferred between different organisms, chromosomal genes may also be transferred in addition to those forming the mobile elements. Most mobile elements also carry genes that confer potentially useful traits on their bacterial hosts. They rarely carry genes that are absolutely essential for the host bacteria, but frequently carry genes that could improve the ‘fitness’ of the host under certain circumstances (van Elsas et al., 2000).

Some plasmids are highly promiscuous and have broad host ranges. This characteristic assists the exchange of genes between different genera or even transfer to different kingdoms (Dionisio et al., 2002; Groisman et al., 1992). They mediate gene transfer between organisms dwelling in various environments including plants, (Arnold et
Genetic elements referred to as genomic islands (GIs) that are capable of horizontal transfer and integration into host chromosomes are key agents in the prokaryotic genome evolution. Pathogenicity islands (PAIs) are specifically referred to as a class of GIs carrying specific gene clusters contributing to a virulence phenotype, e.g. adherence factors, toxins, iron uptake systems, Type III and Type IV secretion systems (Dobrindt et al., 2004; Hacker and Kaper, 2000; Hentschel and Hacker, 2001). PAIs are usually found on genomes of pathogenic strains or variants, but are absent or rarely present in non-pathogenic strains (Hacker et al., 1997). An example is the DNA cassette encoding Vi capsular polysaccharide found on a 134-kb PAI (SPI-7) of Salmonella enterica serovar Typhi (Hacker and Kaper, 2002; Pickard et al., 2003).

Another type of GI observed in prokaryotic genomes is a symbiosis island. This refers to a GI that encodes genes essential for symbiotic interactions with the eukaryotic host. An example is a 502-kb symbiosis island from Mesorhizobium loti strain R7A that was introduced in other M. loti strains from nodulated Lotus plants (Sullivan et al., 2002).

Integration sites for GIs

GIs (including PAIs and symbiosis islands) integrate into chromosomes at a range of specific sites of the tRNA loci (Hacker et al., 1997; Hacker and Kaper, 2000). Hacker and Kaper (2002) hypothesized that the conserved nature of tRNAs across different species, and their occurrence in multiple copies within genomes offer reliable targets for integration of mobile elements (Hacker and Kaper, 2002). Most tRNA genes appear to be
targets for GIs integration. For example, the clc element integrates at tRNA$^{\text{gly}}$ (van der Meer and Senticchilo, 2003), SRL, the PAI of Shigella flexneri 2a integrates at tRNA$^{\text{serX}}$ (Turner et al., 2001), and the three PAIs of E. coli 536 integrate at three different tRNA loci, i.e., tRNA$^{\text{sel}}$, tRNA$^{\text{leu}}$ and tRNA$^{\text{asn}}$ (Dobrindt et al., 2002). The preference of tRNA loci is still unclear, but is believed that specific tRNA may have functions affecting virulence trait and metabolic activity of the GI (Hacker and Kaper, 2002; Reiter et al., 1989).

The recombination of mobile elements such as plasmids and phages with chromosomal DNA is catalysed by site-specific recombinases or integrases, and accessory genes (e.g. excisase, IHF), which encodes their excision and integration activities. Two families of site-specific recombinases have been recognised: the tyrosine and serine recombinase (Stark et al., 1992). The tyrosine recombinase consists of a diverse family of site-specific recombinases found widely distributed in bacteria, plasmids, GIs and bacteriophage. Members of this family usually account for recombination of mobile elements with tRNA genes or their flanking sequences (Esposito and Scocca, 1997b).

1.30. Detection of GIs

Horizontal transfer of genes in prokaryotes can result in the change of microbial genomic composition within a relatively short period (Ochman et al., 2000). The impact could result in an introduction of as much foreign DNA accounting for as much as one-fifth of a bacterial genome (Ochman et al., 2000). The insertion of foreign DNA into the genome can usually be easily recognised by an altered G+C% content that varies from the rest of bacterial genome. They are usually flanked by specific DNA sequences, such as direct repeats. These repeats often resemble the attachment sites for phage integrases and are
often located within tRNA operons (Hacker et al., 1997). GIs also contain genes coding for genetic mobility such as phage genes, insertion sequence elements, integrases, transposases and origins of replication (Hacker et al., 1997; Hacker and Kaper, 2000).

Several methods have been suggested for detecting genes that have recently undergone horizontal transfer. One of the standard methods for detecting GI is to compare two or more genomes of related bacteria; including the pathogenic and non-pathogenic bacteria (Tu and Ding, 2003). The gene clusters present only in pathogenic genomes are candidates for PAIs. Other methods of detection include, guanine-cytosine content (G+C%)(Kerr et al., 1997; Lawrence and Ochman, 1998), codon biases between genomes and GIs (Grocock and Sharp, 2002; Karlin, 2001) and incongruities between phylogenetic trees (Daubin et al., 2002; Daubin et al., 2003).

A prerequisite to understanding the complete biology of mobile elements that allows them to evolve to adapt into new environments is by determination of its entire nucleotide sequence.

1.31. Historical background on sequencing

The ‘Genomic Era’ began when Fred Sanger and his colleagues first sequenced the whole genome of bacteriophage φX174 (5386 bp) in 1977 (Sanger et al., 1977a). Followed soon thereafter by the sequencing of the complete sequence of bacteriophage λ (48,502 bp), cytomegalovirus (229 kb) and 121-kb of chloroplast genomes of Marchantia polymorpha (Oda et al., 1992). With the advancement of high throughput equipment, the first genome sequenced using automated-DNA technology was the 186-kb variola (smallpox) virus in the early 1990s (Massung et al., 1993). In 1995, The Institute for Genomic Research (TIGR; Rockville, USA) completed the sequence of H. influenzae, the first fully genome-sequenced free-living organism of 1.83 Mbp in length (Fleischmann et al., 1995).
1.32. **Importance of complete genomic sequences**

Genomic studies are aimed at the acquisition of knowledge of structure and functions of genomes. The scope of genomic research is wide. It involves the determination of complete nucleotide sequence of an organism; the annotation of the sequences obtained; the analysis of the genetic organisation and the interaction of the gene functions; and the study of how and why genomes evolved the way they did (Lee and Lee, 2000). Some of the rationale of construction of genetic maps includes comparisons for taxonomic and evolutionary studies, location and manipulation of newly discovered genes and identification of homologous genes in other organisms.

Since the completion of the sequence of the whole genome of *H. influenzae* in 1995, many prokaryotic genomes have since been sequenced. The availability of the complete nucleotide sequences of a number of bacterial species has stimulated global bioinformatics and experimental approaches to understanding and identifying previously undiscovered functions (Kapfhammer *et al.*, 2002; McClelland *et al.*, 2001; Parkhill *et al.*, 2001).

The ‘Genomic Era’ has now evolved tremendously and is moving at a rapid pace. To date, genomes of more than 154 bacterial species have been deposited into the nucleotide databases for public access. Amongst others, genome sequences of bacteria such as *Helicobacter pylori, Mycobacterium tuberculosis, Campylobacter jejuni, Neisseria meningitidis, Yersinia pestis, Bordetella pertussis, S. typhi* and *Streptococcus coelicolor* have been completed. Sequence data of other genomes are being continually added and catalogued in online databases.

Analysis of prokaryotic genomes in databases has provided insights (Dobbins *et al.*, 2004; Fleischmann *et al.*, 1995; Parkhill *et al.*, 2001; Tettelin *et al.*, 2000) that include:
(i) amino acid sequence, which can be determined from DNA sequence and genetic code;

(ii) open reading frame (ORF) which are protein coding regions;

(iii) non-coding DNA types, amount, distribution, information content, and functions;

(iv) gene control/regulatory sequences;

(v) coordination of gene expression, protein synthesis, and post-translational events;

1.33. Complete plasmid sequences

To date, 501 bacterial plasmids have also been sequenced and deposited into the NCBI genome database (http://www.ncbi.nlm.nih.gov/genomes/static/eubj3.html). However, the numbers of sequences deposited are rapidly growing each day. The complete plasmid sequences provide information on the biological properties and genetic arrangement that could have contributed to their virulence (Galli et al., 2001), adaptation to environmental changes (Zhong et al., 2002); and also their usefulness as a vector in recombinant DNA technology (Bartosik et al., 1997; Wright et al., 1997). Sometimes, the complete plasmid sequences also provide information on the mechanisms of replication, as demonstrated by the sequence of Marinococcus halophilus (Louis and Galinski, 1997). Comparison of nucleotide sequence data between plasmids has also provided valuable information on the genomic arrangement for evolutionary studies (Katsura et al., 1997; Thomas et al., 1997).

1.34. Various strategies of genomic sequencing

A number of strategies have been used in projects of whole genome sequencing. The strategies used in the projects are broadly primer walking, ‘clone-by-clone’ or hierarchical (HS) shotgun sequencing and Whole Genome Shotgun (WHS) sequencing (Weber and Myers, 1997).
1.34.1. **Sequencing by primer walking**

In primer walking, an initial sequencing reaction is performed using a set of primers of a known vector, usually the universal primers. This is usually achieved by cloning the DNA of interest into a cloning vector. The new sequence obtained is then used to design a new set of primers for sequencing along the molecule. This process is repeated until the molecule has been completely sequenced on both strands.

This strategy however, is not only time consuming (approximately, 400 bases at a time, and 2-6 days between sequencing events), but it is also expensive, as so many different new sets of primers are required. Despite these limitations, this is the standard way used by most non-genome labs for sequencing fragments longer than 400 bases.

1.34.2. **Clone-by-clone sequencing**

In this approach (also known as the hierarchical sequencing (HS) strategy) genomic DNA is cut into large pieces and inserted into BAC vectors to yield a library (Venter *et al.*, 1996). Transformation is usually carried out into *E. coli* for the vector to replicate. The BAC inserts are isolated and mapped by sequence-tagged-sites or by fingerprinting to produce an overlapping tiling path. Each BAC is fragmented randomly into smaller pieces and each piece is cloned into a plasmid and sequenced on both strands. These sequences are aligned so that identical sequences are overlapping and the contiguous sequences are assembled into a finished sequence. This is usually achieved once each strand has been sequenced about four times to produce eight times coverage of high quality data. This strategy has been used for sequencing of bacterial genomes, nematode, *Caenorhabditis elegans* and yeast, *Saccharomyces cerevisiae* (Chen *et al.*, 1993).
1.34.3. **Whole genome shotgun strategy**

This approach does not involve the step of cloning large ordered fragments in BACs. Fred Sanger first introduced this strategy of sequencing in 1982 (Sanger *et al.*, 1977b). It was used to sequence *H. influenzae* Rd in 1995. This strategy was also used in the sequencing of the whole human genome project (Weber and Myers, 1997). In this strategy, the DNA of the entire genome is fragmented randomly. These fragments are cloned into plasmids and sequenced on both strands. Once the sequences are obtained, they are aligned and assembled together by aligning overlaps. The advantage of the shotgun method is that it requires no prior mapping. However, the main disadvantage is the requirement for substantial information technology (IT) resources. Also, unlike the clone-by clone approach, the relationship between ‘contigs’ may not be apparent until near the end of the project.

1.35. **Objective of this thesis**

The aim of this thesis is to investigate the origins of transferable antibiotic resistance in *H. influenzae*. 
CHAPTER TWO

MATERIALS & METHODS

2.1. Bacteria strains and plasmids

2.1.1. Bacteria

*H. influenzae* strain 1056 a capsular type b was isolated from a nasopharynx of an infant with a family outbreak of *H. influenzae* disease in Guildford in 1986 (Brightman *et al.*, 1990). A strain of β-lactamase positive, ampicillin resistance *H. influenzae* type b, strain 299 was isolated from both the CSF and blood of a patient from the Aglaia Kuriakou Children’s Hospital in Athens, Greece in 1991 (Dimopoulou *et al.*, 1997). A streptomycin-resistant, recombination-deficient Rd *H. influenzae* strain nf38 was a recipient used in conjugation experiments, negative for β-lactamase and chloramphenicol acetyl transferase (Setlow *et al.*, 1972). Other *H. influenzae* strains used in this study are summarised in Table 2.1 and *E. coli* strains used are shown in Table 2.2.

2.1.2. Plasmids

Plasmids used and constructed in this study are shown in Table 2.3. Briefly, p1056 (ICE*Hin*1056) was isolated from transconjugant pX1056 that was obtained by mating *H. influenzae* 1056 to nf38 (Dimopoulou *et al.*, 1992), through a mating experiment using the filtration method as described by Stuy (Stuy, 1979). Plasmid of *H. influenzae* 299, p299 (ICE*Hin*299) was isolated through similar method (Dimopoulou *et al.*, 1997).
### Table 2.1. List of *H. influenzae* strains

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<td>amp&lt;sup&gt;+&lt;/sup&gt;, tet&lt;sup&gt;+&lt;/sup&gt;, chlor&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>R. Moxon</td>
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<td><strong>Rd&lt;sup&gt;s&lt;/sup&gt;recA&lt;sup&gt;+&lt;/sup&gt;</strong></td>
<td>Rd strain resistant to streptomycin by selective pressure, recA&lt;sup&gt;+&lt;/sup&gt;</td>
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<td><strong>Rd&lt;sup&gt;t&lt;/sup&gt;recA&lt;sup&gt;+&lt;/sup&gt;</strong></td>
<td>Rd strain resistant to rifampicin by selective pressure, recA&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>D. Crook</td>
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### Table 2.2. List of *E. coli* strains used

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</tr>
<tr>
<td><strong>TOP10</strong></td>
<td>One Shot® Chemically Competent <em>E. coli</em> F&lt;sup&gt;+&lt;/sup&gt;mcrA Δ(mrr-hsdRMS-mcrBC) Δ60lacZAM15 ΔlacX74 deoR recA1 araD139 A(ara-leu)7697 galU galK rpsL (Str&lt;sup&gt;i&lt;/sup&gt;) endA1 nupG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Invitrogen, Holland</td>
</tr>
<tr>
<td><strong>XL1-Blue</strong></td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F&lt;sup&gt;+&lt;/sup&gt; proAB lac&lt;sup&gt;+&lt;/sup&gt;Δ15 Tn10 (Tet&lt;sup&gt;+&lt;/sup&gt;)]</td>
<td>Stratagene, US</td>
</tr>
<tr>
<td><strong>XL10-Gold</strong></td>
<td>Tet&lt;sup&gt;+&lt;/sup&gt; (mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F&lt;sup&gt;+&lt;/sup&gt; proAB lac&lt;sup&gt;+&lt;/sup&gt;Δ15 Tn10 (Tet&lt;sup&gt;+&lt;/sup&gt;) Amy Cam&lt;sup&gt;+&lt;/sup&gt;]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Stratagene, US</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Amy Cam refers to the sensitivity to ampicillin and chloramphenicol.
Table 2.3. List of plasmids/mobile elements and cloning vectors used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Attributes</th>
<th>Size (kb)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICEHin1056</td>
<td>Isolated from H. influenzae 1056, amp', tet', chlor'</td>
<td>60</td>
<td>Guildford, Surrey</td>
</tr>
<tr>
<td>ICEHin299</td>
<td>Isolated from H. influenzae 299, amp'</td>
<td>54</td>
<td>Athens, Greece</td>
</tr>
<tr>
<td>pB7</td>
<td>amp'</td>
<td>12</td>
<td>I. Dimopoulou</td>
</tr>
<tr>
<td>pUC19</td>
<td>amp', lacZ</td>
<td>2.7</td>
<td>MBI Fermentas, Lithuania</td>
</tr>
<tr>
<td>TOPO 2.1</td>
<td>amp' and kan', lacZ</td>
<td>3.9</td>
<td>Invitrogen, The Netherlands</td>
</tr>
</tbody>
</table>

2.1.3. Bacterial sequences

The bacterial sequences of GIs retrieved from the following NCBI sites: (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome), DOE Joint Genome Institute (JGI), California, USA (http://www.jgi.doe.gov/) and The Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). The ICEHin1056 sequences were obtained from the work carried out in Chapter 4.

The seventeen GIs use in this study are shown (Table 2.4). The bacteria containing these GIs were all members of Proteobacteria. Ralstonia metallidurans and Burkholderia fungorum belonged to β-proteobacteria and all other bacterial species are γ-Proteobacteria.
Table 2.4. Bacterial sequences used in this study.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>GI name</th>
<th>Size (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderia fungorum&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LB400</td>
<td>un&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70,906</td>
<td>NZ_AAAJ000000000</td>
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<tr>
<td>Haemophilus influenzae</td>
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<td>ICEHin1056</td>
<td>59,393</td>
<td>AJ627386</td>
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<tr>
<td>Haemophilus ducreyi</td>
<td>35000HP</td>
<td>un</td>
<td>49,075</td>
<td>NC_002940</td>
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<td>Haemophilus somnus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>129PT</td>
<td>un</td>
<td>56,442</td>
<td>NZ_AAB002000009</td>
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<tr>
<td>Haemophilus somnus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2336</td>
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<td>64,831</td>
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<td>Photobacterium luminescens</td>
<td>TT01</td>
<td>un</td>
<td>140,163</td>
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<tr>
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<td>B13</td>
<td>&lt;i&gt;clc&lt;/i&gt;</td>
<td>105,027</td>
<td>AJ617740</td>
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<tr>
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<td>C</td>
<td>pKLC102</td>
<td>103,532</td>
<td>AF257538</td>
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<td>PAP1</td>
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<td>Pseudomonas fluorescens&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>un</td>
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<td>un</td>
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<td>NC_004507</td>
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<tr>
<td>Ralstonia metallidurans&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH34</td>
<td>un</td>
<td>&lt;76,893</td>
<td>NZ_AAAI00000000</td>
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<td>Salmonella enterica serovar Typhi</td>
<td>CT18</td>
<td>SPI-7</td>
<td>134,000</td>
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<tr>
<td>Salmonella enterica serovar Typhi</td>
<td>TY2</td>
<td>un</td>
<td>122,000</td>
<td>NC_004631</td>
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<td>86,019</td>
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<tr>
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<td>8081</td>
<td>un</td>
<td>65,285</td>
<td>NC_003222</td>
</tr>
</tbody>
</table>

<sup>a</sup>sequencing of whole genome is ongoing, <sup>b</sup>un = unnamed

2.2. Media

2.2.1. Solid culture media

Chocolate agar was used to culture <i>H. influenzae</i> strains. <i>H. influenzae</i> type b (Hib) agar was used in conjugation experiments (Barbour <i>et al.</i>, 1993). It was supplemented with nicotinamide adenine dinucleotide (NAD, 10 µg/ml, Sigma) and haemin (15 µg/ml, Sigma). The <i>E. coli</i> strains were grown in Luria Bertani’s (LB) agar. The constituents and procedures for media preparation are listed in detail in Appendix A.
2.2.2. **Liquid culture media**

*E. coli* in broth were grown in LB broth. For the growth and dilution of Haemophilus strains, brain heart infusion (BHI) with NAD (10 µg/ml) and haemin (15 µg/ml) was used.

2.2.3. **Antibiotics selective media**

Stock antibiotic solutions were prepared as shown in Appendix A. LB agar in aliquots of 20 ml in universal bottles were melted in boiling water and allowed to cool to 50°C. For antibiotic selections in matings or growth of antibiotic-resistant strains in liquid media, the desired filter-sterilised antibiotics were added to the media at the same time as the NAD and haemin. For growth of strains on solid agar, the antibiotics were added and gently mixed into the molten agar, and immediately poured were immediately poured into 99-mm disposable plastic Petri-dishes. The final concentrations of antibiotics were as follows: ampicillin, 50 µg/ml; chloramphenicol, 20 µg/ml; rifampicin, 20 µg/ml; streptomycin, 50 µg/ml; and tetracycline, 50 µg/ml. The agar was allowed to set at room temperature and the agar plate was dried for at least 20 minutes before use.

2.2.4. **X-Gal and IPTG plates**

The methods for the preparation of stock X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactosidase, MBI Fermentas, Lithuania) and IPTG (Isopropylthio-β-D-galactosidase, MBI Fermentas, Lithuania) are shown in the Appendix C. For use in transformation procedure, X-Gal agar was prepared by spreading 40 µl of 2% X-Gal onto the surface of a dry LB agar plate with a sterile glass spreader. When IPTG was required, the X-Gal was diluted with some broth (either SOC or BHI) before the addition of 40 µl of 100 mM IPTG (otherwise, the mixture of X-Gal and IPTG produced a white precipitate). Both
solutions were then spread evenly with a glass spreader. However, IPTG was not required when using TOP10 *E. coli* cells in cloning libraries. The mixture was allowed to absorb into the agar for approximately 10 minutes before drying at 37°C for 15 minutes prior to bacterial culture.

### 2.3. Chemical, restriction enzymes and DNA modifying enzymes

Chemicals used in the experiments were mostly obtained from Sigma Biochemicals (Dorset, UK); restriction endonuclease and other DNA modifying enzymes were purchased from MBI Fermentas (Lithuania, France), Gibco (UK) and New England Biolabs (Massachusetts, USA). Media and agar were obtained from Oxoid (Basingstoke, UK), unless otherwise stated. The composition of chemicals and solutions are given in the Appendix A.

Tissue culture sterile water (Sigma, Dorset, UK) was used throughout, and where applicable. Most chemicals were of AnalaR grade and supplied by BDH or Sigma (UK). Exceptions to these suppliers are indicated in the text when the specified reagents are mentioned. The constituents of solutions are described in Sambrook (Sambrook *et al.*, 1989) and exceptions to this are described in the text.

### 2.4. Bacterial growth conditions

#### 2.4.1. Growth of *E. coli* and *H. influenzae* strains

Bacteria, namely *E. coli* were generally sub-cultured on LB agar (without the addition of antibiotics) when recovered from −80°C freezer (Sanyo, Japan). Subsequent culture was made onto agar media containing selective antibiotics, if appropriate. *E. coli* cultured in broth were incubated at 37°C in air with orbital shaking at approximately 100 rpm (New Brunswick Scientific, USA).
*H. influenzae* grown either on chocolate agar or Muller Hinton agar were incubated at 5% CO$_2$ in a 37°C incubator (Sanyo, Japan) while *H. influenzae* strains grown in broth were incubated at 37°C in air. *H. influenzae* colonies are well visualised after 48 hours.

2.4.2. *Culture and storage of bacteria*

Bacterial strains for storage were picked from single colony from fresh agar plates. *H. influenzae* strains were cultured into BHI broth (containing NAD and haemin) and *E. coli* strains were cultured into LB and grown overnight. Equal volume of 30% glycerol was added to the culture and stored at −80°C. Alternatively, fresh pure colonies on agar plates were scraped and generously re-suspended into tryptone soy broth (TSB) with 15% glycerol and stored at −80°C. Commercially prepared competent *E. coli* strains were stored immediately at −80°C upon arrival.

2.5. *Colony counts*

A preliminary estimation of the concentration of the bacterial cells was made. A ten-fold dilution was made by mixing 1 ml of broth culture to 9 ml of the phosphate buffered saline (PBS) used as diluent. When counting colonies of Haemophilus strains, BHI broth was used.

A series of 10-fold dilution was carried out up to the estimated dilution. One hundred micro-litre of each of the diluted cells was plated using a sterile glass spreader onto agar plates containing the appropriate antibiotics. The diluted cells were allowed to absorb into the agar before incubation, by drying at 37°C for 5-10 min. The agar plates were then incubated at 37°C overnight. For each dilution, a colony count of the bacterial
cells was carried out in duplicate. Only agar plates containing between 30-300 colonies were used for colony counting.

2.6. Antibiotic susceptibility test by Etest

Overnight culture of *H. influenzae* strains on chocolate agar were picked and were suspended in phosphate buffered saline (PBS) to a 0.5 McFarland turbidity. A sterile cotton swab was dipped into the inoculum’s suspension and excess fluid was removed by pressing the swab against the inner wall of the glass container. The entire surface of a completely dried Muller Hinton agar (with NAD and haemin) was swabbed twice, by rotating the plate approximately 90 degrees each time to ensure an even distribution of the inoculum. With a pair of sterile forceps, an Etest® strip (AB Biodisk, Sweden) was slowly placed on the surface of the inoculated agar. The agar plate was incubated overnight at 37°C in CO₂ atmosphere.

When the bacterial growth becomes distinctly visible, the minimal inhibitory concentration (MIC) value was read at the point of intersection between the inhibition ellipse edge and the Etest strip.

2.7. Conjugation

2.7.1. Conjugation on solid media

Donor strain (strain 1056) was grown overnight on Muller Hinton agar containing NAD (10μg/ml), haemin (15 μg/ml) and ampicillin (8 μg/ml). Recipient strains were similarly grown on Hib agar containing either streptomycin (20 μg/ml) or rifampicin (20 μg/ml). A dense suspension of cells was made in 1 ml of BHI broth. These cells were harvested by centrifugation and the pellet was re-suspended in BHI broth to a final OD of approximately 0.8 (measured visually with MacFarland’s standard). To 1 ml of BHI
broth, 4 µl of donor cells and 40 µl of recipient cells were added. The cells were gently mixed, and 100 µl of the mixture was spread onto duplicate plates of Hib agar without antibiotics. The inoculum was allowed to dry in a short incubation (5-10 minutes) at 37°C, prior to incubation in CO₂ for 8 hours.

The 'mated cells' were harvested with BHI broth to a volume of approximately 1 ml. Serial dilutions were carried out to determine viable counts and plated on selective agars containing antibiotics for enumeration of colony-forming-unit (cfu) of transconjugants, donors and recipients. The rate of transfer is measured by the number of transconjugants per donor cell.

2.7.2. Conjugation in liquid media
Mating of cells in liquid was carried out concurrently, by incubating the original mixed culture in BHI broth at 37°C for the same period of 8 hours. Viable counts were determined as above.

2.7.3. Measurement of point mutation
Donor or recipient cells that were usually susceptible for a particular antibiotic were tested for point mutation by spreading 100 µl of the re-suspended and washed cells onto Hib agar containing the relevant antibiotic. The plates were incubated for viable counts.

2.7.4. Purification of transconjugants
Approximately 60 transconjugants were plated to Hib agar containing tetracycline, ampicillin or chloramphenicol. Viable counts were carried out following overnight incubation.
2.8. Electron microscopy examination of pili

Presence of pilus by examined by transmission electron microscopy (TEM). Prof. David Ferguson, Dept. of Pathology, at John Radcliffe Hospital, Oxford, kindly undertook the EM examination. In brief, the *H. influenzae* colonies grown overnight on Hib agar were re-suspended with approximately 1 ml of phosphate buffered saline (PBS). Copper grid coated with formavar and carbon was flooded with the suspension and was left for two minutes. Excess fluid from the copper grid was discarded, before a drop of water was added. The grid was negatively stained with 1% methyl tungstate, dried and examined using TEM (JOEL 1200EX, Japan).

2.9. Chromosomal DNA extraction

2.9.1. Extraction of chromosomal DNA from *H. influenzae*

Pure colonies of *H. influenzae* grown on BHI agar were harvested (Sterilin, UK) and were generously re-suspended in 0.2 ml of Tris-EDTA (TE) buffer. A volume of 0.45 ml of GES lysing solution consisting of 5 M guanidium thiocyanate, 100 mM EDTA and 0.5% v/v sarkosyl were added to the suspension and mixed by inversion and allowed to lyse at room temperature for 3 minutes. Cold 7.5 M ammonium acetate (0.25 ml) was added and was mixed by several inversions before it was stored on ice for 10 minutes. Subsequently, 0.25 ml of chloroform: isoamylalcohol (24:1) was added and mixed thoroughly by hand for 3 minutes. The phases were separated by centrifugation at 12,000 g for 20 minutes, and the aqueous upper layer was transferred to a fresh-labelled micro-centrifuge tube. Cold isopropanol of 0.5x volume was added and was gently mixed by inversion for 1 minute or until the DNA threads were visible. Following centrifugation for 5 minutes, the supernatant was discarded and the pellet was re-suspended in 0.2 ml TE buffer.
A 2.5x volume of cold absolute ethanol was added to re-precipitate the DNA. If necessary, the DNA suspension was stored at -20°C for 15-30 minutes. Excess alcohol in the tube was washed off by centrifugation and drained onto a paper towel. The pellet was washed three times with 0.5 ml of 70% ethanol by inversion and centrifugation at 12,000 g for 5 minutes. Finally, the DNA pellet was re-suspended in 0.2-0.3 ml of TE buffer and was allowed to dissolve overnight at 4°C.

2.9.2.  Extraction of crude DNA by boiling method

One colony of bacterial cells from a fresh culture of bacteria was suspended into 50 μl of sterile distilled water and boiled for 10 minutes. The boiling lysate was allowed to cool to room temperature for 5 minutes before it was spun at a 16,000 g for 30 seconds. The supernatant was collected and transferred to a fresh tube. This DNA extract (1 μl) was then used as template in PCR amplifications.

2.10.  Plasmid extraction

2.10.1.  Extraction by mini-prep method

Plasmid extraction was performed according to the instruction provided by a plasmid extraction kit, NucleoSpin Plasmid® kit, (Macherey-Nagel, Germany). Briefly, bacteria from a single colony of an agar plate was picked up and cultured into LB broth with ampicillin (50 μl/ml). The broth was then incubated in a shaking incubator at 80 rpm for 17-18 hours. Subsequently, 1.5 ml of the cultured medium was pipetted into a micro-centrifuge tube, and centrifugation was carried out at 15,000 g (Eppendorf, Hamburg, Germany) for 30 seconds. The supernatant was discarded and the cell pellet was re-suspended by vortexing vigorously in buffer A1 (previously added with Rnase). The suspension was gently mixed with a buffer A2, and was allowed to lyse completely at
room temperature for a maximum of 5 minutes. When binding buffer A3 was added to the lysate, a colloidal precipitate formed was removed by centrifugation for 10 minutes at full speed. Following centrifugation, the supernatant was loaded into a NucleoSpin Plasmid column that had been placed in a 2 ml collecting tube. It was centrifuged again for 1 minute at 16,000 g and the flow-through was discarded.

Pre-warmed (50°C) washing buffer AW, was added to the column and centrifugation was repeated, followed by ethanolic washing buffer A4 for another minute. The residual ethanol in the column was minimised by a further two-minute centrifugation. Finally, buffer AE was added to the column and centrifugation was carried out for 1 minute to elute the plasmid DNA. The extracted plasmids were stored at -20°C until use.

2.10.2. Extraction of plasmid DNA by caesium chloride-ethidium bromide gradients

The method was adapted from the method of Sambrook (Sambrook et al., 1989). This purification of plasmid DNA was carried out according to its buoyant density. A solution of caesium chloride is subjected to a very high centrifugal force such that the ions it contains are forced towards the bottom of a tube. Diffusion counteracts this setting up a gradient of increasing CsCl concentration and thus density down the tube. Any macromolecules present within the CsCl solution will move to, and form bands at that point where the local density is the same as their own buoyant density. Since proteins have a low, RNA a high and DNA an intermediate density, separation is achieved. By incorporating an EtBr into the solution, circular plasmid DNA can be separated from linear chromosomal DNA. This happens because dye binds to and thus displaces fewer of the dense Cs ions from the plasmid DNA. This is thus denser than the chromosomal DNA under these conditions and therefore bands beneath it in the tube.
Following overnight incubation of *H. influenzae* strain X1056 on chocolate agar, the colonies were scraped and emulsified with 1 ml of PBS in a centrifuge tube. The bacterial suspension was then transferred into 500 ml of MH broth supplemented with ampicillin (25 μg/ml), haemin and NAD, and was incubated in a shaking incubator overnight at 800 rpm.

Plasmid DNA was extracted using plasmid extraction kit, Nucleobond AX 500 (Macherey-Nagel, Germany). Briefly, the cultured cells were harvested by centrifugation at 670 g (Sorvall RC, USA) for 10 minutes at 4°C. The cells were re-suspended in the given buffer and then lysed with a buffer containing RNase. Disrupted cells consisting of chromosomal DNA and cell proteins were precipitated in the form of homogenous suspension. The bacterial lysate was then clarified through a NucleoBond filter and the clear lysate was loaded into a NucleoBond BAC 100 column. The flow-through from the column was allowed to adsorb onto the column twice and washed three times before eluting with 6 ml of elution buffer.

To the eluted plasmid DNA, 6 g of solid CsCl was added and allowed to dissolve. Ethidium bromide of 0.48 ml in volume was added to the solution and was mixed well. The refractive index of the mixture was checked to be approximately 1.388 by using a refractometer (Bellingham and Stanley, England). The mixture was then divided and placed into three Beckman polycarbonate centrifuge tubes (size 13 x 15 mm) and was centrifuged overnight (approximately 16 hours) in an ultracentrifuge (Beckman T100, USA) at 215,000 g at 15°C. Following centrifugation, a fine band consisting of plasmid DNA underneath a bright band of chromosomal DNA seen under UV light was collected and put into a fresh micro-centrifuge tube.

Equal amount of water-saturated 1-butanol was added to the collected plasmid DNA. The two phases was thoroughly mixed, and centrifugation was carried out at 670 g
for 3 minutes at room temperature. The aqueous lower phase was removed and the above extraction was repeated until all the pink colour of EtBr in both the upper and lower phase disappeared.

To approximately 200 µl of the extracted plasmid DNA, 5 µl of glycogen (MBI Fermentas, Lithuania), 20 µl of sodium acetate (NaOAc) and 500 µl of cold 95% ethanol was added and mixed gently. The mixture was put in ice for 45 minutes before centrifugation at a 16,000 g for 15 minutes. The pellet was washed twice with 70% ethanol, dried and reconstituted with 25 µl of TE buffer.

2.11. Removal of contaminating chromosomal DNA

Plasmid DNA was precipitated with 1/10 volume of 3M NaOAc; pH 5.2 followed by 1 µl of glycogen (MBF Fermentas, Lithuania) and was mixed gently and cooled in ice for an hour. The mixture was centrifuged for 10 minutes and washed twice with 70% alcohol. The pellet was finally dried and diluted with deionised water. A 1/5 volume of lambda buffer was added to the plasmid, followed by exonuclease I and lambda exonuclease (New England Biolabs, US). The mixture was allowed to react at 37°C overnight and inactivated at 80°C for 20 minutes. To the mixture, 1/10 volume of NaOAc and glycogen were added and incubated in ice for an hour. The mixture was spun at 15,000 g for 10 minutes before washing with 70% alcohol twice. The pellet was dried and diluted with an appropriate amount of distilled water. It was allowed to dissolve at 37°C for 30 minutes.

2.12. Plasmid shearing

In order to obtain fragments of the element of sizes between 500 bp to 2.0 kb, a comparative study was carried out to determine the optimum length of time required to sonicate the DNA of the plasmid. Purified whole plasmid DNA (20 µl) was made up to
1/100 dilution with sterile distilled water and divided into five micro-centrifuge tubes. Each of the tubes was subjected to sonication at various durations of 10, 15 and 20 s. One tube was not sonicated and was kept as a control. The power of sonication was kept constant at 15%. Following sonication, the sheared DNA was gel-electrophoresed (0.7% agarose gel) and the sizes of the sonicated fragments were checked under UV-light. The DNA that produced a heavy band of smearing between 500 bp to 2.0 kb was noted and the sonication time was selected.

2.13. **Agarose gel electrophoresis**

2.13.1. **Preparation of agarose gel**

Most agarose gel electrophoresis used was carried out using 0.7% agarose (Sigma type II; Sigma, UK) prepared in 1x TBE buffer by heat dissolving (see Appendix). Ethidium bromide (final concentration of 0.5 µg/ml) was carefully added to the slightly cooled gel mixture, which was subsequently cast and allowed to set at room temperature.

2.13.2. **Gel electrophoresis**

Plasmid samples (5 µl) were mixed with 2 µl of 6x loading buffer (Sigma, USA) and were loaded into the wells of the agarose gel. A 1-kb ladder (Gibco, UK) was usually used as a molecular weight marker. Electrophoresis was carried out horizontally at 100 volts with 1x TBE as the electrophoresis buffer.

2.13.3. **Observation of DNA in agarose gel**

Following electrophoresis, DNA on agarose gels was viewed using a transilluminator of Gel Doc 1000 image analyser (Bio-Rad, USA). For preparation of DNA for cloning, gels
with DNA separated by restriction enzyme were viewed and excised over 365 nm (long) wavelength of TL33-E Transilluminator (UVP, UK).

2.13.4. Recovery and purification of DNA from agarose gel

DNA fragments on agarose that were digested with restriction enzymes, were excised from the agarose gel using a sterile scalpel into separate clean micro-centrifuge tubes. The DNA in the gel block was eluted using a gel extraction kit, MinElute Gel Extract (Qiagen, USA) with some modifications. Following solubilisation with QG buffer, the dissolved mixture was transferred into the columns and spun for 1 minute at 16,000 g. The flow-through was discarded and the column was washed with PE buffer for 1 minute. The column was dried by centrifuging for another minute, before it was air-dried for another minute to get rid of residual ethanol. The DNA was eluted by adding distilled water to the column and spun at 15,000 g for 1 minute. The DNA obtained was kept at −20°C until use.

2.14. Restriction enzymes digestion

Restriction enzymes used in most of the experiments were purchased from MBI Fermentas (Lithuania). All the restriction endonuclease digests were performed in the presence of buffers supplied by the manufacturer. To every μg of DNA, 5-10 units of enzyme were added and the digestions were performed at 37°C for 1 hour, unless otherwise stated. Restriction digest using BsgI required S-adenosylmethionine for optimal activity. For this particular enzyme, the reaction was heated at 65°C for 20 minutes to inactivate the enzyme.
2.15. **Polymerase chain reaction (PCR)**

2.15.1. **Selection of oligonucleotide primers**

Pairs of PCR primers were selected from sequence data using certain criteria. Firstly to ensure similar $T_m$ (melting temperature) values for paired primers, the length of oligonucleotides should be between 15-22 bp with a similar guanine and cytosine ratio of between 50 to 60%. The nucleotide sequence was tested for secondary structures and complementary pairing using a website: [http://www.basic.nwu.edu/biotools/OligoCalc.html](http://www.basic.nwu.edu/biotools/OligoCalc.html). The primers used were purchased from either Alta Bioscience (The University of Birmingham, UK) or MWG-Biotech AG (Germany).

2.15.2. **Dilution of primers**

Oligonucleotide primers were diluted with sterile distilled water to 100 pmoles/μl according to the instruction of the manufacturer as stock solution and stored at −20°C. The primers used for PCR were diluted to 100 μM as working concentration, while primers used for sequencing were further made to 1:15 dilution. The lists of primers used in the whole study are shown in the Appendix B.

2.15.3. **Preparation of PCR mixture**

For each PCR (20 μl) contained: 2.0 μl of 10x PCR buffer, 3.0 μl of 2 mM dNTP mix (MBI, Fermentas), 1.6 μl of 25 mM MgCl₂, 1.0 μl of 100 pmol/μl forward primer, 1.0 μl of 100 pmol/μl reverse primer, 0.5 μl of 2 unit Taq polymerase (MBI Fermentas, Lithuania), 1.0 μl of the template DNA and 9.9 μl of sterile distilled water.
2.15.4. **Thermal cycling condition**

Amplification of the DNA samples was carried out using an MJ Research Thermal Cycler (USA). The DNA was initially denatured at 94°C for 2 minutes prior to a series of 30 cycles of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 2 minutes. At the end of the cycle, the DNA was given a further extension of another 4 minutes at 72°C before it was cooled at 4°C.

2.15.5. **Observation of amplified products**

Amplified products were separated by electrophoresis in 0.7% agarose gel, and run at 100 mA on a BioRad PowerPac 200 (USA) for approximately 30 minutes. The DNA on the gel were visualised using Gel Doc image analyser (BioRad, US) and the image of the gel was printed to keep as a record.

2.16. **Purification of PCR products and DNA**

2.16.1. **Purification by spin column**

PCR product extracted from agarose was purified using Nucleospin Extract (Macherey-Nagel, Duren, Germany). Briefly, one volume of the excised gel was mixed with four volumes of NT2 buffer and transferred into a column fixed into a collection tube. The column was spun in a centrifuge for 1 minute. All centrifugation was carried out at 15,000 g unless otherwise stated. The flow-through was discarded and NT3 buffer was added into the column and spun for another 1 minute. The column was finally rinsed with NT3 and the buffer was completely removed by a two-minute centrifugation.
2.16.2. Polyethylene-Glycol (PEG) purification

2.16.2.1. Samples in 0.2 ml micro-centrifuge tubes
A volume of 60 μl of 20% polyethylene-glycol 8000 (PEG) solution was added to 20 μl of plasmid DNA or PCR products. It was briefly mixed by vortex and incubated at 37°C for 10 minutes. The mixture was centrifuged for 15 minutes at 15,000 g and the pellet was collected. It was then rinsed twice with 70% ethanol and air-dried. A volume of 20 μl of distilled water was used to re-suspend the DNA pellet. Prior to ligation, agarose electrophoresis was performed to 5 μl of the DNA samples to ensure the presence of the DNA.

2.16.2.2. Samples in microAmp tray
Similar procedure was carried out as above, except that samples in microAmp trays were centrifuged using Allegra GR Centrifuge (Beckman Coulten, USA) at 5,000 g at 4°C for 1 hour and the DNA pellets were washed with 150 μl of 70% ethanol by centrifuging at 5,000 g for 20 minutes. The residual ethanol was drained by inverting the tray on absorbent paper towels and spun at 1,000 g for 1 minute. Washing of the pellets with 70% ethanol was done twice before drying at 65°C in a thermal-cycler for at minimum of 10 minutes.

2.16.3. Ethanol/sodium acetate (NaOAc) precipitation

2.16.3.1. Samples in micro-centrifuge tubes
For sequenced samples that were carried out in 0.2 ml micro-centrifuge tubes, 2.0 μl of 3 M NaOAc, pH 4.6 and 50 μl of 95% ethanol was added to the sample in 1.5 ml micro-centrifuge tube. The mixture was incubated at room temperature for 15 minutes before centrifugation at 15,000 g for 20 minutes. The ethanol was removed and the pellet was washed twice with 150 μl of 70% ethanol by centrifugation for 10 minutes. The
supernatant was discarded and the pellet was dried at 65°C for at least 10 minutes prior to sequencing.

2.16.3.2. **Samples in microAmp tray**

The amount of NaOAc and ethanol used was similar to the samples that were carried out in individual micro-centrifuge tube. However, for samples in microAmp trays, centrifugation was carried out at 5,000 g at 4°C for 1 hour. The pellets were dried by inverting the microAmp tray onto absorbent paper towels and were spun for 1 minute at 1,000 g. The samples were washed with 150 µl of 70% alcohol by centrifuging at 1,000 g for 1 minute and dried at 65°C. The trays were sealed and stored at -20°C until ready for sequencing.

2.17. **DNA manipulations**

2.17.1. **Fragmentation of DNA by sonication**

In a 1.5 ml micro-centrifuge tube, 50 µl of *H. influenzae* plasmid DNA was made up to 200 µl with distilled water. The tube with plasmid solution was placed into an ice-bath and sonicated for 10 s at 15% power (Soniprep 150, MSE, England). Five micro-litres of the sonicated samples were run through agarose gel electrophoresis to determine the sizes of the fragments. The remaining sheared plasmid solution was kept at −20°C until use.

2.17.2. **Creating blunt-ended DNA**

Sonicated DNA plasmid was blunt-ended using T4 DNA polymerase (MBF Fermentas, Lithuania). Briefly, 5x reaction buffer (50 µl), 2 mM dNTPs (10 µl), and T4 polymerase (5 µl) were added to the sheared plasmid DNA (200 µl) and were incubated for an hour at
37°C. The mixture was PEG precipitated with two washings of 70% ethanol. The pellet was dried and re-suspended in 20 µl of distilled water.

2.17.3. Phosphatase treatment of cloning vectors

Following restriction enzyme digestion, the vector DNA was added with 1 µl of calf intestine alkaline phosphatase, CIAP (MBI Fermentas, Lithuania) and 1 µl of CIAP buffer. The incubation at 37°C was continued for 1 hour and the reaction was inactivated at 85°C for 15 minutes.

2.18. DNA ligations

Ligation of fragmented plasmid DNA into pUC19 cloning vector was carried out using T4 DNA ligase and ligation buffer provided by a ligation kit (MBI Fermentas, Lithuania). A ratio of 10:1 of sheared DNA to cloning vector was used in a 20 µl reaction consisting of 2 µl of 10x ligation buffer, 2 µl of PEG 4000, 2 µl of T4 DNA ligase and distilled water. The ligation reactions were performed at 22°C overnight. For ligation of sticky ends, PEG 4000 was omitted and 1 µl of T4 DNA ligase was added.

2.19. Bacterial transformation

2.19.1. Transformation by heat-shock

The E. coli competent cells used in the experiments were mainly obtained from Stratagene (USA) while E. coli TOP10 strains were provided in PCR cloning kit, TOPO TA Cloning (Invitrogen, The Netherlands).

Transformations of DNA into competent E. coli cells by heat-shock were carried out at 42°C for a very short period of time. Frozen competent E. coli cells were thawed on ice and mixed gently by hand. Depending on the strains of the E. coli, between 50-100 µl
of the cells were added to a pre-chilled 15-ml Falcon 2059 polypropylene tube. To the cells, 4 µl of β-mercaptoethanol was added and incubated on ice for 10 minutes. However, for transformation of DNA into *E. coli* XL1-blue subcloning-grade, mercaptoethanol was omitted. Between 4-5 µl of the ligation mix was added to the competent cells. Following 30 minutes incubation in ice, heat pulse treatment was given to the mixture in a water-bath set at 42°C for 45 s for most *E. coli* strains. However, for TOP10 cells heat pulse was carried out for 30 s. Transformed cells were then plated onto selective LB agar plates following instructions of the supplier of the competent cells.

2.19.2. *Transformation of PCR products into TOP10 E. coli*

Prior to transformation of PCR products into TOP10 *E. coli* cells (Invitrogen, Netherlands), TOPO cloning reaction was set up by adding 1.0 µl TOPO vector to a 1.0 µl mixture consisting of PCR product (2.0 µl), salt solution (1.0 µl of 1.2 M NaCl, 0.06 M MgCl₂) and distilled water (1.0 µl). The reaction mixture was gently mixed and incubated for 5 minutes at room temperature.

Subsequently, 2.0 µl of the TOPO Cloning mixture was added to a vial of iced-thawed component cells and was gently mixed before incubating in ice for 5 minutes. The cells were heat shocked at 42°C in a water-bath for 30 s without shaking. Immediately after the heat-shock, the tube was chilled in ice and added with 250 µl of SOC medium. The cap of the vial was tightened and incubated for an hour at 37°C in a shaking incubator at a horizontal speed of 15 rpm. Transformed cells were plated onto LB agar containing X-Gal and ampicillin (50 µg/ml) and incubated overnight.
2.20. **Selection of recombinant clones**

Recombinant colonies were picked based on the colour of the colonies. Ampicillin was used for a selection of bacteria containing the cloning vector. IPTG was used to induce the β-galactosidase gene within TOPO to breakdown the X-Gal into its blue-indole derivative. In the absence of an insert, a functional α-peptide was produced that complemented the gene product of lac\(\beta\)ZΔM15, to produce a functional β-galactosidase protein producing a blue-indole derivative. If an insert was present in the TOPO multiple cloning site, the lacZ α-peptide was interrupted and complementation does not occur leaving the colony to remain white. Therefore, when screening for cloned fragments only white colonies were investigated further. Ten to 15 white colonies were picked for PCR to assess the presence of the inserts using the primers initially used for the amplification of the PCR fragment.

2.20.1. **Confirmation of cloned inserts**

Between 10-15 well-separated bacterial transformants were picked up and sub-cultured onto appropriate agar plates and incubated overnight. The inserts were confirmed by either one of these methods:

2.20.2. **In-well lysis**

This is a rapid method to determine the presence of plasmid following transformation. A loopful of an overnight culture was re-suspended in 100 μl of TE buffer in a well of a micro-titre plate. To the suspension, 20 μl of the mixture of P2 lysis buffer (Qiagen, Germany) and loading dye (Sigma, USA) in the ratio of 3:2 was added. The mixture was agarose electrophoresed and viewed under UV-illumination for presence of plasmid.
2.20.3.  **Amplification of cloned fragment by PCR**
Transformants/recombinants were amplified by PCR using the appropriate primers. Electrophoresis was carried out on the PCR products and observed under UV light. The product size shown on agarose gel determined the fragment size of the cloned inserts.

2.20.4.  **Digestion of clone fragment by restriction endonuclease enzymes**
Plasmids were extracted from the transformants/recombinants using commercial plasmid extraction kit, NucleoSpin Plasmid® kit (Macherey-Nagel, Germany). Appropriate restriction endonuclease enzymes were used to cut the plasmids and fragment size was determined by agarose gel electrophoresis.

2.21.  **Construction of plasmid library**
A random plasmid library was constructed by sonicating the whole plasmid (prepared as described in Chapter 2) at 15% power. A small volume of the fragments was electrophoresed to confirm the sizes of the fragments. The sonicated fragments were end-filled by adding T4 DNA polymerase (MBI Fermentas, Lithuania) for an hour at 37°C prior to polyethylene-glycol (PEG) precipitation. The fragments were ligated into Smal digested pUC19 at 22°C overnight and transformed into XL-10 Gold *E. coli* cells (Stratagene, USA), according to the methods of the manufacturer. Transformed colonies were plated onto ampicillin-LB agar plates (50 µg/ml) that were previously coated with IPTG and X-Gal. White colonies were picked and cultured. Following plasmid extraction, the inserts were sequenced using the M13F and M13R primers.
2.22. DNA sequencing

2.22.1. Principles of DNA sequencing

This procedure utilises base-specific termination during *in vitro* synthesis of DNA and therefore also called the chain termination method. The basis for terminating the elongation of the DNA chain is the use of a nucleoside triphosphate containing a ribose sugar reduced in both the 2' and 3' position. Accordingly, such a nucleoside triphosphate is called dideoxynucleoside triphosphate, or ddNTP. Because of the missing hydroxyl group at the 3' position of the sugar, DNA polymerase cannot form a phosphodiester with the next dNTP, and the growing chain terminates after the incorporation of a ddNTP. The ddNTP, however, still has the 5' position of the sugar free to react with the 3' end of the previously incorporated dNTP. This reaction occurs at a low frequency. But when such a ddNTP is mixed with the four normal dNTPs at the appropriate concentrations, chain termination will occur over a range of hundreds of base positions. Consequently, the four normal dNTPs are mixed with the respective ddNTPs in four different reaction tubes. If all four reactions are compared, termination occurs randomly at all positions of the growing DNA chain. In the reactions, one of the normal dNTPs is labelled at its alpha position with $^{32}$P, in order to incorporate radioactivity with the newly synthesized DNA. When such products are analysed, a balanced level of radioactive signals is achieved for all bands on the ladder gel, since the longer chains incorporate more label but terminate less frequently than the shorter chains.

2.22.2. Templates for sequencing

Templates for sequencing were prepared from either plasmids with inserts, or from PCR products of cloned fragments. Prior to sequencing, plasmids used were purified by using
Nucleospin® Extract 2 in 1 kit (Macherey-Nagel, Germany), while the PCR products were purified by PEG precipitation.

2.22.3. **Cycle sequencing**

Samples for sequencing was prepared in a 5.0 µl reaction volume. Between 200-500 ng of DNA template from a cloned plasmid, or 30-90 ng of a PCR product were added to a reaction mixture consisting of 2.0 µl Terminator Ready Reaction Mix (BigDye Terminator V.3, PE Applied Biosystems, USA) and 1 µl of sequencing primers.

Cycle sequencing was carried out in a MJ Research Thermal Cycler. The DNA underwent a denaturing stage at 96°C for 30 seconds, an annealing stage at 50°C for 10 seconds and an extension stage at 60°C for 4 minutes. The cycle was repeated for 30 cycles and held at 4°C until use. Finally the DNA for sequencing was purified from the BigDye by sodium/ethanol precipitation technique.

2.22.4. **Automated sequencing**

Sequencing analysis was carried out by the chain termination method (Sanger *et al.*, 1977) at the Zoology Department of the University of Oxford using the ABI 377 (PE Applied Biosystems, USA) automated sequence reader.

2.23. **Computer software for sequence assembly**

2.23.1. **Staden**

Sequence data generated from automated nucleotide sequencing were assembled and edited using the Staden programme software on a UNIX platform. This package of programmes was designed for sequence handling and analysis was used in this project.
(Staden, 1996). Staden was developed at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK.

2.23.2. **Phred and Phrap**

Phred and Phrap are programs that have been integrated into the Staden program for database handling (Ewing and Green, 1998; Ewing *et al.*, 1998). Phred reads the raw sequence trace files produced by a DNA sequencer performs base calling and assigns quality values, i.e. error rates for each base. These quality values reflect the estimated probability that a base is erroneously called. Phrap uses the quality information provided by Phred, makes pair-wise comparisons of reads and performs the assembly.

2.24. **Computer programs for sequence annotation**

2.24.1. **GeneMark and Glimmer**

Potential coding sequences were predicted using programs that are available through Internet websites, i.e. GeneMark (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi) and Glimmer (ftp://ftp.tigr.org/pub/software/Glimmer/).

2.24.2. **Artemis**

Artemis is a software package for the annotation of DNA sequences. It is a power-tool that allows visualisation of the sequence features and analyses of the sequence within its six-frame translation (Rutherford *et al.*, 2000). It was developed by The Welcome Trust Sanger Institute, Cambridge (http://www.sanger.ac.uk/Software/Artemis). To determine the potential protein-coding regions, the nucleotide sequence was analysed for the position of the initiation codons (ATG, GTG or TTG) and of stop codons in all possible
reading frames. The coding sequences (CDS) were confirmed by referring to the codon usage table of *H. influenzae* Rd (http://www.kazusa.or.jp/codon). The nucleotide sequences of the element were entered into Artemis and each sequence of the putative CDS were translated into amino acids and searched for homology with other sequences in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl.html) and GenBank (http://www.ncbi.nlm.nih.gov) based on the FASTA (Pearson and Lipman, 1988; Pearson, 1990), BLASTP and BLASTX (Altschul et al., 1990; Altschul et al., 1997) algorithms. The default settings used for FASTA are: Program, fasta3; database, protein; an expectation (E) upper value of 10, and lower value set to the default. The E value represents the number of hits one can ‘expect’ to find by chance when searching a database of a particular size. For the BLAST algorithm, the default settings used was for bacteria; alignment via pairwise, PSI-blast with inclusion threshold of 0.005 and was formatted semi-automatically. Known genes and putative functions were assigned for individual ORFs by inspection of the search outputs.

Potential proteins were examined against the protein feature prediction tools: Transmembrane helix motif (TMHMM) (http://cbs.dtu.dk/services/TMHMM-2.0/), SignalP Prediction Server (http://www.cbs.dtu.dk/services/SignalP/); Protein Motif databases: Prosite (http://www.expansv.ch/prosite/), and Pfam (http://www.sanger.ac.uk/Softwate/Pfam/index.shtml). The analyses were downloaded into Artemis to further characterise the ORFs. The computer program PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) was used to predict protein localisation sites in prokaryotic cells.
2.25. Programs for sequence comparison

2.25.1. GCG

The GCG (Genetics Computer Group Inc., Madison, Wisconsin) is the programme used for sequence comparison including to the sequences from the GenBank and EMBL databases. This package allows the manipulation and analysis of nucleic acid and protein sequences. This package facilitates comparison between two or more sequences, database searching and retrieval, gene finding and pattern recognition, and translation (http://www.molbiol.ox.ac.uk).

2.25.2. Blixem

Blixem was used in the preliminary stage to predict the position and orientation of the ICEHin1056 contigs compared to the position of contigs of another mobile element, ICEHin299 which was also being whole sequenced (Sonnhammer and Durbin, 1994).

2.25.3. Repeat sequences

Tandem repeat sequences on ICEHin1056 were searched using a Tandem Repeat Finder programme available on the website (http://c3.biomath.mssm.edu) (Benson, 1999). Inverted repeats on the sequence were identified using the Palindrome program (http://bioweb.pasteur.fr/seqanal/interfaces/palindrom.html).

2.25.4. Artemis Comparative Tools

The DNA sequences were compared visually by using Artemis Comparison Tool (ACT), developed by the Sanger Institute (http://www.sanger.ac.uk/Software/ACT/). Two or more DNA sequences were compared using ACT that allowed alignment of sequences based on the Artemis program (Rutherford et al., 2000). The nucleotide sequences of the
genomes/plasmids were input and translated by TBLASTX into amino acid for comparison.

2.25.5. **ClustalX and ClustalW**

The sequence alignment and tree estimation programme ClustalX, was used to align two or more DNA sequences or proteins. It provided an integrated environment for performing multiple sequence and profile alignments and analysing the results (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html). This programme is able to match the selected sequences, line them up so that the identities, similarities and differences can be seen. Phylogenetic trees were constructed from a total of 100 bootstrapped replicate re-sampling data sets to provide confidence estimates for tree topologies and viewed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

ClustalW was used to estimate the percentage of sequence homology between genes using pairwise alignment (http://www.ebi.ac.uk/clustalw/).

2.25.6. **Molecular Evolutionary Genetic Analysis (MEGA)**

A programme to estimate evolutionary distances and reconstructing phylogenetic trees from sequence analyses was used (Kumar et al., 2001). Aligned sequences from ClustalX were converted into MEGA files for construction of UPGMA with bootstrap values of 1000 repetitions.

2.25.7. **SplitsTree decomposition**

MEGA files saved as nexus files were downloaded into a programme called SplitsTree (http:bibiserv.techfak.uni-bielefeld.de/splits). This programme uses the split
decomposition method to analyse and visualise distances between gene sequences in the ICEs sequences.
CHAPTER THREE

PHENOTYPIC CHARACTERISTICS *H. INFLUENZAE STRAIN 1056*

3.1. Introduction

Bacterial resistance to antimicrobial drugs is increasing worldwide and is producing an impact on medical practice and antibiotic treatment costs of patients (Cohen, 1992; Cosgrove and Carmeli, 2003). Antibiotic resistance is often associated with mobile elements carrying antibiotic resistance genes.

In clinical isolates of *H. influenzae*, antibiotic resistant genes are most often found integrated into the chromosome, but following transfer by conjugation to a recipient strain, extra-chromosomal plasmid is usually detectable in the recipient (Dimopoulou *et al.*, 1997; Stuy, 1979). This type of element that can integrate and excise, and transfer by conjugation has been termed an integrative and conjugative element (ICE) (Burrus *et al.*, 2002a).

3.2. Objectives

Dimopoulou *et al.* (1992) earlier studied the mobilization of mobile elements present in *H. influenzae* and isolated in the UK (Dimopoulou *et al.*, 1992). In this chapter, the phenotypic characteristics of the mobile element found in *H. influenzae* 1056 were investigated. First, to confirm the earlier studies of Dimopoulou *et al.* (1992) and second to study transfer under a range of additional conditions. It was also important to characterise the conjugative properties prior to fully sequencing a mobile element.
3.3. Materials and Methods

3.3.1. Bacterial strains

Donor and recipient strains used in *H. influenzae* in conjugation studies are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Donor</th>
<th><em>H. influenzae</em> strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1056</td>
<td></td>
</tr>
<tr>
<td>XT3</td>
<td></td>
</tr>
<tr>
<td>XT11</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recipient</th>
<th><em>H. influenzae</em> strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rd\textsuperscript{im}\textit{recA}\textsuperscript{-}</td>
<td></td>
</tr>
<tr>
<td>Rd\textsuperscript{im}\textit{recA}\textsuperscript{-}</td>
<td></td>
</tr>
<tr>
<td>Rd\textsuperscript{if} \textit{recA}\textsuperscript{-}</td>
<td></td>
</tr>
<tr>
<td>XT9</td>
<td></td>
</tr>
</tbody>
</table>

3.3.2. Minimum inhibition concentration test

The minimum inhibition concentration (MIC) was performed, as described in the materials and methods (Chapter 2) on the *H. influenzae* strains 1056 and Rd (strep\textsuperscript{7}) against ampicillin, chloramphenicol, tetracycline and streptomycin.

3.4. Results and discussion

3.4.1. Minimum inhibition concentration (MIC)

The sensitivity/resistance of the strains against antibiotics was carried out prior to mating experiments. The MIC of the isolates is shown in Table 3.2. Strain 1056 is resistant to ampicillin, chloramphenicol and tetracycline.
Table 3.2. Minimum inhibition concentration of strains of *H. influenzae*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ampicillin (ug/ml)</th>
<th>Chloramphenicol (ug/ml)</th>
<th>Tetracycline (ug/ml)</th>
<th>Streptomycin (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1056</td>
<td>96</td>
<td>16</td>
<td>16</td>
<td>0.25</td>
</tr>
<tr>
<td>Rd&lt;sup&gt;sm&lt;/sup&gt;&lt;sup&gt;rec&lt;/sup&gt;</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>&gt;1024</td>
</tr>
</tbody>
</table>

3.4.2. *To determine the rate of plasmid transfer to recA<sup>+</sup> and recA<sup>-</sup> recipients*

In this experiment, the conjugative properties of this mobile element, p1056 was conjugated with Rd strain. To control for the possible role of transformation and homologous recombination, the frequency of transfer between strain 1056 to both recA<sup>+</sup> and recA<sup>-</sup> strains of Rd was studied. The recA<sup>+</sup> gene product in *H. influenzae* is necessary for transformation and homologous recombination (Stuy, 1980b). In its absence, acquisition of resistance genes by transformation will not occur.

Conjugation experiments were carried out both on solid and in liquid media. The result showed in Table 3.3 shows that the rate of transfer of the p1056 by conjugation on solid medium is ~10<sup>-6</sup> per donor cell. This result was in agreement with the findings of Stuy (1979) and Dimopoulou *et al.* (1992), who obtained a frequency of transfer of 10<sup>-7</sup> per donor cell (Dimopoulou *et al.*, 1992; Stuy, 1979). No significant difference in the efficiency of transfer between the recA<sup>+</sup> and recA<sup>-</sup> recipients was observed. This essentially eliminates transformation as the main mechanism of transfer. This is consistent with the findings of Dimopoulou *et al.* (1992) where it was shown that DNase had no effect on transfer efficiency (Dimopoulou *et al.*, 1992).

The efficiency of transfer in liquid medium was 3-log orders lower than on solid media. A similar observation was also obtained during transfer of chloramphenicol and tetracycline resistance into *H. influenzae* recipients by other investigators (van Klingerem *et al.*, 1977). Stuy (1979), however, failed to observe any conjugative transfer in liquid suspension (Stuy, 1979).
Transconjugants selected on tetracycline and streptomycin resistant plates were picked and checked for ampicillin and chloramphenicol resistance. Co-transfer of antibiotic resistance was observed, consistent with the successful transfer of an element as a unit.

Table 3.3. Determination of the rate of transfer of antibiotic resistance of *H. influenzae*. Matings of *H. influenzae* strain 1056 (amp', tet', chlor') with *H. influenzae* Rd (strep') on solid and liquid medium.

<table>
<thead>
<tr>
<th>Donor*</th>
<th>Recipient*</th>
<th>Trans-conjugant</th>
<th>Rate of transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em> 1056 (cfu)</td>
<td><em>H. influenzae</em> Rd (cfu)</td>
<td>(cfu)</td>
<td>(No. of transconjugants per donor cell)</td>
</tr>
<tr>
<td>Selection</td>
<td>Mating experiment on solid medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tet</td>
<td>Sm</td>
<td>Sm</td>
</tr>
<tr>
<td>1056 x Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7.3 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>5.6 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>1056 x Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.3 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>-</td>
<td>2.1 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mating experiment in liquid medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1056 x Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.3 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>2.7 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>1056 x Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.1 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>-</td>
<td>2.9 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tet: tetracycline (2 μg/ml), Sm: streptomycin (20 μg/ml). Results represent the average of experiments done in duplicate twice. Within experimental variation was less than half of a log. *No. of cells resistant to streptomycin ≤ 10<sup>6</sup> cfu; *No. of cells resistant to tetracycline ≤ 10<sup>10</sup> cfu.

3.4.3. **Re-transfer from a transconjugant**

In this experiment, the transconjugant XT3 (amp', tet', chlor' in strep' host) that was obtained from the mating of Rd<sup>sm</sup> recA<sup>+</sup>, was used as the donor and Rd<sup>nif</sup> recA<sup>+</sup> was used as the recipient. The experiment was carried out using either solid or liquid medium. Table 3.4 shows the summary of the results. High frequency transfer of ~10<sup>10</sup> per donor cell was achieved on solid medium. The rate of transfer in liquid medium was ~10<sup>7</sup> per donor cell.
The 5-log increase in the frequency of transfer of the transconjugant XT3 over the original isolate 1056, is dramatic. This difference has been previously reported by Thorne and Farrar (1975), Stuy (1979) and Deneer et al. (1982) (Deneer et al., 1982; Stuy, 1979; Thorne and Farrar, 1975). The difference in frequency of transfer has been inferred to indicate that the resistance element is chromosomally integrated in the primary isolate while predominantly extra-chromosomal in the transconjugant. This is plausible as excision of the element is likely to be rate limiting in conjugal transfer.


<table>
<thead>
<tr>
<th>Selection</th>
<th>Donor*</th>
<th>Recipientb</th>
<th>Trans-conjugant (cfu)</th>
<th>Rate of re-transfer (No. of transconjugants per donor cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating experiment on solid medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XT3 x RdSmrecA+</td>
<td>1.2 x 10^8</td>
<td>3.6 x 10^9</td>
<td>2.0 x 10^7</td>
<td>1.6 x 10^3</td>
</tr>
<tr>
<td>Mating experiment in liquid medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XT3 x RdSmrecA+</td>
<td>5.0 x 10^{10}</td>
<td>9.1 x 10^{11}</td>
<td>2.3 x 10^{4}</td>
<td>4.6 x 10^{7}</td>
</tr>
</tbody>
</table>

Tet: tetracycline (2 μg/ml), Rif: rifampicin (20 μg/ml). Results represent the average of experiments done in duplicate twice. Within experimental variation was less than half of a log. "No. of cells resistant to rifampicin ≤10^9" cfu; b "No. of cells resistant to tetracycline ≤10^{10}" cfu.

3.4.4. Evidence for incompatibility

In this experiment, the effect of the element in the recipient cell on the proficiency transfer was investigated. Mating experiments involved the donor strain XT11 and recipient strain XT9 and was performed as described above. Strain XT11, the donor was mated with strain XT9. Transconjugants were selected on streptomycin and tetracycline. XT11 was mated into RdSmrecA+ as a control.
Table 3.5 shows the rate of transfer. There is 3-log reduction of transfer of the element into a recipient already containing the element over a recipient without the element. Incompatibility is usually associated with both plasmids sharing the same replication of origin (Hardy, 1986). Incompatibility may have different causes including randomisation during replication and partition, and systems that stabilise plasmid maintenance by killing plasmid-free segments from within may give rise to incompatibility effects. Randomisation takes place during replication as well as during partition and leads to distortion of the ratio. However, in this experiment there was no complete exclusion. Such reduced efficiency of transfer has been observed with SXT (M. Waldor, personal communication). The mechanism explaining this reduction is not known.

Table 3.5. Determine of the rate of incompatibility in *H. influenzae*. Matings of *H. influenzae* XT11 (amp<sup>r</sup>, tet<sup>r</sup>, chlor<sup>r</sup>, rif<sup>r</sup>, recA<sup>+</sup>) with *H. influenzae* XT9 (amp<sup>r</sup>, strep<sup>r</sup>, recA<sup>+</sup>) and Rd<sup>sm</sup> recA<sup>+</sup> on solid medium.

<table>
<thead>
<tr>
<th>Selection</th>
<th>Donor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recipient&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Transconjugant&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rate of re-transfer&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>H. influenzae</em> XT11 (cfu)</td>
<td><em>H. influenzae</em> XT9/Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt; (cfu)</td>
<td>Tet&amp;Strep (cfu)</td>
<td>(No. of transconjugants per donor cell)</td>
</tr>
<tr>
<td>XT11 x XT9</td>
<td>8.7 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>6.1 x 10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>XT11 x Rd&lt;sup&gt;sm&lt;/sup&gt; recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.2 x 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>3.9 x 10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>2.0 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Tet: tetracycline (2 μg/ml), Strep: streptomycin (20 μg/ml). Results represent the average of experiments done in duplicate twice. Within experimental variation was less than half of a log. *No. of cells resistant to streptomycin <10<sup>8</sup> cfu; "No. of cells resistant to tetracycline <10<sup>10</sup> cfu.

3.4.5. **Electron microscopy for pilus**

The electronmicroscopist was blinded to the samples. The controls consisted of *H. influenzae* Rd and experimental samples consisted of XT3. Each experiment was done in triplicate and repeated once. Long thin pili as seen in Fig. 3.1 were only observed in grids containing XT3, but all the pili were detached from the cells. The pili observed were
morphologically unlike those reported for piliated *H. influenzae* by Guerina *et al.* (1982) and Pichichero *et al.* (1982) (Guerina *et al.*, 1982; Pichichero *et al.*, 1982). Those pili were thought to be involved in cell adhesions.

Conjugative plasmids usually produce pili (Bradley, 1980). In a conjugative plasmid, the presence of pili is believed to be associated with conjugative activity. For DNA transfer by conjugation to take place, physical contact is required between a recipient cell to a donor cell. However, due to the charges associated with their cell outer layers, bacterial cells are naturally repellent to one another. Thus the cells usually require extracellular filaments to form the initial contacts to allow two cells to interact. During this process, the pilus of a donor cell will retract the surface of a recipient cell for intimate association, followed by the formation of mating bridge to serve as a conduit for DNA (Silverman, 1997; Waters, 1999). The presence of pili in *H. influenzae* XT3 is consistent with its functional role in conjugative DNA transfer.

Conjugative pili fall into three basic morphological types which are described as: thin flexible (*IncF*), thick flexible (*IncI*) and thin rigid filaments or rods (*IncN* and *IncP*) (Bradley, 1980). From the electron-micrograph (Fig 3.1), the pili of *H. influenzae* XT3 appear to be thin and rigid. It is apparently similar to the features of the sex pili of the *IncN* and *IncP* plasmids that are thin and rigid (Bradley, 1983). However, visual observation is not a reliable method to classify the type of pilus.

To predict the type of pilus, Bradley *et al.* (1980) have proposed a criterion by calculating the ratio of solid mating frequency to the ratio of liquid mating frequency. From the results obtained above (Tables 3.2 to 3.5), a comparison of transfer frequency between solid and liquid media is tabulated in Table 3.6. A frequency ratio of approximately 1, indicates that the transfer was as efficient in solid as in liquid, which is typical of plasmids with thin flexible pili. If the frequency ratio ranges between 45 and
470, it indicates thick flexible pili, but a plasmid with rigid pili would have a very high ratio of transfer frequency (>470) (Bradley et al., 1980).

The results obtained from the experiments above, shows that there is a very high ratio of transfer frequency in solid medium compared to that in liquid medium. This also suggests that p1056 possess rigid type pili. This renders them fragile and they fragment. The lack of intact pili on EM is also consistent with such fragile rigid pili.

Fig 3.1. Electron micrographs of pili of *H. influenzae* 1056. The arrows on the figures show the long fine pili. The bar represents 100 nm.
Table 3.6. A comparison of the ratio of frequency of transfer in solid and liquid media.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Rate of transfer</th>
<th>Ratio of transfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(No. of transconjugant per donor cell)</td>
<td>(No. of transconjugant per donor cell)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solid</td>
<td>Liquid</td>
</tr>
<tr>
<td>1056</td>
<td>Rd&lt;sup&gt;sm&lt;/sup&gt;recA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>$2.3 \times 10^6$</td>
<td>$5.2 \times 10^9$</td>
</tr>
<tr>
<td>1056</td>
<td>Rd&lt;sup&gt;sm&lt;/sup&gt;recA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>$1.2 \times 10^6$</td>
<td>$3.5 \times 10^9$</td>
</tr>
<tr>
<td>XT3</td>
<td>Rd&lt;sup&gt;sm&lt;/sup&gt;recA&lt;sup&gt;−&lt;/sup&gt;</td>
<td>$1.6 \times 10^7$</td>
<td>$4.5 \times 10^7$</td>
</tr>
</tbody>
</table>

3.5. Conclusion

The experiments carried out in this chapter showed that the *H. influenzae* 1056 element p1056 was transferred by conjugation to a recipient. The increased efficiency of transfer by a transconjugant as a donor is consistent with excision from the chromosome in the primary isolate and on extra-chromosomal locations in the transconjugant.

The presence of a pilus associated with the presence of p1056 is consistent with it being the sex pilus. From these results, it can be concluded that *H. influenzae* 1056 contains a self-transmissible element. Given the observation of Dimopoulou *et al.* (1992) showing integration at tRNA<sup>Leu</sup> this is an example of an integrative and conjugative element or ICE (Dimopoulou *et al.*, 1992). The element will therefore, be referred to as ICE<sub>Hin1056</sub> (previously p1056).
CHAPTER FOUR

COMPLETE NUCLEOTIDE SEQUENCE OF THE MOBILE

ELEMENT ICEHin1056 OF H. INFLUENZAE 1056

4.1. Introduction

To date, more than 501 complete sequences of plasmid have been deposited into the GenBank, but so far, only four plasmids found in Haemophilus spp. have been completely sequenced. Two of these were from H. influenzae biotype aegyptius (NC_004058 and NC_004846), one each from H. ducreyi (NC_005329) and from H. paragallinarum (NC_005245).

Although both plasmids found in H. influenzae biogroup aegyptius (H. aegyptius) 32-kb pF3028 (Kroll et al., 2002), and 32-kb pF3031 (Mukhopadhyay, S. and Actis, L.A., unpublished) were associated with Brazilian purpuric fever none encode virulence traits. A small 6-kb plasmid pNAD1, isolated from H. ducreyi, contains the nadV gene that confers NAD independence (Martin et al., 2001). Another Haemophilus 6-kb plasmid, p250 was recently deposited in the NCBI and was isolated from H. paragallinarum strain HP250, a strain predominantly found in avian hosts. The complete sequence of this plasmid revealed a haemocin (bacteriocin) operon similar to those of H. influenzae (Terry et al., 2003). To date, no plasmids of H. influenzae type b have been completely sequenced and submitted into the GenBank.

4.2. Objectives of whole sequencing of ICEHin1056

Antibiotic resistance in bacteria has been associated with the presence of plasmids (Khan et al., 1974). Plasmids encoding gene resistance to antibiotics play an important role in the acquisition and transmission of intra-species and inter-species of bacteria. The factors
that influence the rapid spread of antibiotic resistance in bacteria are unclear. An important step in understanding the genetic mechanism involved in the transmission of antibiotic resistance in *H. influenzae*, is the analysis of the complete sequence of an antibiotic resistance element (ICE). The analysis of the nucleotide sequence and the core genes of a resistance plasmid will:

(i) provide an understanding on the genetic relationship between resistant elements;
(ii) provide an understanding on the genetic relationship between elements from different *Haemophilus* spp. from various geographical locations; and
(iii) enable early predictions as to the biological role of the large conjugative *H. influenzae* ICEs.

In this study, the whole nucleotide sequence and annotation of ICE*Hin1056* is produced and investigated.

4.3. Materials and methods

In order to obtain the complete sequence of ICE*Hin1056*, both sequencing strategies: the primer walking and the shotgun methods were used.

Whole ICE*Hin1056* was previously digested with *PstI* and run in an agarose gel (Dimopoulou *et al.*, 1997). The fragments from the gel were excised and ligated into pBluescript vector and transformed into DH5α *E. coli* cells. A 12-kb fragment clone, designated pB7 was initially sequenced using universal M13 primers. The sequences obtained were used to design subsequent primers to ‘walk’ into the insert, towards each end of the cloned fragment. As an alternative, shotgun approached was used. The whole element was sonicated, ligated into pUC19 and plasmid libraries were constructed. The inserts were sequenced, assembled and analysed using computer programs.
4.4. Results and discussion

4.4.1. Primer walking

Sequencing by primer walking on pB7 was simple except that it was time consuming because it required many primers to be designed. New primers could only be designed when the previous sequence results were obtained. Therefore, the shotgun approach was then adopted to expedite whole sequencing.

4.4.2. To determine the optimum length of time for sonication

A comparative study was carried out to determine the optimum length of time for sonication. It showed that a 10-s sonication produced fragments of the required sizes. Prolonged sonication resulted in smaller fragments (Fig. 4.1). Fig 4.1 shows that when DNA plasmid was sonicated for 10 s, the fragment sizes were of the desired size. When sonication was carried out for 20 s, the sheared DNA produced a smear, as shown in lane 5. Thus a 10-s sonication was then adopted.

4.4.3. Construction of plasmid libraries

Six random plasmid libraries for ICEHin1056 were constructed. Most of the inserts were found to be smaller than 500 bp indicating inefficiency during transformation. Only plasmids with inserts between 500 bp to 2.0 kb length were selected for sequencing as shown in Fig. 4.2.
Fig. 4.1. A comparative study to determine the optimum sonication time for plasmid to obtain fragments of sizes between 500 bp to 2.0 kb. Lane 1: 1-kb DNA ladder; lane 2: uncut chromosomal DNA; lane 3-6 shows various sonication durations; lane 3: 10 s; lane 4: 15 s and lane 5: 20 s.

Fig. 4.2. Gel electrophoresis of inserts (by PCR) of plasmid clones derived from the random library. Lanes 1 & 20: 1-kb DNA ladder, lane 2: pUC19 (negative control) and lanes 3-19: pUC19 with random size of inserts.
4.4.4. Sequence assembly of ICEHin1056

All of the sequences obtained from random libraries and by primer walking were assembled. The average usable read length analysed by the ABI 377 sequencer was approximately 500 bp. The sequences were obtained from both forward and reverse strands. In the initial stage of assembly, there were small contigs consisting of one or two sequences and a few contigs with an average length of 8 to 17 kb. When blast searches of these contigs were carried out, most of the small contigs were found to be *H. influenzae* Rd sequences. This suggested that ICEHin1056 DNA was contaminated with chromosomal DNA. Improved EtBr-CsCl purified plasmid DNA was used for the later libraries of ICEHin1056 (Fig. 4.3). Sequences that belonged to *H. influenzae* Rd chromosomal sequences were removed manually.

A total of 31 assemblies were carried out. Ultimately, 679 sequences were needed to assemble the complete element. Before achieving closure of a circular element, manual editing was needed to re-arrange tracefiles belonging to either of the two large repeats. The presence of the repeats was predicted from examining the template display (Fig. 4.4). The assembly adjacent to this ‘pile-up’ contained two species of sequences (Fig. 4.5). When these tracefiles were manually edited the assembly resulted in one large contig of 59,393 bp. The average length of the readings was 569 bp, producing a total length of 386,236 bases, i.e. 6.5 fold of the element.
Fig. 4.3. Agarose gel electrophoresis of chromosomal and plasmid DNAs prepared by CsCl-EtBr gradient centrifugation method. Lane 1: 1-kb DNA ladder, lane 2: chromosomal DNA of *H. influenzae* 1056, lane 3: ICE*Hin*1056 and lane 4: ICE*Hin*299.

Fig. 4.4. Template display of a single contig of ICE*Hin*1056. Note the pile-up of template sequences at 50-kb position.
Fig. 4.5. Part of ICEHin1056 sequences displayed on contig editor. Note the two patterns of sequences highlighted in dark and clear background.

4.4.5. Assignment of potential coding regions of ICEHin1056

The whole sequence of ICEHin1056 was loaded into the Artemis program for annotation. To predict the coding regions, the entire nucleotide sequence was searched for ORFs >100 amino acids. A total of 64 ORFs were obtained by using ATG, GTG or TTG as the translational start codons and TTA, TAG or TGA as the stop codons. Not all of these ORFs were preceded by Shine-Dalgano sequences. This analysis showed that the element contains 64 ORFs with 48 in the forward direction and 16 in the reverse direction. This directional bias therefore, indicates that the ICEHin1056 replication proceeds mostly in forward orientation.

All of the ORFs were translated into amino acid sequences and then subjected to homology search analysis using FASTA, BLASTP and BLASTX, to compare the protein sequences available in NCBI (National Centre Biotechnology Information) and EMBL (European Molecular Biology Laboratory). From the protein homology search, the ORFs of ICEHin1056 were shown to encode 62 complete gene proteins and two pseudo-genes.
Amongst the complete genes, 32 showed similarity to genes of H. ducreyi 35000HP (acc. no.: AE017153). Of the complete genes, 22 showed homology to genes with known functions, while the remaining genes have not been functionally characterised. Six of the hypothetical proteins did not show any significant matches with any sequences deposited in public databases. No ORFs directly related to bacterial pathogenesis were identified.

4.4.6. **General properties of ICEHin1056**

The nucleotide position 1 of the mobile element was orientated to correspond to the start of the attP sequence. This point was chosen because it is the common location for site-specific recombination and is present in many integrative mobile elements. ICEHin1056 has an overall G+C% content of 39.1%, which is slightly higher than the G+C% content of H. influenzae Rd (38.2%). The G+C content is unevenly distributed around the circular mobile element (Fig. 4.6). Two regions showed peaks of G+C% content higher than the mean. The first region lies between position 14,581 to 20,370-bp and has a G+C % content of 45.9%, while the second region lies between 50,667 and 52,733 bp and has a G+C% content of 45.3%. In comparison, the rest of the element has a G+C% content of 36.5%. The anomalies of the G+C content at these regions correlates to the presence of transposons Tn3 and Tn10. Overall percentages of the purines and pyrimidines of the whole element are: A (30.6%), C (19.1%), G (20.0%) and T (30.3%).

Throughout the 60-kb element, nine copies of the 9-bp Haemophilus uptake sequence (5'-AAGTGCGGT) were identified. The genome of H. influenzae Rd has 1,465 copies of uptake sequence (Fleischmann et al., 1995). This means that the element has approximately one copy of uptake sequence for every 6.5-kb of the element, in comparison to one copy of the uptake sequence to every 1.2-kb of the genome. Similar numbers of uptake sequence has also been observed in a 24-MDa plasmid isolated from
*H. influenzae* biotype aegyptius (Kroll *et al.*, 2002). The distribution of the uptake sequence in the whole element is random but is mostly located in the intergenic regions.

The general features of ICE*Hin*1056 are shown in Table 4.1.

![GC Content (%) Window size: 1000](image)

**Table 4.1. Summary of ICE*Hin*1056 properties.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total size</td>
<td>59,393 bp</td>
</tr>
<tr>
<td>G+C content</td>
<td>39.88%</td>
</tr>
<tr>
<td>Predicted coding sequences</td>
<td>62</td>
</tr>
<tr>
<td>Pseudo-genes</td>
<td>2</td>
</tr>
<tr>
<td>Coding density</td>
<td>84.4%</td>
</tr>
<tr>
<td>Average gene length</td>
<td>798</td>
</tr>
<tr>
<td>No. of uptake sequence</td>
<td>9</td>
</tr>
</tbody>
</table>

4.4.7. **Overview of the genetic organisation of ICE*Hin*1056

Computational analysis of the ORFs of ICE*Hin*1056 is illustrated in Fig. 4.7, and the annotations are summarised in Table 4.2. From the annotation of the assembly, it was observed that the genes of the element were arranged in three putative functional modules (Fig. 4.8). The first module that lies from position 1 to 14,719-bp consists of a cluster of putative genes encoding proteins involve in replication. The second module that lies...
between 14,720 to 26,581-bp comprises tetracycline and chloramphenicol resistance genes. The third, being the largest module, lies between position 26,582 to 59,393-bp, consists of a number of hypothetical proteins, five homologues of genes associated with DNA transfer and one being an integrase gene. However, within this region, ampicillin resistance genes homologous to Tn3 is inserted that lies from position 50,985 to 52,585-bp.
Fig. 4.7. Genetic organisation of a 60-kb mobile element of H. influenzae, ICEHin1056 generated by Artemis. Genes are represented by arrow boxes. Putative functions of the genes are described in detail in Table 4.2.
Fig. 4.8. Genetic organisation of ICEHin1056 according to their putative functions. The modules are differently shaded to indicate clusters of genes in the same function module.
Table 4.2. List of predicted genes in ICEHin1056. Amino acid similarity of putative proteins encoded by ICEHin1056 to sequences in public databases.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Coordinates</th>
<th>Gene name</th>
<th>Predicted protein</th>
<th>Database similarity (% aa identity)</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1056.01</td>
<td>141-977</td>
<td>soj</td>
<td>Putative chromosome partitioning protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP possible chromosome partitioning related protein SWALL: AAP95855 (EMBL: AE017153) (83.33 in 276 aa)</td>
<td>1.1e-93</td>
</tr>
<tr>
<td>p1056.02</td>
<td>979-2334</td>
<td>dnaB</td>
<td>Replicative DNA helicase</td>
<td>Similar to <em>H. ducreyi</em> 35000HP replicative DNA helicase DnaB1 or HD0973 SWALL: AAP95854 (EMBL: AE017153) (70.94 in 444 aa)</td>
<td>5.2e-119</td>
</tr>
<tr>
<td>p1056.3</td>
<td>2327-4015</td>
<td>Conserved hypothetical protein</td>
<td>Similar to central region of <em>H. ducreyi</em> 35000HP hypothetical protein HD0971 SWALL: AAP95852 (EMBL: AE017153) (70.18 in 218 aa)</td>
<td>2.5e-53</td>
<td></td>
</tr>
<tr>
<td>p1056.04</td>
<td>4015-4566</td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0969 SWALL: AAP95850 (EMBL: AE017153) (79.0 in 181 aa)</td>
<td>2.6e-56</td>
<td></td>
</tr>
<tr>
<td>p1056.05</td>
<td>4716-5939</td>
<td>Conserved hypothetical protein</td>
<td>Similar to N-terminal region of <em>H. ducreyi</em> 35000HP hypothetical protein HD0968 SWALL: AAP95849 (EMBL: AE017153) (68.35 in 237 aa)</td>
<td>1e-71</td>
<td></td>
</tr>
<tr>
<td>p1056.06</td>
<td>6164-6919</td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0966 SWALL: AAP95847 (EMBL: AE017153) (72.44 in 254 aa)</td>
<td>5.3e-05</td>
<td></td>
</tr>
<tr>
<td>p1056.07</td>
<td>6919-7407</td>
<td>intrR</td>
<td>Putative integrase regulator R</td>
<td>Low similarity to <em>H. ducreyi</em> hypothetical protein SWALL: AAP958445 (EMBL: AE017153) (74.41 in 43 aa), and to <em>Pseudomonas sp.</em> B13 integrase regulator R SWALL: Q8341 (EMBL: AJ536665) (28.5 in 134 aa)</td>
<td>6.9e-23</td>
</tr>
<tr>
<td>p1056.08</td>
<td>7654-8076</td>
<td>ssb</td>
<td>Single-strand binding protein</td>
<td>Similar to <em>H. influenzae</em> single-strand binding protein ssb or H10250 SWALL: P44409 (EMBL: HI04997) (66.66 in 168 aa)</td>
<td>5.1e-40</td>
</tr>
<tr>
<td>p1056.09</td>
<td>8100-8621</td>
<td></td>
<td>Putative lipoprotein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0961 SWALL: AAP95842 (EMBL: AE017153) (46.51 in 172 aa)</td>
<td>8.6e-08</td>
</tr>
<tr>
<td>p1056.10c*</td>
<td>8837-9394c</td>
<td>osa</td>
<td>Hypothetical protein</td>
<td>Similar to the C-terminal region of <em>S. flexneri</em> protein Osa SWALL: P29772 (EMBL: PP30471) (36.97 in 119 aa)</td>
<td>1.3e-139</td>
</tr>
<tr>
<td>p1056.11</td>
<td>9482-11530</td>
<td>topB</td>
<td>DNA topoisomerase III</td>
<td>Similar to <em>H. ducreyi</em> 35000HP DNA topoisomerase III topB1 or HD0954 SWALL: AAP95835 (EMBL: AE017153) (79.11 in 450 aa)</td>
<td>5.4e-33</td>
</tr>
<tr>
<td>p1056.12</td>
<td>12415-12843</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0951 SWALL: AAP95832 (EMBL: AE017153) (57.63 in 144 aa)</td>
<td>3e-20</td>
</tr>
<tr>
<td>p1056.13</td>
<td>12919-13599</td>
<td></td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p1056.14</td>
<td>13738-14208</td>
<td>radC</td>
<td>Putative DNA-repair-like protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP conserved possible DNA repair protein HD0945 SWALL: AAP95827 (EMBL: AE017153) (41.02 in 156 aa)</td>
<td>0.87</td>
</tr>
<tr>
<td>p1056.15</td>
<td>14301-14780</td>
<td></td>
<td>Hypothetical protein</td>
<td>Very low similarity to <em>H. ducreyi</em> 35000HP hypothetical protein HD0944 SWALL: AAP95826 (EMBL: AE017153)</td>
<td></td>
</tr>
</tbody>
</table>

* c: reverse direction
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Coordinates</th>
<th>Gene name</th>
<th>Predicted protein</th>
<th>Database similarity (% aa identity)</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1056.16</td>
<td>14720-15838</td>
<td>IS/10</td>
<td>Transposase of IS/10</td>
<td>Identical to <em>E. coli</em> and <em>Salmonella Typhi</em> transposase (IS/10 transposase) R0085</td>
<td>1e-154</td>
</tr>
<tr>
<td>p1056.17</td>
<td>15964-16896</td>
<td>IS5</td>
<td>Transposases for IS5</td>
<td>Similar <em>E. coli</em> transposase insH for insertion sequence element IS5 SWALL:PO3837 (EMBL:U000399) (55.66 in 300 aa)</td>
<td>1.8e-53</td>
</tr>
<tr>
<td>p1056.18</td>
<td>17570-18211</td>
<td><em>cat</em></td>
<td>Chloramphenicol acetyltransferase</td>
<td>Identical to <em>Pasteurella piscicida</em> chloramphenicol acetyltransferase CAT SWALL:Q8RQP2 (EMBL:AB082569) (100 in 213 aa)</td>
<td>3e-63</td>
</tr>
<tr>
<td>p1056.19</td>
<td>18374-19321</td>
<td>IS30</td>
<td>Transposase IS30</td>
<td>Similar to <em>N. meningitidis</em> transposase, IS30 family SWALL:Q9JS28 (EMBL:AE002563) (41.5 in 318 aa)</td>
<td>2.3e-44</td>
</tr>
<tr>
<td>p1056.20</td>
<td>19653-20438</td>
<td>IS5</td>
<td>Transposase of IS5</td>
<td>Similar to <em>Vibrio cholerae</em> IS5 transposase VCA0472 and VCA0282, SWALL:Q9K218 (EMBL:AE004379) (58.30 in 259 aa)</td>
<td>2.4e-60</td>
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<tr>
<td>p1056.21</td>
<td>20822-21142</td>
<td>ybeB</td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>Salmonella Typhimurium</em>, <em>S. flexneri</em>, <em>E. coli</em>, and <em>S. Typhi</em> YbeB protein or YebB or YdcD or R0078 or HCM1.246c SWALL:Q9S457 (EMBL:AP005147) (100 in 106 aa)</td>
<td>7.7e-42</td>
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<tr>
<td>p1056.22</td>
<td>21024-21521</td>
<td>ybeA</td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>S. Typhi</em>, and <em>S. flexneri</em> 2A hypothetical protein R0079 SWALL:Q9K2K3 (EMBL:AF250878) (100 in 165 aa)</td>
<td>6e-63</td>
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<tr>
<td>p1056.23</td>
<td>21529-22215</td>
<td>ybdA</td>
<td>Putative transcriptional protein</td>
<td>Similar to <em>S. Typhimurium</em>, <em>S. flexneri</em>, <em>E. coli</em>, and <em>S. Typhi</em> YbdA protein or YebB or R0080 SWALL:Q9S456 (EMBL:AP005147) (97.8 id in 228 aa)</td>
<td>1.1e-90</td>
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<tr>
<td>p1056.24c</td>
<td>22193-22815c</td>
<td>tetA</td>
<td>Tetracycline repressor protein (pseudogene)</td>
<td>Pseudogene. Identical to <em>E. coli</em> tetracycline repressor protein class B from transposon Tn10 TetR SWALL:PO4483 (100 in 207 aa)</td>
<td>3.1e-85</td>
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<td>p1056.25</td>
<td>22897-24102</td>
<td>tetC</td>
<td>Tetracycline resistance protein</td>
<td>Identical to <em>S. Typhimurium</em>, <em>S. flexneri</em> 2a TetC protein SWALL:Q9K2Y4 (EMBL:AP005147) (100 in 401 aa)</td>
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<td>p1056.26c</td>
<td>24215-24808c</td>
<td>tetD</td>
<td>Putative transcriptional regulator</td>
<td>Identical to <em>S. Typhimurium</em>, and <em>S. flexneri</em> 2a putative transcriptional regulator TetD SWALL:Q9S45F25 (EMBL:AP005147) (100 in 197 aa)</td>
<td>9.5e-73</td>
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<tr>
<td>p1056.27</td>
<td>24896-25312</td>
<td>tetD</td>
<td>Putative transcriptional regulator of Tn10</td>
<td>Highly similar to <em>E. coli</em> transposon Tn10 TetD protein (SWALL:P28816) (98.55 in 138 aa)</td>
<td>1.6e-53</td>
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<tr>
<td>p1056.28c</td>
<td>25322-26530c</td>
<td>IS/10</td>
<td>Transposase of Tn10</td>
<td>Identical to <em>Citrobacter freundii</em> transposase Tnp SWALL:Q8GC95 (EMBL:AJ508060) (100 in 402 aa)</td>
<td>1.9e-170</td>
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<tr>
<td>p1056.29</td>
<td>26582-26931</td>
<td>Conserved hypothetical protein (pseudogene)</td>
<td>Pseudogene. Low similarity to central region of <em>H. ducreyi</em> 35000HP hypothetical protein HD0944 SWALL:AP95826 (EMBL:AE017153) (40.74 in 81 aa)</td>
<td>2.4e-08</td>
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<tr>
<td>p1056.30c</td>
<td>26989-27753c</td>
<td>Putative conserved membrane protein</td>
<td>Low similarity to <em>P. syringae</em> membrane protein, putative PSPT00411 SWALL:Q88AH9 (EMBL:AE016857) (26.19 in 252 aa)</td>
<td>1.3e-08</td>
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<td>27852-28481</td>
<td>pilL</td>
<td>Putative lipoprotein</td>
<td>Similar to the N-terminal of <em>P. aeruginosa</em> pilL2 or Ri086 SWALL: AAP98211 (EMBL:AY273989) (32.98 in 191 aa)</td>
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<td>p1056.32</td>
<td>28485-29225</td>
<td>Putative membrane protein</td>
<td>Similar to <em>S. Typhi</em> putative exported protein STY4558 SWALL: Q8Z1K6 (EMBL:AL627282) (35.77 in 246 aa)</td>
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<td>p1056.34</td>
<td>29978-30484</td>
<td>TraD</td>
<td>DNA transfer protein</td>
<td>Similar to <em>P. aeruginosa</em> hypothetical protein SWALL:Q8BPT3 (EMBL:AF440524) (42.51 in 167 aa)</td>
<td>4.1e-19</td>
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<tr>
<td>p1056.35</td>
<td>30481-32730</td>
<td>ttd</td>
<td>Hypothetical protein</td>
<td>Similar to <em>P. putida</em> hypothetical 80.3 kDa protein SWALL:Q8WM3 (EMBL:AJ344068) (59.04 in 713 aa), and similar to <em>Providencia rettgeri</em> TraD SWALL:Q8RL4 (EMBL:AY090559) (31.64 in 610 aa)</td>
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<td>p1056.36</td>
<td>32741-33049</td>
<td>H. ducreyi 35000HP hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0942 SWALL: AAP9824 (EMBL:AE017153) (46.72 in 107 aa)</td>
<td>7.2e-17</td>
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<td>p1056.37</td>
<td>33049-33735</td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0941 SWALL: AAP9823 (EMBL:AE017153) (74.56 in 228 aa)</td>
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<td>p1056.38</td>
<td>33866-34216</td>
<td>Putative export protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0940 SWALL: AAP9822 (EMBL:AE017153) (68.26 in 104 aa)</td>
<td>1.4e-20</td>
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<tr>
<td>p1056.39</td>
<td>34906-35277</td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0941 SWALL: AAP9820 (EMBL:AE017153) (60.16 in 123 aa)</td>
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<tr>
<td>p1056.41</td>
<td>36817-38217</td>
<td>Putative export protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0935 SWALL: AAP9817 (EMBL:AE017153) (61.23 in 485 aa)</td>
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<td>p1056.42</td>
<td>38227-38634</td>
<td>Putative lipoprotein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0934 SWALL: AAP9816 (EMBL:AE017153) (64.34 in 129 aa)</td>
<td>2.1e-30</td>
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<tr>
<td>p1056.43</td>
<td>38650-41520</td>
<td>Conserved hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0933 SWALL: AAP9815 (EMBL:AE017153) (75.40 in 797 aa)</td>
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<tr>
<td>p1056.44</td>
<td>38227-38634</td>
<td>Putative lipoprotein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0934 SWALL: AAP9816 (EMBL:AE017153) (64.34 in 129 aa)</td>
<td>2.1e-30</td>
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<tr>
<td>p1056.45</td>
<td>38650-41520</td>
<td>VirB4</td>
<td>Putative Type IV secretory pathway, <em>VirB4</em> components</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0933 SWALL: AAP9815 (EMBL:AE017153) (75.40 in 797 aa); and to <em>H. somnus</em> 2336 Type IV secretory pathway, <em>VirB4</em> components (acc. no. ZP_00132732)</td>
<td>0</td>
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<td>p1056.46</td>
<td>41528-41935</td>
<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0931 SWALL: AAP9813 (EMBL:AE017153) (47.40 in 135 aa)</td>
<td>1.8e-26</td>
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<td>p1056.47c</td>
<td>41945-42268c</td>
<td>Hypothetical membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0930 SWALL: AAP9812 (EMBL:AE017153) (39.80 in 103 aa)</td>
<td>5.7e-12</td>
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<tr>
<td>p1056.48</td>
<td>42277-43770</td>
<td>Putative membrane protein</td>
<td>Similar to <em>S. Typhi</em> putative membrane protein STY4579 SWALL: Q8Z1I6 (EMBL:AE016848) (42.8 in 500 aa)</td>
<td>6.4e-89</td>
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<th>Database similarity</th>
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<tr>
<td>p1056.49c</td>
<td>43826-44155c</td>
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<td>Hypothetical membrane protein</td>
<td>No significant database matches</td>
<td></td>
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<td>p1056.50c</td>
<td>44575-44982c</td>
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<td>Hypothetical exported protein</td>
<td>No significant database matches</td>
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<td>p1056.51c</td>
<td>44998-46998c</td>
<td></td>
<td>Putative exported protein</td>
<td>Similar to N-terminal of <em>H. ducreyi</em> 35000HP hypothetical protein HD0922</td>
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<td>p1056.52c</td>
<td>47013-47939c</td>
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<td>Putative exported protein</td>
<td>Similar to C-terminal of <em>H. ducreyi</em> 35000HP hypothetical protein HD0921</td>
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<tr>
<td>p1056.53c</td>
<td>47951-48388c</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Low similarity to C-terminal of <em>H. ducreyi</em> 35000HP hypothetical protein HD0920</td>
<td>3.9e-08</td>
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<tr>
<td>p1056.54c</td>
<td>48763-49125c</td>
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<td>Hypothetical membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0919</td>
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<tr>
<td>p1056.55c</td>
<td>49210-49443c</td>
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<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0918</td>
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<tr>
<td>p1056.56c</td>
<td>49985-50890c</td>
<td><em>traC</em></td>
<td>Putative primase protein</td>
<td>Similar to the <em>H. ducreyi</em> 35000HP probable DNA primase Trac or HD0917</td>
<td>9.8e-90</td>
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<td>p1056.57c</td>
<td>50985-51845c</td>
<td><em>bla</em></td>
<td>beta-lactamase precursor</td>
<td>Identical to <em>Acinetobacter baumannii</em> beta-lactamase blaTEM-1</td>
<td>1.7e-11</td>
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<tr>
<td>p1056.58c</td>
<td>52028-52585c</td>
<td><em>tnpR</em></td>
<td>Transposon Tn3 resolvase</td>
<td>Identical to <em>Salmonella enteritidis</em> resolvase</td>
<td>7.6e-60</td>
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<tr>
<td>p1056.59c</td>
<td>52884-53201</td>
<td></td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
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<tr>
<td>p1056.60c</td>
<td>53491-54189</td>
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<td>Hypothetical protein</td>
<td>Similar to <em>P. putida</em> hypothetical protein</td>
<td>8.8e-13</td>
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<tr>
<td>p1056.61c</td>
<td>54706-55260c</td>
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<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
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<tr>
<td>p1056.62c</td>
<td>55497-57404</td>
<td><em>tral</em></td>
<td>Conjugative relaxase</td>
<td>Similar to N-terminal of <em>H. ducreyi</em> 35000HP hypothetical protein HD0900</td>
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<td>p1056.63c</td>
<td>57495-58307</td>
<td><em>int</em></td>
<td>Putative site-specific recombinase</td>
<td>Similar to <em>H. ducreyi</em> 35000HP integrase/recombinase HD0897</td>
<td>2.7e-70</td>
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<tr>
<td>p1056.64c</td>
<td>58302-59078</td>
<td></td>
<td>Conserved phage-related hypothetical protein</td>
<td>Similar to, although shorter than, Bacteriophage phi CTX ORF28</td>
<td>6.9e-08</td>
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</table>

* c: reverse direction
Putative replication module

The nucleotide sequence from 1 to 14,719 bp contains 15 genes, only five of which have homology to genes of known functions. Thirteen of fifteen amino acid sequence in this region shares homology to sequences found in *H. ducreyi*. Their ORFs are in syntheny.

The first gene, p1056.01 is highly similar to the putative chromosome partitioning related protein (*soj*) of *H. ducreyi* 35000HP (acc. no. AE017153). *Soj* is also known as chromosome partitioning related protein and belongs to the *parA* family of genes that encode a protein that actively divides and distributes plasmid copies upon cell division (Autret *et al.*, 2001). The second gene, ORF p1056.02 is predicted to encode for a gene homologous to the helicase, *dnaB*. The enzyme encoded by this gene is responsible for unwinding helical DNA strands. The helicase binds DNA to a molecule of adenosine triphosphate (ATP) to begin the unwinding process (Subramanya *et al.*, 1996). The ATP/GTP binding (P-loop) motif is featured in this protein. The protein motifs, pfam indicate the presence of a DnaB helicase-like domain. Little is known about the five conserved hypothetical proteins that lie downstream of the helicase protein; however, it is thought that they are most likely involved in replication. One of these genes, p1056.07 curiously has low similarity (E value 2.6e-05) to the integrase regulator R, *inrR* of *Pseudomonas* sp. B13 (acc. no. CAD60671). This gene is responsible for the activation of transcription of integrase expression (Sentchilo *et al.*, 2003).

The eighth gene in this module, p1056.08, has homology to *ssb* gene that encodes for single-stranded binding protein. The function of this protein is to bind the lagging single-stranded DNA created by DnaB helicase to prevent them from re-annealing so that DNA replication can continue (Lewin, 1990). Two ORFs downstream of the single-stranded protein are hypothetical proteins. One of the genes, p1056.09 is believed to encode membrane lipoprotein based on a motif typical of a lipoprotein-binding site. The
other gene, p1056.10 encodes hypothetical protein, and has low similarity (E value 8.6e-08) to the homologues of C-terminal region of S. flexneri Osa protein (acc. no. 1560781). This protein suppresses the oncogenicity activity in Agrobacterium tumefaciens but its function in H. influenzae is rather doubtful (Chen and Kado, 1994).

The gene, p1056.11 is highly similar to gene that encodes DNA topoisomerase III, topB protein of H. ducreyi 3500HP (acc. no. AE017153). Topoisomerase is the enzyme that changes a DNA’s superhelical. During replication of DNA, the enzyme facilitates the unwinding of the double-helical structure of DNA (Zhang et al., 1995). It recognizes and cleaves regions of single-stranded DNA and holds and protects both free ends of the broken strand from random ligation reactions that could be detrimental to the organism. Homologues of the four genes mentioned, parA, dnaB, ssb and topB genes are recognised to play a role together in plasmid replication (Espinosa et al., 2000).

Genes p1056.12 and p1056.13 encode hypothetical proteins with unknown functions, while ORF p1056.14 possibly encodes a DNA repair protein, similar to H. ducreyi 3500HP RadC protein (acc. no.: AE017153). This protein functions specifically in the recombination repair that is associated with a replication fork (Saveson and Lovett, 1999). p1056.15 has low similarity to a gene that encode hypothetical protein of H. ducreyi follows; however, the amino acid of the protein is predicted by PSORT to be possibly a lipoprotein.

4.4.9. Antibiotic resistance module

The second module is approximately 12 kb and is a transposon Tn10 variant. It contains tetracycline and chloramphenicol resistance genes. In this region, three types of insertion sequences, namely IS10, IS5 and IS30 were identified. The IS10 are encoded by genes p1056.16 and p1056.27, which flanks both ends of this composite transposon. Within the
IS10, lies insertion sequences IS5 (p1056.17 and p1056.20), which flanks chloramphenicol acetyltransferase gene, cat (p1056.18) and IS30 (p1056.19) (Fig. 4.9). This represents a variation on the typical Tn10 structure.

A cluster of genes (p1056.21 to p1056.27) that lies within the Tn10 like structure comprises genes involved in the regulation of tetracycline resistance. Gene p1056.21 that encodes ybeB or jemB protein, at present has no known function, but is believed to be a member of a family of functional proteins (Chalmers et al., 2000). Next to this gene is p1056.22, which is predicted to be a homologue of Ybe protein. A comparison between the Tn10 of S. flexneri and ICEHin1056 (Fig. 4.10) shows that ICEHin1056 does not have the jemA. JemA is a gene that is homologous to the sodium-dependent glutamate permease of Salmonella sp. and E. coli. Analysis has shown that the functional residue involved in sodium-dependent glutamate interactions is glycine and this is important for protein flexibility (Lawley et al., 2000). Absence of jemA suggests that ICEHin1056 depends on tetA as the only transport system. Downstream is p1056.23, which encodes putative transcriptional protein, YbdA. The N-terminal of this protein contains a metal/DNA-binding domain; but the C-terminal domain is of unknown function (Chalmers et al., 2000).

Unlike the Tn10 of S. flexneri and S. Typhi, the Tn10 of ICEHin1056 probably does not have a functional tetracycline repressor (Chalmers et al., 2000; Lawley et al., 2000) as p1054.24 is a pseudogene. Since the repressor gene is mutant, this suggests that tetracycline resistance in ICEHin1056 would be constitutively expressed. A membrane-bound efflux pump produces tetracycline resistance and is encoded by tetA gene p1056.25 in ICEHin1056. The Tn10 contains two additional structural genes, tetC and tetD, however, their functions are still unknown.
Fig. 4.9. Genetic organisation of Tn10 insert in ICEHin1056. The Tn10 transposon consists of cat gene and tet genes flanked by two inverted IS10. Within the Tn10, the cat gene is flanked by two IS5. Note that the tetR is a pseudogene.

Fig. 4.10. ACT diagram showing comparison of Tn10 of S. flexneri (acc. no. AF162223) and Tn10 of ICEHin1056. Note that ICEHin1056 has cat gene flanked by IS5, but is devoid of the jemA gene.

4.4.10. Putative transfer module

The majority of ORFs that lie between 26,582 and 59,393-bp encodes hypothetical proteins. The gene, p1056.29 has low similarity to a gene that encodes hypothetical
protein of *H. ducreyi*, however, it is a partial gene. Next to this gene ORF p1056.30, is a gene that has low similarity (E value 1.3e-08) to homologues of *Pseudomonas syringae* membrane protein (acc. no.: 12928499) and also to the *S. Typhi* TraX (acc. no.: 2877970). The *traX* is required in pilin acetylation in conjugative transfer (Manchak *et al.*, 2002; Maneewannakul *et al.*, 1995). This feature is confirmed by the prediction of THMM2.0 that showed an array of eight transmembrane helices and Pfam motif prediction of the TraX protein. The TraX protein is required to process the translation of *traA* (propilin polypeptide) for the formation of F-pilus (Manchak *et al.*, 2002).

Downstream of the TraX are series of ORFs that are membrane-associated. They consist of genes encoding proteins involved in export functions (ORFs p1056.31, p1056.32, p1056.33, p1056.38, p1056.41, p1056.50, p1056.51 and p1056.52), and genes that encode membrane protein (p1056.34, p1056.37, p1056.39, p1056.47, p1056.48, p1056.49, p1056.54, and p1056.55). Gene p1056.35 shows similarity to *traD* of *Providencia rettgeri* conjugative genomic island R391 (acc. no. AY090559). Other genes in this region include genes encoding lipoproteins (ORFs p1056.42, p1056.44) and hypothetical proteins (p1056.36, p1056.40, p1056.43, p1056.46, and p1056.53). The motifs of membrane-associated proteins in this region have features similar to the Type IV pilus biosynthesis locus (Srimanote *et al.*, 2002). ORF p1056.31 is a lipoprotein that has high similarity to the N-terminal region of PilL protein of the pathogenicity island PAP1 of *P. aeruginosa* PA14 (acc. no.: AAP84211). The pilin gene, *pilL* is a Type IV pilus and has a short leader sequence. The *pilL* is a gene involved in production of sex pilus and is responsible for the transfer of DNA by conjugation (Sakai and Komano, 2000). The Type IV pilus gene has also been associated with pathogenicity of *Yersinia pseudotuberculosis* (Collyn *et. al*, 2002), however, the Type IV pilus in ICEHin1056 is for conjugative transfer rather than for pathogenicity since no virulence genes are found.
p1056.59 did not show matches to any genes in the database. The adjacent p1056.60 (53,491–54,189-bp position) showed similarity to a homologue of *P. putida* hypothetical protein (acc. no. AAP57242). This gene has helix-turn-helix motif (HTH) that suggests it to encode a putative DNA binding protein. The p1056.61 encodes hypothetical protein while ORF p1056.62 encodes protein similar to the hypothetical protein of *H. ducreyi* 35000HP (acc. no. AAP95784). This ORF is also similar to *tral* that encodes putative relaxase of *P. rettgeri* (acc. no. AY090559). p1056.63 appears to be a homologue of integrase/recombinase protein of *H. ducreyi* 35000HP. This integrase gene belongs to the XerC integrase lineage and is most similar to the xerC of *P. aeruginosa* (acc. no.: AF285416).

### 4.4.11. Ampicillin resistance module

This module (coordinates 50,985 and 52,585 bp) consists of genes that encode β-lactamase resistance. ORF p1056.57 is identical to *Acinetobacter baumannii* TEM-1 β-lactamase *bla* precursor (acc. no. AAP20891). Adjacent to this gene, ORF p1056.58 is identical to *Salmonella enteritidis* resolvase *tnpR* (acc. no. BAC77164). This gene also shows a HTH DNA binding motif. However, unlike the Tn3 of *E. coli*, the Tn3 of ICEHin1056 is truncated because *tnpA* is missing. Also, only one copy of the 38-bp inverted repeat (5′-GGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAG-3′) is present. Fig. 4.11 depicts the truncated Tn3 of ICEHin1056 in comparison to the complete Tn3 of *E. coli* (acc. no. V00613). Although *tnpA* is absent in ICEHin1056, the β-lactamase activity is detectable and 1056 is ampicillin resistant with an MIC of 96 μg/ml.
4.4.12. Identification of putative origin of replication

One of the insights from analysing the bacterial genome or plasmids is that, the base composition of each chromosomal or plasmid strand changes at the origin and terminus of replication (Freeman, 1998; Lobry, 1996a, b). This phenomenon is known as GC skew, which can be produced by calculating (G-C)/(G+C) values for a given length of the entire sequence. The site where there is a switch of the polarity of GC skew often corresponds with \textit{oriV} where initiation of DNA replication takes place. This pattern was well observed in genome sequences such as \textit{Borellia burgdorferi} (Picardeau et al., 1999). The GC skew of the whole sequence of a plasmid of \textit{H. influenzae} biotype \textit{aegyptius} was also obvious, resulting in the recognition of the \textit{oriV} (Kroll et al., 2002). However, the whole genome of \textit{H. influenzae} Rd did not show an obvious GC-skew, as shown in Fig. 4.12 (Uno et al., 2000).
The sequences of ICEHin1056 were also analysed for GC skew. The GC skew plot of this mobile element did not reveal clear bioinformatics evidence of oriV (Fig. 4.13). The inability to obtain a GC skew in ICEHin1056 is most probably due, in part, to the presence of insertions that could have interfered with the GC bias. It is reported that, in the absence of transposition and insertion, a bias favours the accumulation of purines in the leading strand (Freeman, 1998).

Although the GC skew in ICEHin1056 is unclear, a possible oriV site of the element is predicted to be located at approx. 6000-bp. This prediction is based on the presence of multiple inverted sequences and direct tandem (two 33-bp and four 36-bp) repeats that lie within AT-rich region (Fig. 4.14).

A program Oriloc has been developed to predict the putative origin and terminus of replication in prokaryotic genomes (Frank and Lobry, 2000). It successfully predicted the origin of replication in the complete genomes of bacteria lacking genes involved in replication (dnaA, priA and recA), such as, Bifidobacterium longum (Schell et al., 2002) and Blochmannia floridanus (Gil et al., 2003). The sequences of ICEHin1056 were downloaded into this program, but were unable to show a clear signal for the origin of replication. The tentative position of a skew reversal is at position 0, and at approximately at 42-kb position (Fig. 4.15). This is opposite the putative oriV deduced from sequence structure data discussed above.

The author of the Oriloc program suggests that the oriV for this element is at position 0 (Lobry, personal communication). This programme fails to locate the oriV of ICEHin1056 probably because the element has low G+C (<50%) content and is further complicated by the presence of transposons. However, it was able to detect the termination of replication (ter) at 42-kb position. Existence of ter site in ICEHin1056 sequence suggests that the element undergoes theta type replication, consistent to the
Initiation of DNA replication occurs at oriV and, during theta replication, the replication forks, moving unidirectionally or bidirectionally, are arrested at sequence-specific replication termini (Bussiere and Bastia, 1999; del Solar et al., 1998). No sequences resembling IHF ((A/T)ATCAANNNNTT(A/G) where N is A/C/T/G) was detected in the whole sequence.

Fig. 4.12. GC skew of the whole genome of *H. influenzae* Rd taken from Uno et al. (Uno et al., 2000). Non-overlapping window of 10 kb was used for this analysis. Arrows indicate the replication origin and terminus. No clear shift point is observed in *H. influenzae* Rd.

Fig. 4.13. GC deviation [(G-C)/(G+C)] calculated for ICE.Hin1056 based on a 5000-bp window. The GC skew is obscured by the insertion elements. This graph was obtained by using Artemis program.
Fig. 4.14. Putative oriV of ICEHin1056. Presence of interons in an AT-rich region indicative of possible oriV site. This diagram was obtained by using Artemis program.

![Putative oriV](image)

Fig. 4.15. Cumulative composite skew of ICEHin1056. The oriV of this element is unclear, however, the site for termination of replication terminus (ter), is approximately at 42-kb position shown by arrow. This graph was obtained by using Oriloc program.

![Cumulative composite skew](image)

4.4.13. **Identification of putative origin of transfer**

During conjugation, initiation of DNA transfer occurs at a site known as origin of transfer or oriT, whereafter cleavage by a nickase, a single-stranded DNA molecule is released.
which will ultimately be transferred to a recipient cell. Usually the region close to the _tral_ gene contains the possible _oriT_.

From the sequences of the element, it is suggested that the putative _oriT_ of the _ICEHin1056_ is between gene p1056.60 and p1056.61 (54190 to 54714-bp position). This assumption is based on reports of others that the _oriT_ of conjugative plasmid is usually located in (i) the vicinity of their DNA processing genes, (ii) a non-coding region, and (iii) the presence of repeats (Galli _et al._, 2001). In _ICEHin1056_ this region contains a 114-bp DNA domain-containing 10.3 sets of 13-bp perfect direct repeats. The _tral_ gene, p1056.62, (similar to _P. rettgeri tral_, acc. no. AY090559) and a putative DNA-binding protein gene, p1056.60, (similar to _P. putida_, acc. no. AY299015) are located immediately adjacent to this region (Fig. 4.16). Besides, this region also has a lower G+C content of 34.3% (i.e. higher A+T content) typical of an _oriT_.

![Fig. 4.16. Putative _oriT_ of _ICEHin1056_. Presence of multiple interons, DNA binding site and close to _tral_, indicative of possible _oriT_ at 54,396 bp position shown by an arrow. The small arrowheads represent repeats. This diagram was obtained by using Artemis program.](image)

4.4.14. Nucleotide sequence accession number

The complete sequence data of _ICEHin1056_ was deposited in the EMBL/GenBank DNA databases under the accession number AJ627386.
4.5. Conclusion

The complete nucleotide sequence of ICEHin1056 was determined in this study. This is the first report of a complete sequence of a conjugative and integrative element encoding multi-drug resistance in \textit{H. influenzae}. The complete sequence of ICEHin1056 has provided new understanding of its structure and organisation from which insight into its successful transmission of antibiotic resistance in \textit{H. influenzae} can be predicted. This circular 59,393-bp element was preferably integrated into the chromosome of \textit{H. influenzae} strain 1056 at the 3' end of the tRNA\textsuperscript{leu} site (Dimopoulou \textit{et al.}, 2002). The annotation of the sequenced circular element reveals that the element consists of gene clusters of related functions likely to be involved in integration, conjugative transfer and probable replication and maintenance of a plasmid form. Besides these genes, ICEHin1056 also contains genes encoding resistance to antibiotics. No evidence of genes involved with virulence was found on analysing the nucleotide sequence of this element.

The antibiotic resistance genes on ICEHin1056 are contained in a Tn10-like structure encoding tetracycline-resistance and chloramphenicol resistance. This finding is in agreement with the findings of Jahn \textit{et al.} and Levy \textit{et al.} (Jahn \textit{et al.}, 1979; Levy \textit{et al.}, 1984). Ampicillin resistance is encoded by the TEM \(\beta\)-lactamase gene and is present in a truncated Tn3 missing \textit{TnpA}.

During the annotation of the genes of ICEHin1056, many of the genes matched the genes of \textit{H. ducreyi} 35000HP and are organised in the same order. A comparison between these two sequences was carried out in chapter seven.
CHAPTER FIVE

NUCLEOTIDE SEQUENCE AND ANALYSIS OF ICE\textit{Hin}299

5.1. Introduction

Large conjugative plasmids of \textit{H. influenzae} type b carrying the β-lactamase gene, isolated from Greece and the UK, have been analysed for their genetic relatedness (Dimopoulou \textit{et al.}, 1992). Southern hybridisation techniques, using UK ICEs as probes show that elements cut with \textit{PstI} and \textit{EcoRI} shared some homology. However, the extent and degree of relatedness is not known.

5.2. Objectives

In this chapter, molecular characteristics of a mobile element of a \textit{H. influenzae} strain 299 isolated in Greece will be investigated by sequencing the whole element ICE\textit{Hin}299. The information on the complete sequence of this element will be compared to ICE\textit{Hin}1056, and if possible to other \textit{Haemophilus} ICEs, to better understand the properties of these elements and how they may have evolved.

5.3. Materials and methods

5.3.1. Extraction of ICE\textit{Hin}299

The ICE\textit{Hin}299 was grown on chocolate agar for 48 hours before being cultured into MH broth for plasmid extraction. Extraction of the element was carried out using CsCl gradient centrifugation as described in Chapter 2.
5.3.2. Sequencing of ICEHin299

As in ICEHin1056, shotgun-sequencing method was performed on ICEHin299. Nucleotide sequences obtained were assembled, analysed and the contigs were ordered in the same order as ICEHin1056. Gaps between the contigs were filled by ‘primer walking’ procedure. In regions where nucleotide sequences were shared between ICEHin299 and ICEHin1056, primers of ICEHin1056 were used to close the gaps, and to improve the quality of the sequences.

5.4. Results and discussion

5.4.1. Overall organisation of ICEHin299

Two random plasmid DNA libraries and six sequence assemblies were carried out. The total number of readings used in the final assembly was 787, with an average length for each sequence being 503 bases. The complete sequence of ICEHin299 produced a length of 53,902 bases. It had 38.59% G+C content consisting of 31.8% adenine, 18.4% cytosine, 20.9% guanine and 28.9% thymine. The total number of bases sequenced was 396,396 bases producing approximately 7.4 fold coverage. Of the 67 predicted genes, 53 showed no homology to genes of known functions. As in ICEHin1056, ICEHin299 also has nine copies of Haemophilus uptake sequence (Table 5.1). This number of uptake sequence is also similar to the uptake sequence of a plasmid of H. aegyptius (Kroll et al., 2002). Whether all other mobile elements of Haemophilus spp. constantly have nine copies of the Haemophilus uptake sequence remains to be shown.

As in ICEHin1056, the start of the sequence was positioned at the site of recombination, attP. The whole element contains 67 ORFs with 52 ORFs in the forward and 15 in the reverse direction (Fig. 5.1). Unlike ICEHin1056, ORFs of ICEHin299 has only one pseudo-gene. Thirty-five of the predicted proteins showed homology to proteins
of *H. ducreyi*. Fourteen genes on this element did not have matches with any sequences in databases. Properties of ICE*Hin299* are shown in Table 5.1.

Table 5.1. Summary of ICE*Hin299* properties.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total size</td>
<td>53,902 bp</td>
</tr>
<tr>
<td>GC content</td>
<td>38.59%</td>
</tr>
<tr>
<td>Coding sequences</td>
<td>67</td>
</tr>
<tr>
<td>Pseudo-genes</td>
<td>1</td>
</tr>
<tr>
<td>Coding density</td>
<td>88.1%</td>
</tr>
<tr>
<td>Average gene length</td>
<td>709 bp</td>
</tr>
<tr>
<td>No. of uptake sequence</td>
<td>9</td>
</tr>
</tbody>
</table>

*Comparison of properties between ICE*Hin299* to ICE*Hin1056* is shown in chapter 6."
Fig. 5.1. Genetic organisation of ICEHin299. Genes are represented by arrow boxes. Putative functions of the genes are described in Table 5.2.
Table 5.2. Putative ORFs of ICEHin299. Amino acid similarity of putative proteins encoded by ICEHin299 to sequences in public databases.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Coordinates</th>
<th>Gene name</th>
<th>Predicted protein</th>
<th>Database similarity (aa identity)</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p299.01</td>
<td>141-977</td>
<td>soj</td>
<td>Chromosome partitioning related protein</td>
<td>Similar to H. ducreyi 35000HP possible chromosome partitioning related protein HD0974 SWALL: AAP95855 (EMBL: AE017153) (83.33 in 276 aa)</td>
<td>8.3e-94</td>
</tr>
<tr>
<td>p299.02</td>
<td>979-2334</td>
<td>dnaB</td>
<td>DNA helicase</td>
<td>Similar to H. ducreyi 35000HP DNA helicase DnaB or HD0973 SWALL: AAP95854 (EMBL: AE017153) (71.62 in 444 aa)</td>
<td>1.8e-120</td>
</tr>
<tr>
<td>p299.03</td>
<td>2327-4027</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Similar to N-terminal of H. ducreyi 35000HP hypothetical protein HD0971 SWALL: AAP95852 (EMBL: AE017153) (72.30 in 195 aa)</td>
<td>6.3e-49</td>
</tr>
<tr>
<td>p299.04</td>
<td>4027-4578</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Similar to H. ducreyi 35000HP conserved hypothetical protein HD0969 SWALL: AAP95850 (EMBL: AE017153) (79.0 in 181 aa)</td>
<td>2.7e-56</td>
</tr>
<tr>
<td>p299.05</td>
<td>4728-5951</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Similar to, although longer than H. ducreyi 35000HP hypothetical protein HD0968 SWALL: AAP95849 (EMBL: AE017153) (68.77 in 237 aa)</td>
<td>1.1e-58</td>
</tr>
<tr>
<td>p299.06</td>
<td>5968-6231</td>
<td></td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p299.07</td>
<td>6248-7003</td>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Similar to the central region of H. ducreyi 35000HP hypothetical protein HD0966 SWALL: AAP95847 (EMBL: AE017153) (72.44 in 254 aa)</td>
<td>1e-71</td>
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<tr>
<td>p299.08</td>
<td>7003-7491</td>
<td>inrR</td>
<td>Putative integrase regulator</td>
<td>Similar to, although longer H. ducreyi 35000HP hypothetical protein HD094 SWALL: AAP95845 (EMBL: AE017153) (74.41 in 43 aa)</td>
<td>5.3e-06</td>
</tr>
<tr>
<td>p299.09</td>
<td>7737-8159</td>
<td>ssb</td>
<td>Single-strand binding protein</td>
<td>Similar to A. actinomycetemcomitans single strand binding protein Ssb SWALL: Q9F278 (EMBL: AF302424) (72.41 in 145 aa)</td>
<td>5.6e-41</td>
</tr>
<tr>
<td>p299.10</td>
<td>8186-8704</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to H. ducreyi 35000HP hypothetical protein HD0961 SWALL: AAP95842 (EMBL: 017153)(47.09 id in 172 aa)</td>
<td>2.9e-23</td>
</tr>
<tr>
<td>p299.11c*</td>
<td>8920-9477c</td>
<td>osa</td>
<td>Putative osa protein</td>
<td>Low similarity to C-terminal region of S. flexneri protein Osa SWISSPROT: OSA_SHIFL (38.05 in 119 aa)</td>
<td>9.6e-08</td>
</tr>
<tr>
<td>p299.12</td>
<td>9553-11613</td>
<td>topB</td>
<td>DNA topoisomerase III</td>
<td>Similar to H. ducreyi DNA 35000HP DNA topoisomerase III TopB1 or HD0954 SWALL: AAP95835 (EMBL: AE017153) (8.66 in 450 aa)</td>
<td>3.9e-139</td>
</tr>
<tr>
<td>p299.13</td>
<td>11750-11974</td>
<td></td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p299.14</td>
<td>12498-12926</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to H. ducreyi 35000HP hypothetical protein HD0951 SWALL: AAP95832 (EMBL: AE017153) (57.63 in 144 aa)</td>
<td>5.5e-33</td>
</tr>
<tr>
<td>p299.15</td>
<td>13041-13682</td>
<td></td>
<td>Hypothetical protein</td>
<td>Low similarity to C-terminal region Rhizobium meliloti hypothetical 50.7 kDa protein SWALL: Q91UT8 (EMBL: PPP304453) (28.98 in 138 aa)</td>
<td>0.0052</td>
</tr>
<tr>
<td>p299.16</td>
<td>13705-14124</td>
<td></td>
<td>Putative lipoprotein</td>
<td>No significant database matches</td>
<td></td>
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</table>

*c: reverse direction
<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Coordinates</th>
<th>Gene name</th>
<th>Predicted protein</th>
<th>Database similarity</th>
<th>E value</th>
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<tr>
<td>p299.17</td>
<td>14225-14695</td>
<td>radC</td>
<td>Putative DNA repair protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP conserved possible DNA repair protein HD0945 SWALL:AAAP95827 (EMBL:AE017153) (41.02 in 156 aa)</td>
<td>5.2e-20</td>
</tr>
<tr>
<td>p299.18</td>
<td>14788-15384</td>
<td></td>
<td>Putative lipoprotein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0944 SWALL:AAAP95826 (EMBL:AE017153) (36.06 in 183 aa)</td>
<td>7.1e-15</td>
</tr>
<tr>
<td>p299.19</td>
<td>15442-16203</td>
<td>traX</td>
<td>Conserved hypothetical glycin-rich protein</td>
<td>Similar to <em>P. syringae</em> putative membrane protein PSPTO0411 SWALL:Q88AH9 (EMBL:AE016857) (25.49 in 251 aa)</td>
<td>3.7e-08</td>
</tr>
<tr>
<td>p299.20</td>
<td>16302-16931</td>
<td>pillL</td>
<td>Putative exported protein</td>
<td>Similar to N-terminal region of <em>P. aeruginosa</em> Pill2 or RL086 SWALL:AAAP84211 (EMBL:AY273869) (32.98 in 191 aa)</td>
<td>1.1e-14</td>
</tr>
<tr>
<td>p299.21</td>
<td>16935-17675</td>
<td></td>
<td>Putative exported protein</td>
<td>Similar to <em>S. Typhi</em> putative exported protein STY4558 or T4256 SWALL:Q8ZIK6 (EMBL:AL27282) (35.77 in 246 aa)</td>
<td>1.6e-24</td>
</tr>
<tr>
<td>p299.22</td>
<td>17654-18412</td>
<td></td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p299.23</td>
<td>18428-18934</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>P. aeruginosa</em> hypothetical protein SWALL:Q8GPT3 (EMBL:AF440524) (42.51 in 167 aa)</td>
<td>4.1e-19</td>
</tr>
<tr>
<td>p299.24</td>
<td>18931-21180</td>
<td>traD</td>
<td>Putative transfer protein</td>
<td>Similar to <em>P. putida</em> hypothetical 80.3 kDa protein SWALL:Q8VMJ3 (EMBL:AJ344068) (59.18 in 713 aa), and to <em>P. rettgeri</em> conjugative genomic island R391 TraD SWALL:Q8RL14 (EMBL:AY90559) (31.0 670 in 610 aa)</td>
<td>7e-15; 2e-76</td>
</tr>
<tr>
<td>p299.25</td>
<td>21215-21499</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0942 SWALL:AAAP95824 (EMBL:AE017153) (44.0 in 100 aa)</td>
<td>2.5e-15</td>
</tr>
<tr>
<td>p299.26</td>
<td>21499-22185</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0949 SWALL:AAAP95823 (EMBL:AE017153) (74.12 in 228 aa)</td>
<td>7.9e-73</td>
</tr>
<tr>
<td>p299.27</td>
<td>22316-22666</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0940 SWALL:AAAP95822 (EMBL:AE017153) (40.44 in 89 aa)</td>
<td>3.2e-05</td>
</tr>
<tr>
<td>p299.28</td>
<td>22670-22915</td>
<td></td>
<td>Putative exported protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p299.29</td>
<td>22935-23288</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0939 SWALL:AAAP95821 (EMBL:AE017153) (50.0 in 120 aa)</td>
<td>2.1e-18</td>
</tr>
<tr>
<td>p299.30</td>
<td>23355-23726</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0938 SWALL:AAAP95820 (EMBL:AE017153) (60.97 in 123 aa)</td>
<td>6.9e-30</td>
</tr>
<tr>
<td>p299.31</td>
<td>23738-24382</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein SWALL:AAAP95819 (EMBL:AE017153) (67.74 in 186 aa)</td>
<td>4.2e-52</td>
</tr>
<tr>
<td>p299.32</td>
<td>24382-25251</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0936 SWALL:AAAP95818 (EMBL:AE017153) (67.01 in 285 aa)</td>
<td>2.3e-74</td>
</tr>
</tbody>
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*c: reverse direction*
<table>
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<tr>
<th>Gene ID</th>
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<th>Predicted protein</th>
<th>Database similarity (aa identity)</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p299.33</td>
<td>25262-26662</td>
<td>virB4</td>
<td>Putative type IV secretion</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0935 SWALL: AAP95817 (EMBL: AE017153) (61.44 in 485 aa), and to <em>H. influenzae</em> R2866 Type IV secretory pathway, COG3451: VirB4 components (ZP_00157627) (97.0 id in 956 aa)</td>
<td>1.1e-68, 0.0</td>
</tr>
<tr>
<td>p299.34</td>
<td>26672-27079</td>
<td></td>
<td>Putative exported protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0934 SWALL: AAP95816 (EMBL: AE017153) (63.56 in 129 aa)</td>
<td>8.7e-33</td>
</tr>
<tr>
<td>p299.35</td>
<td>27095-29965</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0933 SWALL: AAP95815 (EMBL: AE017153) (75.65 id in 797 aa)</td>
<td>0</td>
</tr>
<tr>
<td>p299.36</td>
<td>29937-30380</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0931 SWALL: AAP95813 (EMBL: AE017153) (47.61 in 147 aa)</td>
<td>1.4e-27</td>
</tr>
<tr>
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<td>30390-30713</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0930 SWALL: AAP95812 (EMBL: AE017153) (39.8 id in 103 aa)</td>
<td>5.5e-12</td>
</tr>
<tr>
<td>p299.38</td>
<td>30722-32215</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>S. Typhi</em> membrane protein STY4579 SWALL: Q8Z1I6 (EMBL: AL627282) (42.6 in 500 aa)</td>
<td>5e-89</td>
</tr>
<tr>
<td>p299.39c</td>
<td>32271-32600c</td>
<td></td>
<td>Putative membrane protein</td>
<td>No significant database matches</td>
<td></td>
</tr>
<tr>
<td>p299.40c</td>
<td>32758-35763c</td>
<td>tnpA</td>
<td>Transposase</td>
<td>Identical to <em>S. enteritidis</em> transposase SWALL: BAC77163 (EMBL: AB103092) (100 in 1001 aa)</td>
<td>0</td>
</tr>
<tr>
<td>p299.41</td>
<td>35927-36484</td>
<td>tnpR</td>
<td>Tn3 resolvase</td>
<td>Identical to <em>S. enteritidis</em> resolvase SWALL: BAC77164 (EMBL: AB103092) (100 in 175 aa)</td>
<td>4.1e-61</td>
</tr>
<tr>
<td>p299.42</td>
<td>36532-37392</td>
<td>bla</td>
<td>Beta-lactamase TEM1</td>
<td>Identical to <em>S. flexneri</em> beta-lactamase SWALL: AAC97980 (EMBL: U48775) (100 in 286 aa)</td>
<td>1.7e-111</td>
</tr>
<tr>
<td>p299.43c</td>
<td>37840-38248c</td>
<td></td>
<td>Putative exported protein</td>
<td>Low similarity to <em>Neisseria gonorrhoeae</em> Gly1ORF1 precursor Gly1ORF1 precursor SWALL: O30392 (EMBL: AF003941) (22.38 in 134 aa)</td>
<td>0.13</td>
</tr>
<tr>
<td>p299.44c</td>
<td>38264-40264c</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0922 SWALL: AAP95805 (EMBL: AE017153) (61.49 in 561 aa)</td>
<td>2.5e-120</td>
</tr>
<tr>
<td>p299.45c</td>
<td>40279-41253c</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to, although longer than <em>H. ducreyi</em> 35000HP hypothetical protein HD0921 SWALL: AAP95804 (EMBL: AE017153) (74.77 in 218 aa)</td>
<td>6.6e-75</td>
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<tr>
<td>p299.46c</td>
<td>41217-41660c</td>
<td></td>
<td>Putative exported protein</td>
<td>Similar to, although longer than <em>H. ducreyi</em> 35000HP hypothetical protein HD0920 SWALL: AAP95803 (EMBL: AE017153) (62.50 in 48 aa)</td>
<td>9.4e-09</td>
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<tr>
<td>p299.47</td>
<td>42029-42391</td>
<td></td>
<td>Putative membrane protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0919 SWALL: AAP95802 (EMBL: AE017153) (52.58 in 116 aa)</td>
<td>1.6e-17</td>
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<td>p299.48</td>
<td>42476-42709</td>
<td></td>
<td>Hypothetical protein</td>
<td>Similar to <em>H. ducreyi</em> 35000HP hypothetical protein HD0918 SWALL: AAP95801 (EMBL: AE017153) (37.68 in 69 aa)</td>
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<tr>
<td>p299.49</td>
<td>42712-42960</td>
<td></td>
<td>Hypothetical protein</td>
<td>Low similarity to the central region of <em>H. ducreyi</em> 35000HP HD0947 hypothetical protein SWALL: AAP95829 (EMBL: AE017153) (34.0 in 50 aa)</td>
<td>0.35</td>
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<td>No significant database matches</td>
<td></td>
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*c: reverse direction*
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<tr>
<th>Gene ID</th>
<th>Coordinates</th>
<th>Gene name</th>
<th>Predicted protein</th>
<th>Database similarity (aa identity)</th>
<th>E value</th>
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<tr>
<td>p299.51</td>
<td>43253-44206</td>
<td><em>traC</em></td>
<td>DNA primase</td>
<td>Similar to <em>H. ducreyi</em> 35000HP probable DNA primase or HD017 SWALL:AAP95800 (EMBL:AE017153) (71.92 in 317 aa)</td>
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<td>p299.52</td>
<td>44310-45071</td>
<td>Possible type I restriction enzyme M subunit</td>
<td>Similar to <em>H. ducreyi</em> 35000HP possible type I restriction enzyme M subunit HD0914 SWALL:AAP95798 (EMBL:AE017153) (66.27 in 252 aa)</td>
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<tr>
<td>p299.54c</td>
<td>45627-46211c</td>
<td>Resolvase/integrase</td>
<td>Similar to <em>H. aegyptius</em> resolvase/integrase-like protein BPL01 SWALL:Q8VRE8 (EMBL:AF447808) (55.27 in 199 aa)</td>
<td>5.6e-28</td>
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<tr>
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<td>46226-46417c</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
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<td></td>
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<td>p299.56c</td>
<td>46432-46737c</td>
<td>Putative membrane protein</td>
<td>Low similarity to <em>Fusobacterium nucleatum subsp. vincentii</em> ATCC49256 hypothetical protein FNV1196 SWALL:AAA24216 (EMBL:AAF01000046) (31.46 in 89 aa)</td>
<td>0.79</td>
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<td>p299.57c</td>
<td>46741-46935c</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
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<tr>
<td>p299.58c</td>
<td>46997-47455c</td>
<td>Putative lipoprotein</td>
<td>Very low similarity to <em>Synechococcus elongatus</em> Thr2451 protein SWALL:Q8DG72 (EMBL:AP005377) (34.83 in 89 aa)</td>
<td>3.3</td>
<td></td>
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<td>47481-47603c</td>
<td>Putative membrane protein</td>
<td>No significant database matches</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p299.60c</td>
<td>47627-48109c</td>
<td>Hypothetical protein</td>
<td>Low similarity to the N-terminal region of <em>Yersinia pestis</em> hypothetical protein YP01174 SWALL:Q8ZGV3 (EMBL:AJ414146) (26.38 in 144 aa)</td>
<td>0.29</td>
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<td>p299.61</td>
<td>48363-48650</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
<td></td>
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<td>p299.62</td>
<td>48710-48844</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
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<tr>
<td>p299.63</td>
<td>48817-48978</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p299.64</td>
<td>48975-49636</td>
<td>Conserved hypothetical protein (pseudogene)</td>
<td>Pseudogene. Similar to <em>P. putida</em> hypothetical protein SWALL:AAP57242 (EMBL:AY299015) (32.57 in 221 aa)</td>
<td>2e-12</td>
<td></td>
</tr>
<tr>
<td>p299.65c</td>
<td>49900-50604c</td>
<td>Hypothetical protein</td>
<td>No significant database matches</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p299.66</td>
<td>50841-52745</td>
<td><em>tral</em></td>
<td>Putative relaxase</td>
<td>Similar to C-terminal region of <em>Xylella fastidiosa</em> hypothetical Xf1753 SWALL:Q9PC5M (EMBL:AE003998) (36.79 in 606 aa), and to <em>P. rettgeri</em> (acc. no. AAM08003) (31.0 in 231 aa)</td>
<td>4.9e-77; 4e-18</td>
</tr>
<tr>
<td>p299.67</td>
<td>52932-53648</td>
<td><em>int</em></td>
<td>Integrase</td>
<td>Similar to <em>A. actinomycetecomitans</em> integrase SWALL:Q9ZAB5 (EMBL:AF006830) (69.74 in 265 aa)</td>
<td>8.6e-70</td>
</tr>
</tbody>
</table>

*c: reverse direction*
5.4.2. **Description of ICEHin299 sequences based on functional module**

The gene organisation reveals four main modules (Fig. 5.2 and Table 5.2). The first module (coordinates 1-15384) shows a cluster of putative genes involved in replication, and the second domain (15385-32600) believed to be the region responsible for gene transfer. The third domain (32601-37392), inserted between the third and fourth domain is responsible for ampicillin resistance. The fourth domain (37840-38248) contains a region putatively involved in transfer and integration (i.e. *int* is present).

![Genetic organisation of ICEHin299](image)

**Fig. 5.2. Genetic organisation of ICEHin299, according to putative functions.**

5.4.3. **Putative replication module**

The complete sequence analysis shows that approximately 140 bases downstream of *attP* is a gene similar to the partitioning protein *soj* of *H. ducreyi* (p299.01), followed by the helicase gene *dnaB* (p299.02). Following these two genes are five ORFs (p299.03 to p299.07), which encodes for hypothetical proteins, of which four are conserved. These four genes not only share homology to genes of *H. ducreyi* 35000HP (NC_002940), but they are also in synteny with ICEHin1056. The p299.08 is similar to the putative integrase regulator *inrR* gene of *H. ducreyi*, associated with the excisive recombination of...
the element. Adjacent to this, is a gene similar to the ssb of A. actinomycetemcomitans encoded by p299.09, followed by a gene with homologue to conserved hypothetical protein of H. ducreyi (p299.10). Gene p299.11 encodes for protein that has low similarity (E value 9.6e-08) to Osa protein of S. flexneri (SWISSPORT:OSA_SHIFL). How such gene product with oncogenic suppression has a role in this element is unclear. Next to the Osa protein, is a gene similar to H. ducreyi topoisomerase III topB (p299.12). Genes p299.13 to p299.16 are predicted to produce proteins of unknown functions. Gene p299.17 has homology to RadC DNA repair protein that functions specifically in recombinational repair associated with replication. Presence of soj, dnaB, ssb and topB in this region is consistent with this module being involved with replication, presumably of the plasmid form of ICEHin299. Surprisingly, no parB and dnaA genes were detected during the annotation of this element.

5.4.4. Putative transfer module

This is the biggest module (p299.18 to p299.39) consisting of a cluster of hypothetical proteins putative for DNA transfer. The presence of the lipoprotein gene (p299.18), glycine-rich protein (p299.19), PilL (p299.20), TraD (p299.24), VirB4 of Type IV secretory pathway (p299.33) and a series of membrane and exported proteins (p299.21, p299.22, p299.23, and p299.25 to p299.39), are consistent with this module being involved in DNA conjugative transfer.

5.4.5. Putative transfer/integration module

Genes p299.43 to p299.67 consist of 25 ORFs of which many encode hypothetical proteins with unknown functions. Only three ORFs in this module have homologues with known functions. Gene p299.51 encodes a homologue to DNA primase TraC of H.
ducreyi 35000HP (acc. no. AAP95800). The gene product of p299.66 is homologous to a conserved hypothetical gene of *Xylella fastidiosa* (acc. no. AE003998) and is also predicted to be a putative relaxase (similar to the Tral of *P. rettgeri* (acc. no. AAM08003). Gene p299.67 is similar to the *int* gene of *A. actinomycetecomitans* (EMBL AF006830). Presence of these genes likely explains the capability of ICEHin299 to carry out integrative recombination with its host's chromosome.

5.4.6. *Antibiotic resistance module*

This module consists of a cluster of genes belonging to the Tn3 transposon, which has been inserted into the transfer module. Unlike ICEHin1059, ICEHin299 has a complete copy of Tn3. The organisation of the genes is similar to the archetypal Tn3 transposon (Grindley, 2002). It is flanked by two 38-bp inverted repeats. However, these two inverted repeat sequences are not identical. Other investigators have also reported similar observation (Takeya et al., 1979). The β-lactamase TEM-1 *bla* gene is identical to the *bla* gene of *S. flexneri* (acc. no. U48775). The *tnpA* gene of Tn3 on this element is found immediately adjacent to the one end of the transposon and is transcribed towards it. The TEM-1 β-lactamase gene of ICEHin299 is similar to the β-lactamase gene of ampicillin resistant *H. influenzae*, which were commonly isolated (Hasegawa et al., 2003; Ling et al., 1989; Scheifele et al., 1982).

5.4.7. *G+C content of ICEHin299*

Fig. 5.3 depicts the distribution of G+C content in the whole element of ICEHin299. Between positions 32,401 to 37,666 bp, the G+C% was higher than the average percentage. This increased G+C% correlates with Tn3. This difference in G+C% is
typical of an insertion of a “foreign DNA” into another DNA sequence (Hacker et al., 1997).

Fig. 5.3. G+C content of ICEHin299, based on a 1000-window. The region between 32,401 and 37,666 bp has high G+C% indicating a recent DNA insertion. This graph was obtained by using Artemis program.

5.4.8. Determination of putative oriV and oriT

The oriV of most extrachromosomal DNA are usually characterised by structural elements such as, DnaA box sequences, multiple repeat sequences (interons), and an AT-rich region (Galli et al., 2001). The DnaA box sequences act as binding sites for the host DnaA initiation protein. Binding of DnaA protein to the DnaA box consensus sequence, localised within a promoter region, can stimulate or inhibit transcriptional activity, hence the regulation of gene expression and/or stimulation of the initiation of DNA replication (Messer and Weigel, 1997). However, in ICEHin299, no DnaA box (5’-TT(A/T)TNCACA) sequence was detected. If an oriV is present it would function independent of a DnaA box. Examples where DNA replication occurs without a DnaA box are plasmid RSF1010 and bacteriophage λ (Konieczny, 2003).

It is estimated that the oriV of ICEHin299 lies between positions 4,583 to 6,195-bp. This prediction is based on the presence of multiple indirect repeats, two 33-bp and four 36-bp direct repeats in an AT-rich region (Fig. 5.4). This region also correlates to the G+C% skew of the element. The process of replication is predicted by bioinformatics to
terminate at approximately 32,000-bp position, as predicted by the Oriloc program (Fig. 5.5) (Frank and Lobry, 2000). This is opposite to the predicted oriV. The existence of specific replication termini suggests that ICEHin299 undergoes theta-type replication (Bussi
erie and Bastia, 1999).

Also from the graph depicted in Fig. 5.4, it is predicted that the ICEHin299-specific oriT is located upstream of p299.66, at approximately 48,439-bp. This area has multiple repeats, two 13-mer direct repeats and two sets of inverted repeats. This region is adjacent to a gene similar to a relaxase gene of P. rettgeri (acc. no. AAM080030). This location is consistent to the observation by Murata et al. (2002), that oriT is usually located at or close to the end of the tra gene cluster (Murata et al., 2002) and adjacent to a conjugative relaxase (Mills et al., 1998). These are consistent to some of the common features found in transfer origins (Lanka and Wilkins, 1995).

The determination of oriV and oriT based on the characteristics of the nucleotide sequences is a prediction, and therefore, not confirmative. Experimental data is necessary to demonstrate the presence of oriV and oriT of an element (Mills et al., 1998).

Fig. 5.4. G+C% content (above) and GC deviation [(G – C)/(G + C)] (below) of ICEHin299, based on a 2000-bp window. Position 4,780-bp and 48,439-bp correlates to putative oriV and oriT respectively. This graph was obtained by using Artemis program.
Fig. 5.5. An output of the Oriloc program showing the terminus of ICEHin299. Replication of ICEHin299 is estimated to terminate at position 32,000-bp position.

5.5. Conclusion

The primary objective in determining the complete sequence of ICEHin299 was to provide a basis to further understand the characteristics of ICEs as the vehicles for dissemination of antibiotic resistance in H. influenzae strains. Although ICEHin1056 was sequenced as described in Chapter four, the sequencing of another ICE of H. influenzae is useful in comparative analysis of this family of element as described in Chapter six.

The complete nucleotide sequence data of ICEHin299 yielded a 53,902-bp element. It possesses only one antibiotic resistance gene *bla*, while the rest of the genes on this element are predicted to be mainly concerned with replication, transfer and integration. No genes were predicted to be associated with virulence.

The complete sequence of ICEHin299 further emphasizes that the genes of this mobile element are likely arranged in clusters according to functional modules. The detection of clusters of gene domains essentially equivalent to those of ICEHin1056
strengthens the findings that these genes are required for transfer, propagation and integration of this family of ICEs.
CHAPTER SIX

COMPARATIVE STUDIES ON MOBILE ELEMENTS OF 

HAEMOPHILUS SPP.

6.2. Objectives

In the previous chapters, the molecular characteristics of two mobile elements of *H. influenzae* were described. Preliminary analysis of ICEHin1056 and ICEHin299 shows that these epidemiologically unrelated mobile resistance elements of *H. influenzae* share extensive sequence homology. The main aim of this chapter is to investigate the relationship between mobile elements found in *Haemophilus* spp. to better understanding the extent to which they share common origins.

6.3. Materials and Methods

6.3.1. Detection of additional elements on *Haemophilus* spp.

All genomes and plasmids of *Haemophilus* spp. in the NCBI that have been completely sequenced were compared to the sequences of ICEHin1056 using Artemis Comparison Tool (ACT). Those sequences that share sequence homology with ICEHin1056 were extracted and compared.

6.3.2. *Haemophilus* spp. sequences of ICEs

The complete sequence of ICEHin1056 and ICEHin299 obtained from Chapters 5 and 6 were used in this study. A nucleotide sequence of non-typeable *H. influenzae* R2866 genome, which is still incomplete, was kindly made available by Dr. Arnold Smith from Seattle Biomedical Research Institute, USA. Dr. Thomas Inzana, Virginia Tech, Blacksburg, USA provided *H. somnus* 2336 genome sequences, which is also incomplete.
A complete sequence of an element of *H. parainfluenzae* ICEHpa8F was provided by Dr. Lucielle Mansfield, from the Nuffield Dept. of Clinical Laboratory Sciences, University of Oxford, UK.

Nucleotide sequences of complete genomes of *H. ducreyi* 35000HP (NC_002940) and *H. somnus* strain 129PT (NZ_AAB002000009); plasmids of *H. ducreyi*, pNAD1 (NC_005239), *H. aegyptius* pF3028 (NC_004058) and pF3031 (NC_004046), and *H. paragallinarum* p250 (NC_005245) were extracted from the NCBI database. A sequence of a 16-kb genomic island of *H. influenzae*, HiGI1 was obtained from accession number AF198256 (Chang *et al.*, 2000).

### 6.4. Results

#### 6.4.1. Comparison of mobile elements of Haemophilus spp.

During the search for sequence homology of ICEHin1056 to the sequences of *Haemophilus* spp. genomes and plasmids, *H. ducreyi* 35000HP and *H. somnus* 129PT were found to show sequence homology to ICEHin1056. However, only human *Haemophilus* spp. that inhabit the nasopharynx were used for comparative studies. Relationships of ICEHin1056 to the elements of *H. ducreyi* and *H. somnus* will be described in Chapter 7.

The nucleotide sequences of four mobile elements of *Haemophilus* spp., ICEHin1056, ICEHin299, ICEHpa8F and ICEHin2866 were viewed with Artemis. In Table 6.1, the general features of the four *Haemophilus* spp. are shown. Amongst the four elements, ICEHin1056 is the largest in size and has the highest G+C% content. This is attributed to the presence of Tn10 in ICEHin1056 that is absent in the other elements. ICEHin299 has the most ORFs (67 predicted genes), but most of these additional ORFs are conserved hypothetical and hypothetical proteins with unknown functions. Although
the element of *H. influenzae* 2866 has the least ORFs, the average length of its predicted genes was the longest (838 bp).

The nucleotide sequences of these four elements were compared using ACT. Two different combinations of sequences were performed to ensure fair comparison between the sequences. Figs. 6.1 and 6.2 show that all the elements share extensive sequence homology with each other, and the genes are arranged mostly in the same order (syntheny). High sequence homologies (95-99%) were observed in the replication and conjugative modules, but in the conjugation/integration module, the percentage of homologies ranged widely. The lowest homology (32%) of sequences in this module was between ICEHin1056 and ICEHpa8F, while ICEHin1056 shared the highest (98%) homology with ICEHin2866.

Individual genes of each element were compared by pairwise alignment, and the presence of genes unique to an ICE was recorded; antibiotic resistance genes (ampicillin, chloramphenicol and tetracycline in ICEHin1056 and ampicillin gene in ICEHin2866, ICEHin299 and ICEHpa8F) were excluded in this analysis (Fig. 6.3). The figure shows that 46 predicted genes are conserved in all of the elements. These genes are possibly the essential genes (core genes) in ICEs of human oropharyngeal *Haemophilus* spp. In ICEHin2866, ICEHin299 and ICEHpa8F, there are three to six genes that are non-conserved. All of these genes are located 3' of *traC* and 5' to *traI* genes. None of these genes have known functions. These non-conserved genes are most likely accessory genes and have no predicted function. They may also mark a hot spot for insertion of accessory genes. In all the elements except ICEHin1056, the insertion of Tn3 occurred at the same location.
<table>
<thead>
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<th>General features</th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHin2866</th>
<th>ICEHpa8F</th>
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<td>53,902 bp</td>
<td>53,115 bp</td>
<td>52,725 bp</td>
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<td>Per cent of genome coding</td>
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<td>89.0%</td>
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<td>Functionally classified*</td>
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<td>66% (44/67)</td>
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<td>61.6% (37/60)</td>
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<td>Conserved with no function</td>
<td>18.8% (12/64)</td>
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<td>15% (9/54)</td>
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<td></td>
</tr>
<tr>
<td>Direct</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Inverted</td>
<td>56</td>
<td>44</td>
<td>42</td>
<td>45</td>
</tr>
</tbody>
</table>

* as determined by Psort, Pfam, Prosite, HTH motif, HMTMM, FASTA and BLASTP, e.g. transmembrane or DNA binding site etc.
Fig. 6.1. Schematic diagram of ACT of four mobile elements of *Haemophilus* spp. Percentage sequence homology of each functional module between ICE*Hin*1056, ICE*Hin*2866, ICE*Hin*299 and ICE*Hpa*8F was compared. The small insert of actual diagram of ACT shows that the genes are arranged in syntheny.
Fig. 6.2. Schematic diagram of ACT of four mobile elements of *Haemophilus* spp. (arranged in a different combination). Percentage sequence homology of each functional module between ICEHin299, ICEHin1056, ICEHpa8F, and ICEHin2866 were compared. The small insert of actual diagram of ACT shows that the genes are arranged in syntheny.
Fig. 6.3. An array of predicted genes present in mobile elements of four *Haemophilus* spp. ICEs. Absence or presence of genes was recorded to reveal conserved genes and differences between ICEs.
6.4.2. *Phylogenetic relationship between the genes in the* *Haemophilus* *spp.*

Relationships between 12 genes present in all of the elements were determined. Nucleotide sequences of four genes in the replication module, six genes in the conjugation module and two genes in the integration/integration module were chosen. Phylogenetic relationship between the genes was determined using MEGA and SplitsTree decomposition. The results are shown in Table 6.2 and pairwise distances between the genes, created by MEGA, are shown in Appendix C.

Overall, results showed that genes in ICEHin1056 are constantly found to be more distant than genes of other elements. Although the genes in the replication module *soj, dnaB, ssb* and *topB* are congruent to each other, the genes in ICEHin1056 seem to be relatively more distant. Similarly in the conjugation module, the *traD* gene of ICEHpa8F was the only gene showing a slight change in the congruence of the tree topologies. However, in the conjugation/integration module, the relaxase and the primase of ICEHin1056 and ICEHin2866 were, by comparison, very distantly related to those found in ICEHin299 and ICEHpa8F.
Table 6.2. Phylogenetic relationship of genes in mobile elements of four Haemophilus spp.

UPGMA output

SplitsTree decomposition output

soj

DNA

soj

BF soj

1056 soj

1056 ssb

2866 ssb

299 ssb

BF ssb

1056 ssb

dnaB

DNA

soj

BF soj

1056 soj

1056 dnaB

2866 dnaB

299 dnaB

BF dnaB

1056 dnaB

ssb

DNA

soj

BF soj

1056 soj

1056 ssb

2866 ssb

299 ssb

BF ssb

1056 ssb

topB

DNA

soj

BF soj

1056 soj

1056 topB

2866 topB

299 topB

BF topB

1056 topB
UPGMA output

Gene 52

tral

int

SplitsTree decomposition output

traC


6.4.3. *Comparison of ampicillin resistance genes*

Sequence homology of the genes on the Tn3 transposon of the elements was investigated. The nucleotide sequences of these genes were compared using ACT and pairwise alignment for the percentage of sequence homology were carried out using ClustalW (Fig. 6.4).

One major difference between the Tn3 ICEHin1056 to the other elements is that it does not have the gene that encodes transposase. With the exception of ICEHin1056, the *tnpA*, *tnpR* and *bla* genes of ICEHin299, ICEHpa8F and ICEHin2866 are identical. ICEHin1056 shares an identical *bla* gene with these ICEs, but has 3% base difference in its *tnpR* gene. However, the ampicillin resistance gene *bla* of the *Haemophilus* spp. is between 1-6% different in nucleotide sequence to the Tn3 associated *bla* found in *E. coli*.

The sequences (100-bases) flanking the Tn3 were investigated to determine the preferred site for insertion of the transposon. The truncated Tn3 of ICEHin1056 was located at a different location to those found in the other three ICEs. Tn3 inserted at the identical site in ICEHin299, ICEHin2866 and ICEHpa8F, as deduced from analysing the sequences at the point of insertion (the ampicillin genes of ICEHin1056 were excluded in the comparison because ICEHin1056 does not have complete sequences). See the nucleotide sequences at the left junction between Tn3 and the three ICEs, Fig. 6.5. A similar observation was noticed at the right junction of Tn3.

A phylogenetic relationship of ampicillin genes between *Haemophilus* spp. and *E. coli* (acc. no. V00613 and plasmid pIGAL1, acc. no. AY424310) are shown in Table 6.3. The ampicillin resistance genes within *Haemophilus* spp. were congruent but are not relatively different from those of *E. coli*. 

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Fig. 6.4. Schematic diagram of Tn3 transposons of four *Haemophilus* spp. in comparison to *E. coli* (acc. no. V00613).
Fig. 6.5. Part of the nucleotide sequences of left junction between Tn3 of three ICEs. Conserved core ICE sequences observed at the junction. Sequences were aligned using ClustalW.

Table 6.3. Phylogenetic relationship of ampicillin resistance genes of *Haemophilus* spp. and *E. coli*

<table>
<thead>
<tr>
<th>UPGMA output</th>
<th>SplitsTree decomposition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tnpA</em></td>
<td></td>
</tr>
<tr>
<td><em>tnpR</em></td>
<td></td>
</tr>
<tr>
<td><em>bla</em></td>
<td></td>
</tr>
</tbody>
</table>
6.4.4. Comparisons of oriV, ter and oriT

The putative regions for oriV of the Haemophilus ICEs were predicted and shown as depicted in Fig. 6.6. This prediction was based on the presence of iterons type sequences, multiple indirect repeats in an AT-rich region and located in an intergenic region. The sequences 200-bp upstream of the iterons are fairly conserved with three bases differences, however, the nucleotide sequences of the presumed iterons are variable (Fig. 6.7)

Replication terminates at a predicted ter site. In all of the elements, the predicted ter sites are indicated by the G+C% skew as shown in Fig. 6.8. At this site, all of the elements have multiple indirect repeats. Presence of TMHMM motif, signal peptide with cleavage site and ATP/GTP binding site are predictive of ter-associated genes. The nucleotide sequences immediately before and after the predicted ter site are highly conserved.

The presence of predicted genes with the following: HTH motifs, TMHMM motifs, the presence of homologues to primase and relaxase and inverted repeats in an AT-rich region predicts a putative oriT (Fig. 6.9). This position also maps asymmetrically to the putative conjugative module, thereby, conforming to another of the features of an oriT (Lanka and Wilkins, 1995). The indirect repeats predicted to represent oriT have a core 8-bp (ATGGGGAG) conserved in all ICEs. However, there are sequences variations between the ICEs (Fig. 6.10). The nucleotide sequence flanking the putative oriT of ICEHin1056, ICEHin299 and ICEHin2866 are highly conserved, but in ICEHpa8F, only the sequences containing the indirect repeat of the putative oriT and those immediately adjacent to it are conserved (Fig. 6.10).
Fig. 6.6. Putative origin of replication (oriV) of four mobile elements of Haemophilus spp. Presence of direct repeats and multiple indirect repeats in low G+C% region, indicative of the region of an oriV (circled). The small arrowheads represent indirect repeats and the short arrow within the circle represents interons. This diagram was obtained by using Artemis program.
Fig. 6.7. Schematic diagram of the oriV region of ICEs.
Fig. 6.8. Putative replication termination (ter) site of the four mobile elements of *Haemophilus* spp. The ter sites are bracketed. The small arrowheads represent indirect repeats and the short arrow within the circle represents interons. This diagram was obtained by using Artemis program.
Fig. 6.9. Putative origin of transfer (oriT) of four mobile elements of *Haemophilus* spp. Presence of direct repeats and multiple indirect repeats in low G+C% region, indicative of the region of an oriT (bracketed). The small arrowheads represent indirect repeats and the short arrow within the circle represents interons. This diagram was obtained by using Artemis program.
Alignment of sequences of the four elements closed to the oriT. Note that the sequences are highly conserved in ICEHin1056, ICEHin2866 and ICEHin299 but in ICEHpa8F, only the site of oriT is conserved. Alignment performed by ClustalW.
6.4.5. Comparison of ICE Hin1056 to other Haemophilus plasmids and genetic islands

6.4.5.1. Genetic island of HiG1

H. influenzae strain Eagan has been reported to possess a 16-kb genetic island, HiG1 is inserted into the genome at the 3' end of tRNA_{leu} and possesses an integrase gene located downstream of the tRNA_{leu} (AF198256) (Chang et al., 2000).

An ACT was performed for HiG1 against ICE Hin1056 and the result is shown in Fig. 6.11. No significant matches between these two sequences were observed. In addition, pairwise comparison between the sequences did not give any significant similarity. Although this locus has several features of a genetic island, it does not show an organisation or sequence homologies that suggests it shares features in common with the ICEs reported here other than inserting in tRNA_{leu} and possessing an integrase.

![ACT diagram of sequence comparison between genetic island HiG1 and ICE Hin1056. The vertical lines denote amino acid homology of >25%. No gene similarity was observed.](image)
6.4.5.2. *H. aegyptius* plasmids

Two plasmids (pF3028 and pF3031, acc. no. NC_004058 and NC_004846 respectively) have been isolated from *H. influenzae* biotype aegyptius causing Brazilian Purpuric Fever in children. An ACT comparison was carried out between the sequences of ICE*Hin*1056 and the plasmids. Fig. 6.12 shows that only *topB* of the *H. aegyptius* plasmids was homologous to ICE*Hin*1056. In addition, no significant similarities at nucleotide level were observed by pairwise alignment.

Fig. 6.12. An ACT diagram of sequence comparisons between pF3028, pF3031 and ICE*Hin*1056. The vertical lines denote amino acid homology of >25%. The thick line indicates the gene similarity between the sequences.

6.4.5.3. *H. ducreyi* pNAD1

The sequences of a 5.6-kb plasmid pNAD1, isolated from *H. ducreyi* strain ATCC27722 were compared to ICE*Hin*1056. The ACT diagram in Fig. 6.13 shows that pNAD1 do not have any gene similarities to ICE*Hin*1056.
Fig. 6.13. An ACT diagram of sequence comparison between pNAD1 and ICEHin1056. The vertical lines denotes amino acid homology of >25%. No gene similarity between the sequences is observed.

6.4.5.4. *H. paragallinarum p250*

The nucleotide sequence of a plasmid isolated from an avian Haemophilus was compared to ICEHin1056. Plasmid p250 encodes haemocin in *H. paragallinarum* HP250. It contains a putative integrase, a putative replication protein (*repB*) and five haemocin genes (Terry *et al.*, 2003). Haemocin in *H. paragallinarum* is believed to aid in colonization by inhibiting other Gram-negative bacteria in the respiratory tracts of chickens.

The result of ACT between p250 and ICEHin1056 is shown in Fig. 6.14. No genes of p250 were similar to ICEHin1056. This observation indicates that p250 does not have shared sequences with ICEHin1056.
6.5. Discussion

The mobile elements encoding antibiotic resistance ICEHin1056, ICEHin299, ICEHpa8F and ICEHin2866 have been shown to possess nucleotide sequences that are highly homologous and are arranged in the same order. The predicted genes with homology to genes with known functions are highly conserved and are organised into putative modules for replication, conjugative transfer and integration.

The phylogenetic relationships of the genes in the replication and conjugation modules of the four elements were essentially congruent as demonstrated by the conserved tree topologies. Some of the ICEHin1056 and ICEHpa8F genes have measurably diverged, but it is not possible to determine whether this has been a result of point mutation or recombination. The genes in the conjugation/integration module are divergent and, showed different tree topologies to those in the other modules. This strongly suggests that the genes in this module have undergone recombination and re-assortment. These genes are located between the putative oriT and the integration/recombination site, attP. The data available in this comparative study suggests...
that the conjugation/integration module consists of one of two divergent lineages that have recombined with the rest of the highly conserved ICE core sequences. How this has happened is unclear, but may relate to the ICEs preferred host species. The ICEHin1056 variant module is over-represented in *H. influenzae* compared to *H. parainfluenzae* (Dr D. Crook, personal communication).

In all the ICEs, the *bla* gene are carried on Tn3. Tn3 is inserted in the same specific site in all the ICEs, except ICEHin1056. This site specificity for insertion of Tn3 is not surprising, as transposons have preferred sites of insertion. The event that has led to the truncated Tn3 in ICEHin1056 is unclear. Though, such defective transposons have been frequently observed in other organisms. The highly conserved nucleotide sequence of the Haemophilus derived Tn3 transposons when compared with those found in *E. coli* is consistent with a recent acquisition by Haemophilus. It also suggests that *E. coli* has not recently donated Tn3 to oropharyngeal haemophili.

The sequences at the oriV region were highly conserved. It is located in the replication module, between dnaB and ssb. Though this is in a region predicted to contain a gene, there are no clear functional homologues for the predicted gene. Therefore, this may represent an intergenic region without any stop codons. In this AT-rich region, multiple inverted repeats and two regions of putative iterons were observed. This is consistent with the features of an oriV, described in various reports (del Solar *et al.*, 1998; Espinosa *et al.*, 2000). Iterons play important role in the binding site of Rep proteins. Surprisingly, no Rep protein and DnaA protein-binding sequence have been detected in this region. It is possible that ICEs of *Haemophilus* spp. does not have DnaA box, and are similar to that reported for *P. aeruginosa* plasmid R18, which carries out replication without a DnaA box (Shah *et al.*, 1995). Despite the lack of a box, the deletion
of dnaA in P. aeruginosa reduced the plasmid copy number. Therefore, the lack of a DnaA box does not necessarily mean that DnaA has no role in replication.

The replication process in all the elements putatively terminates at a common ter site, indicated by the descending G+C% in the GC-skew graph. At this site, the presence of predicted genes with a TMHMM motif, a predicted cleavage site and multiple inverted repeats are features typical of where replication terminates (Lanka and Wilkins, 1995). Presence of a predicted ter site on all of the elements suggests that the ICEs carry out theta replication (Bussiere and Bastia, 1999; del Solar et al., 1998; Espinosa et al., 2000).

The sequences of the oriT region were conserved in ICEHin1056, ICEHin299, and ICEHin2866, but in ICEHpa8F only sequences around the inverted repeat of the predicted oriT were conserved. There can be variation in the sequences of oriT between related plasmids from different species and this may explain why ICEHpa8F differs from the other three ICEs all found in H. influenzae. Also, it is possible that the minimal oriT consists of only the core 8-bp shared by all the inverted repeats. Besides the inverted repeats, the high A+T content of this region, proximity to the predicted oriT to traC, and tral at the extreme end of the putative conjugative module suggest that DNA transfer process begins at this region (Lanka and Wilkins, 1995).

The predictions for the location of the oriV, ter and oriT of the elements are mainly based on the features and motifs provided through bioinformatics, and are not really necessarily reliable predictions. The ultimate test of whether these are the predicted structures will depend on experimental proof.

Other species of Haemophilus, H. ducreyi 35000HP and H. somnus 129PT have sequences on their genome that share sequences homologous to ICEs, indicating that the strains of H. ducreyi and H. somnus apparently carry mobile elements that are still
integrated on their chromosome. Further analysis of these elements is described in Chapter 7.

In contrast, the genetic island of *H. influenzae*, HiG1 and plasmids of *H. aegyptius*, *H. ducreyi* and *H. paragallinarum* do not share homology to ICEs. Although HiG1 has inserted into tRNA<sub>leu</sub>, it clearly has no detectable homologies using the TBLASTX algorithm.
CHAPTER SEVEN

ICEHin1056 AND ITS RELATIONSHIP WITH OTHER GENOMIC ISLANDS

7.1. Introduction

GIs which are part of the horizontal or flexible gene pool often constitute a large part (over 10%) of bacterial genomes (Hacker et al., 1997; Hacker and Carniel, 2001; Spencer et al., 2003). Genes important in habitat specific adaptation cluster on these island (Dobrindt et al., 2004; Hacker and Carniel, 2001). Well-recognised examples include island with genes involved in pathogenesis (Hacker and Kaper, 2000), avirulence (Alfano et al., 2000), chemical degradation (van der Meer and Sentchilo, 2003), antibiotic resistance (Klockgether et al., 2004) and metabolic functions (Larbig et al., 2002). Hitherto, the evolutionary origin of these islands has not been elucidated (Dobrindt et al., 2002).

7.2. Objectives

The objective of this study was to investigate the relationship between ICEHin1056 and other GIs among all bacteria. The presence of a coherent conserved core structure was investigated among homologous GIs.

7.3. Materials and Methods

7.3.1. Identification of GIs

The identification of sequences homologous to ICEHin1056 was achieved by searching the NCBI database using the TBLASTX algorithm. The input ICEHin1056 sequence consisted of the entire element without the antibiotic-resistance associated (Tn10 and
Tn3) sequences. Sequences producing significant alignments (i.e. E value <10<sup>-5</sup>) were individually interrogated by TBLASTX for contiguous sequences homologous to ICEHin1056. Twenty candidates GIs were identified.

Preliminary analysis of the ORFs of the candidate GIs were compared for amino acid sequence homology using ACT. However, four potential GIs that were found to contain short sequence of shared homology, or were from incompletely sequenced genomes were excluded for further analysis. The remaining 17 GIs (including ICEHin1056) with extensive homology were used for further investigation. A preliminary investigation of phylogenetic relationships between these 17 GIs was undertaken using ClustalX for multiple alignment and tree estimation. The amino acid sequences of two genes present in all 17 GIs were aligned. This yielded four clusters. One well-known representative GI (ICEHin1056, SPI-7, PAP1 and PAGI-3) of each cluster was chosen to determine all genes shared by these GIs and to identify potential core GI genes.

### 7.3.2 Construction of virtual core GI sequences

Core GI sequences were defined as those present (BLASTP E value <10<sup>-5</sup>) in at least three of the four GIs SPI-7, PAP1, ICEHin1056 and PAGI-3. The presence or absence of GI genes was visually scored by pair-wise comparison between each of the GIs using ACT and individually aligning potential homologues using the BLASTP algorithm. Thirty-three core genes fitting the definition were identified (numbered 1 to 33, Fig. 7.1). The sequence of each gene was used to construct four virtual core GIs each representative of ICEHin1056, SPI-7, PAP1, and PAGI-3. Virtual core GIs consisting of either amino acid sequences or nucleotide sequences was constructed for each of the four GIs respectively. For any of the genes missing from a GI, the homologue from PAP1 was used.
7.3.3. **Identification of core genes of other GI candidates**

The concatenated nucleotide sequence of core genes (containing 33 core genes) from each of these four representative GIs were used to re-interrogate the databases using TBLASTX to ensure that no additional GIs were missed during the preliminary screening using ICEHin1056 sequences. No other GIs were found.

A virtual coherent GIs were used to compare to each candidate GI using ACT. The presence and absence of core genes was visually scored by pair-wise comparison between the candidate GIs and the virtual coherent element. A coherent evolutionary related GI was defined as possessing ≥25 (~75%) of the 33 core genes. Of the 33 core genes, only 15 genes were shared by all the GIs.

7.3.4. **G+C% content**

The percentages of G+C% content of each GIs and their core sequences was determined through Artemis, and the G+C% content of the host’s whole genomes was accessible through GenBank website (http://www.ncbi.nlm.nih.gov/genomes/static/eub_g.html)

7.3.5. **Phylogenetic relationship between GIs**

Phylogenetic relationships between the amino acid sequences of each of the 15 core genes common in all the GIs were analysed using ClustalX. The concatenated amino acid sequences of all 15-shared genes in each GI were aligned. The analysis used the following settings: exclude position with gaps, correct for multiple substitutions, phylip format and bootstrap neighbour-joining tree of 100. The output of the analysis was viewed using TreeView (Page, 1996). The congruence of tree topologies was compared to determine whether there was evidence of divergent evolution.
7.4. Results

7.4.1. Identification of related coherent GIs

Twenty potential GIs with homology to the non-resistance associated sequences of ICEHin1056 were identified from interrogation of the NCBI database using the TBLASTX algorithm. Four of these with extensive homology (ICEHin1056, SPI-7, PAP1 and PAGI-3) and representing the diversity of these GIs were selected to identify the predicted core element (i.e. genes present in at least three of four) of these GIs. Thirty-three genes fulfilled this definition. Virtual core elements of 33 genes each representing these four GIs were constructed and used in a pair-wise comparison with the 20 candidate and related GIs. Sixteen GIs of the 20 met the definition of a coherent element (24 of the 33 (~75%) core genes being present). The species and strains harbouring the coherent GIs are listed Table 2.4. As the GI in B. fungorum, (acc. no. NZ_AAAJ00000000) shares nucleotide sequence identity to the clc element of Pseudomonas sp. B13 (van der Meer and Sentchilo, 2003), it was not analysed as a separate GI.

The following complete genomes contained contiguous sequences homologous to < 24 of the 33 genes present in a virtual coherent GI: X. fastidiosa 9a5c, (acc. no. NC_002488, 11 genes); and P. syringae pv. tomato DC3000 (acc. no. NC_004578, 17 genes). These, therefore, did not meet the definition of a coherent GI. An incomplete genome sequence of A. vinelandii (NZ_AAAU00000000) contained sequences suggesting the presence of a homologous GI, but the unfinished sequence was unsuitable for determining whether it contained a coherent GI. Lastly, a P. fluorescens strain currently undergoing whole genome sequencing contained a homologous GI, but was not analysed further due to publication restrictions. The three GIs of E. coli strain 536 (PAI I536, PAI II536, and PAI III536, acc. nos.: AJ488511, AJ494981, X16664, respectively),
which were investigated as negative controls, exhibited no homology with a virtual core element when viewed with ACT.

7.4.2. **Features of core genes**

The elements were all predicted to, or are known to, integrate into tRNA genes as listed in Fig. 7.1. There were five different tRNA genes that were sites of insertion for these GIs, with six of 15 inserting in tRNA\textsubscript{gly}. The core genes of each of the GIs were largely in the same order (i.e. syntheny) (Fig. 7.2). The amino acid sequence homology between GIs at the extremes of homology was as low as 25% to 30% for many of the shared core genes indicating wide evolutionary divergence. Of the 33 core genes, ten had homology to genes with known function. These included four genes, \textit{parA}, \textit{dnaB}, \textit{ssb} and \textit{topB} among genes 1 to 10 (Figs. 7.1 and 7.2). Homologues to these genes are recognised to play a role together in plasmid replication (Espinosa \textit{et al.}, 2000; Gerdes \textit{et al.}, 2000). One of the genes in this region \textit{inrR} has recently been shown to regulate integrase function in the \textit{clc} element and controls excisive and integrative recombination of that element with tRNA\textsubscript{gly}. Genes 11 to 32 are mostly of unknown function, but four exhibit homology to \textit{pilL} (core gene 11), \textit{virB4} (core gene 27), \textit{traD} (core gene 17) and a relaxase or \textit{tral} (core gene 32); homologues of these four are recognized to play a role in conjugative DNA transfer (Zechner \textit{et al.}, 2000).

Integrases were found in all GIs other than that in \textit{R. metallidurans}. As the DNA sequence of this organism was not complete, it is premature to conclude that an integrase associated with this GI is absent. The remaining GIs possess an integrase of either the P4 or XerC/D lineages. SPI-7, the pathogenicity island found in \textit{S. Typhi}, possesses copies of both types of integrase (Fig. 7.2). The XerC/D integrase that was immediately adjacent to the innermost tRNA\textsubscript{phe} sequence present at the right end of the GI (the putative \textit{attR}) was
selected as the integrase shown in Fig. 7.2, because only this integrase was consistently present in SPI-7 like GIs identified in a range of *Salmonella enterica* serovars Typhi (Pickard *et al.*, 2003).

Fifteen of the core GI genes were conserved across all the GIs (Fig. 7.1). The G+C% content of these were determined and ranged widely from 40.2% for *H. somnus* 2336 to 69.6% for *X. axonopodis*. Compared to the average G+C% of their host’s genome, nine GIs had higher percentage content. These consisted of all those found in the *Pasteurellaceae* and *Enterobacteriaceae* (except SPI-7 in *S. Typhi*, which had a lower content than its host genome). Similarly, the GIs found in *Pseudomonas* sp. B13, B. *fungorum*, (genomic G+C% = 61.8%), *R. metallidurans* and *X. axonopodis* had higher G+C% content than their host’s genome. However, the GIs found in both *P. fluorescens* and the GIs PAGI-3, PAP1 and pKLC102 found in *P. aeruginosa* had lower G+C% content than their hosts.
<table>
<thead>
<tr>
<th>Bacteria (strain/GI name)</th>
<th>tRNAb</th>
<th>G+C%c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(GI/Genome)</td>
</tr>
<tr>
<td>H. ducreyi (3500HP/un^3)</td>
<td>gly</td>
<td>43.5/38.7</td>
</tr>
<tr>
<td>H. influenzae (1056/ICE Hin1056)</td>
<td>leu</td>
<td>41.3/38.1</td>
</tr>
<tr>
<td>H. somnus (129PT/un)</td>
<td>gly</td>
<td>41.9/38.1</td>
</tr>
<tr>
<td>H. somnus (2336/un)</td>
<td>leu</td>
<td>40.2/38.1</td>
</tr>
<tr>
<td>S. typhi (CT18 and TY2/SPI-7)</td>
<td>phe</td>
<td>49.3/52.1</td>
</tr>
<tr>
<td>P. luminescens (TT01/un)</td>
<td>phe</td>
<td>50.2/42.8</td>
</tr>
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<td>Y. enterocolitica (8081/un)</td>
<td>gly</td>
<td>50.0/47.3</td>
</tr>
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<td>P. fluorescens (P90-1/un)</td>
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<td>59.7/60.6</td>
</tr>
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<td>P. fluorescens (SBW25/un)</td>
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<td>P. aeruginosa (PA14/PAPI)</td>
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</tr>
<tr>
<td>P. aeruginosa (C/pKLC102)</td>
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<td>62.7/66.6</td>
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<tr>
<td>R. metallidurans (CH34/un)</td>
<td>cys</td>
<td>68.2/63.5</td>
</tr>
<tr>
<td>Pseudomonas sp (B13/cle^d)</td>
<td>gly</td>
<td>66.2/65.0</td>
</tr>
<tr>
<td>P. aeruginosa (SG17M/PAGI-3)</td>
<td>gly</td>
<td>66.1/66.6</td>
</tr>
<tr>
<td>X. axonopodis (306/un)</td>
<td>leu</td>
<td>69.6/64.7</td>
</tr>
</tbody>
</table>

Fig. 7.1. An array of predicted genes present in related coherent GIs found in Proteobacteria. ^unnamed, ^tRNA site of GI integration, ^G+C% content of GI and its host genome, ^cle element is present in the complete sequence of B. fungorum.
Fig. 7.2. A modified ACT diagram of the homologous regions identified between four GIs ICEHin1056, SP-7, PAP1, clc and the core genes of the virtual GI. Homologous sequences (>25% amino acid identity) are indicated by red lines joining regions on the five schematic representations of the GIs.
7.4.3. *Phylogenetic relationship between GIs*

The phylogenetic relationships between each of the 15 genes common to all the GIs exhibited congruent structure with a few minor exceptions. The trees for each of four representative core genes (genes 1, 6, 18, 27) are available in Appendix E. The phylogenetic relationships of the GIs as determined by alignment of the concatenated amino acid sequences of all 15 conserved genes is depicted in Fig. 7.3 and is congruent with that for each of the individual genes.

![Diagram of phylogenetic relationships]

Fig. 7.3. A SplitTree generated by ClustalX analysis of concatenated core genes of 15 GIs. The amino acid sequences of the 15 predicted genes common to all 15 GIs were concatenated and aligned using ClustalX. The alignment of each of the genes alone was congruent to the one illustrated here. The names of the GIs, where available, are given in the parentheses.
The size of the coherent virtual element was approximately 18 kb, while the whole GIs ranged from 49 kb for *H. ducreyi* to 140 kb for *P. luminescens* (Table 7.1). The variation in the sizes of these related GIs was attributably largely to differences in accessory gene content.

Nestled between conserved core genes of each GI was a wide range of different accessory genes (Figs. 7.1 and 7.2). The sites of insertion were largely clustered in two regions, either between core genes 9 to 14 or between core genes 31 to 33. The major attributes of the accessory genes are listed in Table 7.1 and include: antibiotic, metal and antiseptic resistance, degradation of chemicals, Type IV secretion systems, two component signalling systems, biofilm regulation, Vi antigen capsule synthesis, cytolethal toxin production, anti-restriction systems and a wide range of metabolic functions. Many of the accessory genes do not share homology to genes of known function (Fig. 7.2). The resistance genes found in *ICEHin1056* were inserted at two different sites. A TnJ0-like transposon that contains the *tetA* and *cat* genes lies between core genes 10 and 11. Tn3 that contains the *bla* gene is inserted between core genes 31 and 32 (Figs. 7.1 and 7.2).
Table 7.1. Molecular and phenotypic characteristics of GIs of bacterial pathogens causing diseases in human, animals and plants.

<table>
<thead>
<tr>
<th>Bacteria (strain/GI name)</th>
<th>Size (bp)</th>
<th>Accession No.</th>
<th>Major attributes of accessory genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. ducreyi</em> (35000HP/un)</td>
<td>49,075</td>
<td>NC_002940</td>
<td>Type I antirestriction, cytolethal distending toxin</td>
</tr>
<tr>
<td><em>H. influenzae</em> (1056/ICEHin1056)</td>
<td>59,393</td>
<td>AJ627386</td>
<td>Antibiotic resistance</td>
</tr>
<tr>
<td><em>H. somnus</em> (129PT/un)</td>
<td>56,442</td>
<td>NZ_AAB002000009</td>
<td>Type I antirestriction, virulence protein</td>
</tr>
<tr>
<td><em>H. somnus</em> (2336/un)</td>
<td>64,831</td>
<td>NZ_AAC00000000</td>
<td>Antibiotic, metal and antiseptic resistance</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> (8081/un)</td>
<td>65,285</td>
<td>NC_003222</td>
<td>Type IV secretion, arsenic resistance, hemagglutinin-related protein</td>
</tr>
<tr>
<td><em>S. typhi</em> (CT18 and TY2/SPI-7)</td>
<td>134,000</td>
<td>NC_003198</td>
<td>Type IV secretion, UV protection, Vi antigen</td>
</tr>
<tr>
<td><em>P. luminiscens</em> (TT01/un)</td>
<td>140,163</td>
<td>NC_005126</td>
<td>Metabolic enzymes, Type IV secretion</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (PI0-1/un)</td>
<td>70,421</td>
<td>NZ_AAAT00000000</td>
<td>Biofilm regulators, fimbral proteins, metabolic enzymes</td>
</tr>
<tr>
<td><em>P. fluorescens</em> (SBW25/un)</td>
<td>101,233</td>
<td>NC_004507</td>
<td>Metabolic enzymes</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (PA14/PAP1)</td>
<td>106,518</td>
<td>AY273869</td>
<td>Pyocin, type IV secretion, fimbral protein, two component signal system</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (C/pKLC102)</td>
<td>103,532</td>
<td>AY225738</td>
<td>Type IV secretion, virulence protein</td>
</tr>
<tr>
<td><em>R. metallidurans</em> (CH34/un)</td>
<td>&gt;76,893</td>
<td>NZ_AAA100000000</td>
<td>Degradative enzymes, metabolic enzymes, pathogenesis-related protein</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> (B13/e/c)</td>
<td>105,027</td>
<td>AJ617740</td>
<td>Metabolic enzymes, degradative enzymes</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (SG17M/PAGI-3)</td>
<td>114,632</td>
<td>AF440524</td>
<td>Plasmid transfer proteins, type I restriction-modification, metabolic enzymes</td>
</tr>
<tr>
<td><em>X. axonopodis</em> (306/un)</td>
<td>86,019</td>
<td>NC_003919</td>
<td>Phage-related proteins, efflux pump, hemolysin</td>
</tr>
</tbody>
</table>

*un = unnamed, *cic element is present in the complete sequence of *B. fungorum.*
7.4.5. Hot spots

Some ‘hot spot’ areas were apparent where insertion of accessory genes was mainly concentrated (Fig. 7.1). Most of the insertion of accessory genes occurred in two regions: between the ssb and pilL genes (core genes 9 and 14); and the region close to the int gene (core genes 31 and 33). It is noted that all of the islands have insertions in the region close to the int gene. The reason for their preference is unknown.

The regions where no or few insertions occurred are apparently made up of clusters of genes that are cohesive. It is possible that the functions of these clusters of genes would be interrupted by insertion of accessory genes. The region to the left of the first ‘hot spot’ contains gene cluster putatively involved in replication. It has been previously shown in enterobacteria that the order of genes close to replication origins is more conserved (Sharp et al., 1989). The following region between core genes 14 to 31 contains a gene cluster likely encoding DNA transfer. Insertion of accessory genes in this region may disrupt conjugative transfer.

7.5. Discussion

7.5.1. A coherent core GI

A family of related and coherent GIs with a common evolutionary origin among diverse Proteobacterial hosts has been recognized for the first time. These GIs have diverged widely as demonstrated by as much as 30% divergence (40% to 70%) in the G+C% content between GIs and amino acid homologies as low as 25% to 30% between homologous GI genes. These GIs have acquired diverse accessory genes with habitat specific adaptive functions typical of GIs or PAIs (Dobrindt et al., 2004; Hacker and Carniel, 2001). Other features typical of Proteobacterial GIs are insertion into tRNA genes and a G+C% content different to their host’s genome (Dobrindt et al., 2004).
It has been hypothesized that GIs are mosaics of independently evolving genes or clusters of genes (Burrus et al., 2002a; Toussaint and Merlin, 2002). This is referred to as modular evolution. In the reports by Burrus et al., it was demonstrated that heterogeneous modules were assorted between different GIs (Burrus et al., 2002a, b). This lack of coherent structure is probable following repeated recombination arising from frequent horizontal spread of GIs. This would be expected to erase coherent structures between GIs over time. The finding reported here, at least, identifies one family of related GIs that challenges this hypothesis. A coherent element with a common evolutionary origin has been found. The cut-off value of 75% (core GI sequences in at least three of the four GIs) may have limited the identification of only one family of GI. Perhaps, by reducing the cut-off value, more than one family of GI could be identified, is yet to be determined.

The accessory genes of these related GIs are inserted in a manner apparently consistent with modular evolution. However, variable modules consisting of accessory genes nestle in a conserved module that is a coherent core GI. This forms the apparent mosaic structure of this family of GIs. The presence of a coherent core GI suggests a fitness property of the conserved core genes acting collectively that has ensured their survival as a coherent whole. It is unclear what this may be; yet it is reasonable to surmise that it is the ability of these GIs to transmit and propagate better as a coherent whole. The role of accessory genes in the survival of this coherent element appears to be interwoven with allowing organism specific adaptation to habitats. This will necessarily involve complex interactions between the core GI, accessory genes and the survival of the host bacterium in new hostile environments.

Bio-informatics analysis of the core genes and review of the functional properties of some of the well-characterised examples of this family of GI provides insight on the possible function of the core GI genes. An integrase is present in all but one (R.
metallidurans) of these GIs. These integrases are likely to encode integration with tRNA genes and all belong to the family of tyrosine integrases. The only GI where this has been conclusively shown is the clc element found in Pseudomonas sp. B13, which contains a homologue of the P4 integrase (Ravatn et al., 1998). A curious aspect of the tyrosine integrases found in these GIs is that they belong to two different lineages, a P4 lineage or a XerC/D lineage. Both types of integrase are known to encode recombination with tRNA genes in other mobile elements (Mantri and Williams, 2004). How members of these GIs have acquired either of these two divergent lineages of integrases is intriguing and the available data provides no obvious explanation. The observation that SPI-7 found in S. Typhi contains both integrase lineages, while SPI-7 like GIs found in other S. enterica serovars do not (Pickard et al., 2003) may suggest that there are ready opportunities for exchanging integrase lineages.

How GIs transfer horizontally has not been clearly explained (Dobrindt et al., 2004). Though, it has been recognised that the clc element is a GI and is capable of conjugative transfer (van der Meer and Sentchilo, 2003). The observation made here that another conjugative element, ICEHin1056, found in H. influenzae, is also a related GI strengthens this observation. Both these GIs transfer by conjugation at frequencies of $10^{-6}$ to $10^{-7}$ (transconjugants/donors) (Dimopoulou et al., 1992; Ravatn et al., 1998). The genes encoding this property have not been identified in either GI. However, the genes encoding this function are likely to be present among the core genes as none of the accessory genes present in either GI is shared. Also, none of their accessory genes is known to encode conjugative functions. Furthermore, four of the core GI genes are homologous to pilL, traD, virB4 and tral and are interspersed along a contiguous 22-gene segment of the GI. These genes are known to be involved in DNA conjugative transfer systems (Zechner et al., 2000). Therefore, it can be inferred that core genes are likely to
encode transfer functions that would explain how this family of GI transfers between hosts. Before this can be firmly concluded, however, this needs to be formally investigated and demonstrated experimentally.

Autonomous replication would be an unexpected property of GIs (Dobrindt et al., 2004). However, four of the first 10 genes are homologues of *parA, dnaB, ssb* and *topB*, genes known to be associated with plasmid replication (Espinosa et al., 2000; Gerdes et al., 2000). Furthermore, both ICE*Hin1056* found in *H. influenzae* and pKLC102 found in *P. aeruginosa* C appear to exist as extra-chromosomal closed circular plasmid under some conditions (Dimopoulou et al., 1997, Dimopoulou et al., 1992, Klockgether et al., 2004; Leaves et al., 2000). ICE*Hin1056* or other related Haemophilus conjugative elements are found largely in plasmid form in transconjugants immediately following conjugation (Dimopoulou et al., 1992). This is supported by two observations: first, ready isolation of plasmid from transconjugants and not from parent donor clinical isolates (Dimopoulou et al., 1992). Second, the appearance of Southern blot hybridisation patterns consistent with a closed circular form in transconjugants and a pattern indicating chromosomal integration in parent donor strains (Dimopoulou et al., 1992; Dimopoulou et al., 1997; Leaves et al., 2000). The pKLC102 has not been formally shown to replicate autonomously; however, Klockgether et al. reported the presence of an extra-chromosomal form and sequences close to the *ssb* gene with features typical of an *oriV* (Klockgether et al., 2004). These data suggest that some, if not all, these GIs may be capable of autonomous replication under some conditions, but this needs to be formally determined experimentally before this can be concluded. Were this to be found, it would be a new, and hitherto unrecognised, dimension to GIs (Dobrindt et al., 2004; Hacker and Carniel, 2001) or integrating and conjugating elements reviewed by Burrus et al. (Burrus et al., 2002a). Furthermore, an element that was capable of integrative and excisive
recombination, conjugative transfer and autonomous replication would require finely coordinated regulation of these functions to ensure their timing was appropriate to the relevant state of the GI.

The origin and host range of this family of GI is not apparent from the data available in this investigation. They were only present in organisms belonging to *Proteobacteria*, but the investigation is restricted by the limitations of bio-informatics dependent on algorithms relatively poor at detecting weak homologies across contiguous multi-gene structures necessary for recognizing these related GIs and also the relatively small number of whole genome sequences available for analysis. If this family of GIs is truly restricted to *Proteobacteria*, some insight into the origin of this GI family may come from simple consideration of its average G+C% content. The wide range in the G+C% content from 40% to 70% for the shared core GI genes is not substantially different to the range found for β- and γ-Proteobacteria (Krieg and Holt, 1984). This could be interpreted to suggest a longstanding relationship with this Phylum. This interpretation is based on the proposition, first, that GIs are constrained to this Phylum and, second, that horizontally acquired DNA with a different G+C% equilibrates over time to that of its host’s genomic G+C% content through a process referred to as amelioration (Lawrence and Ochman, 1998). It follows that for this family of GIs to have evolved such divergent G+C% contents; it would have necessitated substantial time in hosts with similarly divergent G+C% contents. It would also require limited horizontal transfer and recombination between divergent members of this family of GI, as this would tend to homogenise G+C% contents.

The presence of two GIs with G+C% of 68.2% and 69.6% that is markedly higher than their respective host’s genomic G+C%, namely, *R. metallidurans* (63.5%) and *X. axonopodis* (64.7%), is curious. In the case of *X. axonopodis*, this is remarkable as there
are only three regions of the genome with such a highly divergent G+C% content. In addition, the most extensive region is accounted for by the core genes of this GI (28 genes), the others being one or three genes in extent (data are accessible through TIGR http://www.tigr.org/tigr-scripts/CMR2/GCDisplay.spl?asmbl_id=226). One interpretation is that these two high G+C% content GIs have been acquired from hosts with even higher G+C% content than them. This would suggest an origin among organisms with >70% G+C% average content for their genomes. One possible host and organism common to Proteobacteria is the environmental organism, Derxia spp. a β-Proteobacteria, with a genomic G+C% greater than 70% (Becking, 1984). Few, if any, other known Proteobacteria have such a high G+C% content (Krieg and Holt, 1984). An alternative and more speculative possibility is that these high G+C% GIs may originate in high G+C% content Gram-positive bacteria such as Streptomyces spp. These Gram-positive organisms have a G+C% content as high as 73% (Bentley et al., 2002; Omura et al., 2001). This would then suggest a wider host range than just the Proteobacteria. Conclusive evidence of this may become apparent from the growing number of whole genomic sequences available for analysis and improved algorithms for interrogating these databases for large contiguous and weakly homologous sequences. The origin, more precise age and host range of this family of related GIs remains speculative, but future comparative investigations of codon usage and the G+C nucleotides at the third codon position may go a long way to answering these questions.

7.5.2. Antibiotic resistance accessory genes and this family of GIs.

The observation of an evolutionarily related coherent GI provides new insight into how the recent emergence and spread of β-lactamase positive Apf and/or tetracycline and chloramphenicol antibiotic resistance in H. influenzae has arisen. The phylogenetic
relationship of ICEHin1056 to other distantly related GIs indicates that transferable resistance in *H. influenzae* has deep evolutionary origins. The resistance genes, conveyed by transposons (e.g. Tn10 or Tn3) (Elwell *et al.*, 1977, Kaulfers *et al.*, 1978), form clusters of accessory genes on the core element that have apparently evolved stable relationships. No other accessory genes with different properties are apparent from analysis of the whole sequence. The emergence of this resistance element in pathogenic *H. influenzae* only became readily detectable in the early 1970s (Brunton *et al.*, 1986; Elwell *et al.*, 1975; Powell, 1988; Syriopoulou *et al.*, 1976; Syriopoulou *et al.*, 1978). Curiously, resistance then rapidly emerged over the next few years worldwide among pathogenic *H. influenzae* and rose to high prevalence (20% to 30%) in many countries (Powell, 1988; Sentchilo *et al.*, 2003). Evidence indicates that this resistance is accounted for by the appearance of ICEs (GIs) highly related to ICEHin1056 (Dimopoulou *et al.*, 1992; Dimopoulou *et al.*, 1997; Dimopoulou *et al.*, 2002; Leaves *et al.*, 2000; Powell, 1988; Powell and Livermore, 1988; Schito *et al.*, 2000). The epidemic expansion of this family of ICEs among pathogenic *H. influenzae* and high prevalence among other commensal haemophili (Leaves *et al.*, 2000; Scheifele and Fussell, 1981b; Scheifele *et al.*, 1982) suggest that this GI is well adapted to these bacterial hosts and offers them a survival advantage under antibiotic exposure. An intriguing question is whether the acquisition of resistance transposons and their intimate and apparent stable relationship with these GIs in haemophili has recently evolved or has deeper origins preceding the antibiotic era. The latter supposition would support the notion that antibiotic resistance is an ancient adaptive response that had pre-evolved in bacteria. Modern day intensive use of antibiotics only serves to change the distribution or organization of these pre-evolved structures.
The association of resistance genes to this family of GIs is not unique to *H. influenzae*. GIs found in both *H. somnus* and *P. aeruginosa* contain accessory genes with homology to resistance genes. In the case of *H. somnus* 2336, homologues of *tetA* and *romA* (multi-drug resistance gene) are present and the strain is tetracycline resistant (minimum inhibitory concentration to tetracycline is 8 mg/ml, T. Inzana, personal communication). In *P. aeruginosa* strain C, the GI, pKLC102, contains *aadB* that encodes tobramycin and gentamicin resistance (Klockgether *et al.*, 2004). This gene is found in a large complex class I transposon named TNCP23 inserted into pKLC102 (Klockgether *et al.*, 2004). As this strain was isolated from a case with cystic fibrosis who regularly received treatments of tobramycin, it was surmised that this variant had been selected by the tobramycin exposures (Klockgether *et al.*, 2004). The relationship between this family of GIs and resistance is unpredictable. For example, the related GI, SPI-7, found in the antibiotic multi-resistant (including tetracycline resistant) strain *S. Typhi* CT 18 does not contain resistance genes, even though tetracycline resistance in this strain is encoded by a Tn10-like structure (Parkhill *et al.*, 2001). This Tn10-like structure along with other antibiotic resistance genes is found on the large unrelated conjugative plasmid, pHCM1 (Wain *et al.*, 2003). Therefore, this family of related GIs does not simply and predictably acquire resistance genes even when transposons containing resistance genes are present in trans.

7.5.3. **Major properties of other accessory genes**

The accessory genes conveyed by this family of GI have a wide range of attributes other than antibiotic resistance that contribute to the habitat specific survival of their host organisms. Another and most common associated attribute is Type IV secretion found in five of the GIs, two of which, PAP1 (He *et al.*, 2004) and SPI-7 (Pickard *et al.*, 2003), are
well described pathogenicity islands. The Type IV secretion system in SPI-7 is involved in the adhesion of *S. Typhi* to macrophages and is important to *S. Typhi*’s intracellular life cycle and in disease causation (Morris *et al.*, 2003; Tsui *et al.*, 2003; Zhang *et al.*, 2000). The role of genes encoding the Type IV secretion system in PAP1 is less clear. These genes were not reported as contributing to the virulence of *P. aeruginosa* PA14 (He *et al.*, 2004). The Type IV secretion system present in the GI found in *Y. enterocolitica* is probably important in the organism’s survival in animal hosts. This can be indirectly inferred from investigations into *Yersinia pseudotuberculosis* (Collyn *et al.*, 2002). The *Y. enterocolitica* Type IV secretion system is highly homologous to the one investigated in *Y. pseudotuberculosis* (comparison performed using ACT, data not shown). The Type IV secretion system found in *Y. pseudotuberculosis* was shown to be crucial in mouse invasion models (Collyn *et al.*, 2002). Furthermore, the distribution of the Type IV secretion system among isolates of *Y. pseudotuberculosis* was restricted to pathogenic strains and its variable presence was regarded as consistent with it being on a mobile element (Collyn *et al.*, 2002), perhaps another of the GI family described here. The data available is incomplete, but it is consistent with the Type IV secretion systems present in this family of GI being involved in adherence of the host organisms to specific targets in their habitat.

Other genes important in virulence are present in these GIs. The Vi antigen encoded by SPI-7 is a well-known capsular antigen characteristic of *S. Typhi* that contributes to the pathogenesis of typhoid fever and is a valuable vaccine antigen (Pickard *et al.*, 2003). PAP1 contains a number of virulence associated accessory genes including two-component regulators that may be involved in regulating pathogenicity activities (He *et al.*, 2004). The island found in *H. ducreyi* contains the cytolethal distending toxin genes. Their role in pathogenesis have been studied and despite *in-vitro*
evidence of causing cell damage and death, these genes do not appear to be essential for causing disease in an animal model or in a human challenge study (Gelfanova et al., 1999; Stevens et al., 1999; Young et al., 2001). Nevertheless it remains an intriguing gene cluster likely involved in bacterial human host interactions.

Accessory genes important in the survival of organisms in environmental habitats include a range of genes encoding metabolic functions. These are found in species that live in potentially nutrient deficient environments such as water or soil and include *P. aeruginosa* SG17M, *Pseudomonas* sp. B13, *B. fungorum* and *R. metallidurans*. Another attribute of accessory genes of some of these environmental species are highly processive degradative enzymes. In particular, the *clc* element found in *Pseudomonas* sp. B13 and *B. fungorum* encodes a number of genes that inactivate toxic chemicals such as chlorocatechols (van der Meer and Sentchilo, 2003). Organisms possessing these enzymes and related DNA elements have become widely distributed, presumably through selection, in environments heavily contaminated with toxic waste (Chatterjee et al., 1981; Chatterjee and Chakrabarty, 1983; van der Meer and Sentchilo, 2003).

7.6. Conclusions

There is a very large and diverse population of GIs present in bacteria (Dobrindt et al., 2004) most of which do not share detectable homology with the family of related GIs described here. However, the methods used here to identify and characterise one family of GI may be used in principle to identify other families of coherent GI among these many apparently unrelated GIs. It seems possible that there will be both examples of mosaics without evidence of a coherent core GI structure and other GIs which have coherent core genes and a common evolutionary origin. This will be increasingly possible to investigate with the improving bio-informatics techniques and the rapidly increasing
sequence data available from complete bacterial genomes. Such a systematic classification of GIs is likely, in turn, to necessitate a commensurate systematic naming scheme.
CHAPTER EIGHT

CONCLUDING REMARKS AND FUTURE RESEARCH

The main objective of this work was to investigate the origin of antibiotic resistance in *H. influenzae*. The principal and unexpected finding was that the ICEs that transfer antibiotic resistance in *H. influenzae* have a common origin with a diverse family of coherent GIs found in β- and γ-Proteobacteria. Analysis of the core genes shared between these related GIs has given new insight into how they may conjugate and, thereby, explain how some GIs horizontally transfer between their bacterial hosts. A surprising finding was that these GIs may be capable of autonomous replication given the presence of genes associated with replication and the prediction that an *oriV* is present in some of them.

Future investigation of this family of GIs will focus on experimentally identifying genes encoding conjugation, replication and integrative and excisive recombination. Identifying and understanding the regulatory genes involved in controlling these properties will be particularly interesting and may reveal novel mechanisms of gene control. These studies will, therefore, contribute to our understanding of how GIs propagate and survive in bacterial populations. An intriguing possibility is that these GIs are capable of mobilising whole chromosomes in an Hfr type manner. If this is the case they may contribute to the diversification of bacteria and this may explain how large segments of DNA (e.g. capsular genes) are acquired and exchanged by bacteria in nature.

Another direction of future research will be to determine the extent to which GIs in general are classifiable using the methods described in this thesis. In order to systematically study the large number of GIs present in the many whole genomes now available for analysis, new improved search algorithms will be needed for identifying contiguous sequences with weak homologies. These investigations may not only produce
a systematic classification of GIs, but may give new insight into the host range of the family of related GIs described in this thesis.

The study of the antibiotic resistance ICEs in oropharyngeal haemophili revealed that these elements are highly related. Curiously, the sequences adjacent to and including the integrase gene derived from either of two divergent origins. The explanation for how these divergent regions evolved and how they have recombined with highly conserved modules (replicative and conjugative modules) is unclear. Also, whether this will inform on the recent emergence of antibiotic resistance in *H. influenzae* is unclear. However, the sequence variant that associates with ICEHin1056 and ICEHin2866 is over represented in *H. influenzae* compared to *H. parainfluenzae* (Dr. Crook, personal communication). One hypothesis is that this variant sequence is better adapted to *H. influenzae* and may have been recently acquired and in part accounts for the successful emergence of transferable resistance in pathogenic *H. influenzae*. In addition, the near identity in sequence found among the Tn3 sequences from epidemiologically unrelated isolates is consistent with this transposon being relatively recently acquired by this family of ICEs.

By investigating the population structure of these oropharyngeal ICEs, a better understanding of how they have evolved and spread through the population of haemophili will be possible. Methods have been developed in the laboratory to sequence multiple genes conserved in these ICEs and their bacterial hosts. Large collections of isolates from different parts of the world and from people on antibiotics have been assembled. These can be used to investigate whether there has been a recent clonal expansion of a few ICE variants in the population of pathogenic *H. influenzae*. It will also be possible to determine if there is geographical structuring of ICEs, which would be consistent with local evolution rather than rapid, worldwide spread of these ICEs. This experimental approach will allow predictions about the routes of transmission and spread of these ICEs.
to be made. This will give insights into the evolution of ICEs in haemophili and how they have acquired resistance transposons and spread to become so prevalent worldwide.
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APPENDIX A

MEDIA AND ANTIBIOTICS

Composition of Media

Brain heart infusion (BHI) broth

To prepare a litre of the broth, 37 g of brain heart infusion (BDH/Merck, Poole, UK) was added to 1 L of deionised water. The broth was mixed and autoclaved for 20 minutes at 121°C. When the broth has cooled to 55°C, 3.0 ml of stock haemin (5 mg/ml) and 1.5 ml of stock NAD (10 mg/ml) was added and dispensed into Petri-dishes.

Chocolate agar

Chocolate agar was prepared by adding 40 g of blood agar base No. 2 (Oxoid, UK) to 1 L of distilled water, and mixed until completely dissolved. Following autoclaving for 15 minutes at 121°C, the medium was cooled to 55°C before the adding 100 ml of horse blood (previously heated to 75°C).

Hib agar

Hib agar was made by adding 39 g of Columbia base (Oxoid, UK) and 5 g of yeast extract (Oxoid, UK) in 1 L of distilled water. It was autoclaved for 15 minutes at 121°C. After the medium had cooled to 55°C, 1.5 ml of stock NAD (10 mg/ml) and 3.0 ml of stock haemin (5 mg/ml) were added before dispensing into Petri-dishes.
**Luria Bertani (LB) broth and agar**

To prepare 1 L volume of LB broth, 10 g of typtone, 5 g of yeast extract and 10 g of NaCl were dissolved in 950 ml deionised water. The pH of the solution was adjusted to 7.0 with NaOH and brought up to 1 L volume. It was autoclaved for 20 minutes at 121°C.

For preparation of LB agar, 15 g/L of agar was added to the LB medium before autoclaving for 20 minutes at 121°C. Antibiotic/s was/were added when the agar had cooled to 55°C, and dispensed into Petri-dishes.

**Muller Hinton Broth**

For the preparation of a litre of the media, 35 g of Muller Hinton dehydrated medium (BioMerieux, USA) and 5 g of yeast extract were suspended in 1 L of deionised distilled water and autoclaved for 20 min at 115°C. When it had cooled to 55°C, 3.0 ml of stock haemin and 1.5 ml of stock NAD (10 mg/ml) were added.

For the preparation of Muller Hinton agar, 1.5% of agar was added to the medium before autoclaving. Haemin and NAD were added when the agar had cooled at 55°C prior to dispensing.

**SOC Medium**

This medium was prepared by adding 20 g of tryptone, 5 g of yeast extract and 0.5 g of NaCl to 1 L of distilled water, before autoclaving for 20 minutes at 121°C.

**Tryptone soy broth (TSB)**

To 1L of distilled water, 30 g of a dehydrated tryptone soya broth media (Oxoid, UK) was added and mixed before sterilising by autoclaving at 121°C for 15 minutes.
Additives to culture media

**IPTG**

For the preparation of a 100 mM stock solution, 238 mg of isopropylthio-β-D-galactosidase (IPTG) was dissolved in 10 ml deionised distilled water. The solution was filter-sterilised and stored in 1 ml aliquots at -20°C. To prepare agar with IPTG, a volume of 40 μl of IPTG stock solution was pipetted onto the surface of a LB plate and smeared with a sterile spreader. The solution was allowed to diffuse into the plate by drying at 37°C for 10-15 minutes.

**X-Gal**

To prepare a 40 mg/ml X-Gal stock solution, 400 mg 5-Bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) was dissolved in 10 ml dimethylformamide (Sigma). The solution was stored in a brown bottle at -20°C to protect from light. To a previously made LB agar plate, 40 μl of the stock solution was pipetted onto the surface of the agar and evenly spread with a glass spreader and dried for 15 minutes.

**Growth supplements**

Table 1. Growth supplements for the culture of *H. influenzae*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Role</th>
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<tr>
<td></td>
<td>Stock (mg/ml)</td>
<td>Final (μg/ml)</td>
</tr>
<tr>
<td>Haemin in DMSO</td>
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<td>10</td>
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<tr>
<td>Nicotinamide adenine dinucleotide (NAD)</td>
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## Antibiotics solutions

### Table 2. List of antibiotics used

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<th>Stock (mg/ml)</th>
<th>Final (μg/ml)</th>
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<td>Ampicillin</td>
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<td>50</td>
</tr>
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<td>20 -50</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10</td>
<td>Various</td>
</tr>
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<td>Streptomycin</td>
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<td>50</td>
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<td>Tetracycline</td>
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## Composition of stock solution and buffers

### Table 3. List of buffers used

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<th>Composition</th>
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<td>Phosphate buffered saline (PBS)</td>
<td>150 mM NaCl, 60 mM NaPO₄, pH 7.0</td>
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<td>x10 Alkaline phosphatase buffer (MBI Fermentas)</td>
<td>500 mM Tris-HCl (pH 7.9), 1 M NaCl, 100 mM MgCl₂, 10 mM DTT</td>
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<tr>
<td>x10 Ligation buffer (MBI Fermentas)</td>
<td>500 mM Tris-HCl (pH 7.4), 100 mM MgCl₂, 10 mM DTT, 10 mM ATP, 250 μg/ml BSA</td>
</tr>
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<td>10x PCR buffer without MgCl₂ (MBI Fermentas)</td>
<td>100 mM Tris-HCl, pH 8.3, 500 mM KCl</td>
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### Table 4. List of chemicals and compositions used

<table>
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<th>Composition</th>
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<tbody>
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<td>Tris-borate EDTA, TBE buffer (10x)</td>
<td>89 mM Tris base, 89 mM boric acid, 2 mM EDTA</td>
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<tr>
<td>Tris EDTA, TE buffer (pH 7.4, 7.6 or 8.0)</td>
<td>10 mM Tris-HCl (pH 7.4, 7.6 or 8.0), 1 mM EDTA (pH 8.0)</td>
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<tr>
<td>6x Loading buffer, type III (Sigma, Dorset, England)</td>
<td>Bromophenol Blue 0.25% (w/v), Xylene cyanol FF 0.25% (w/v), sucrose in water 40% (w/v)</td>
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<td>2.5x Sequencing buffer</td>
<td>200 mM Tris-HCl, 5 mM MgCl₂ (pH 9.0)</td>
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<tr>
<td>Ethidium bromide, EtBr (10 mg/ml in water)</td>
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APPENDIX B

PRIMER SEQUENCES

Table 1. List of primers used for PCR and sequencing.

<table>
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<tr>
<th>Primer</th>
<th>Sequence (from 5' to 3')</th>
<th>Annealing Temp °C</th>
<th>Length (bases)</th>
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* p & s refer to M13 primers for PCR and sequencing, respectively

Table 2. Primers sequences for plasmid sequencing

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<td>Annealing Temp °C</td>
<td>Length (bases)</td>
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<td>CAA TAA CCC ATC TAC TCG GC</td>
<td>54.2</td>
<td>24</td>
</tr>
<tr>
<td>PPG421R</td>
<td>GTA CAT AAC ATT TGC ATT AGA TCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## APPENDIX C

### PAIRWISE ALIGNMENT OF GENES IN *HAEMOPHILUS* SPP. ELEMENTS

Table 1. Pairwise distance of 12 genes in *Haemophilus* spp. calculated using MEGA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHpa8F</th>
<th>ICEHin2866</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>soj</em></td>
<td>0.006</td>
<td>0.008</td>
<td>0.002</td>
<td>0.006</td>
</tr>
<tr>
<td><em>dnaB</em></td>
<td>0.010</td>
<td>0.010</td>
<td>0.001</td>
<td>0.010</td>
</tr>
<tr>
<td><em>sub</em></td>
<td>0.024</td>
<td>0.024</td>
<td>0.000</td>
<td>0.024</td>
</tr>
<tr>
<td><em>topB</em></td>
<td>0.020</td>
<td>0.016</td>
<td>0.008</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHpa8F</th>
<th>ICEHin2866</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pilL</em></td>
<td>0.010</td>
<td>0.010</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td><em>traD</em></td>
<td>0.017</td>
<td>0.034</td>
<td>0.033</td>
<td>0.017</td>
</tr>
<tr>
<td><em>traC</em></td>
<td>0.02</td>
<td>0.000</td>
<td>0.002</td>
<td>0.02</td>
</tr>
<tr>
<td><em>virB4</em></td>
<td>0.006</td>
<td>0.006</td>
<td>0.001</td>
<td>0.006</td>
</tr>
<tr>
<td><em>traI</em></td>
<td>0.227</td>
<td>0.250</td>
<td>0.037</td>
<td>0.228</td>
</tr>
<tr>
<td><em>int</em></td>
<td>0.345</td>
<td>0.340</td>
<td>0.011</td>
<td>0.343</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHpa8F</th>
<th>ICEHin2866</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>traI</em></td>
<td>0.227</td>
<td>0.250</td>
<td>0.037</td>
<td>0.228</td>
</tr>
<tr>
<td><em>int</em></td>
<td>0.345</td>
<td>0.340</td>
<td>0.011</td>
<td>0.343</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHpa8F</th>
<th>ICEHin2866</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>traI</em></td>
<td>0.227</td>
<td>0.250</td>
<td>0.037</td>
<td>0.228</td>
</tr>
<tr>
<td><em>int</em></td>
<td>0.345</td>
<td>0.340</td>
<td>0.011</td>
<td>0.343</td>
</tr>
</tbody>
</table>
Table 2. Pairwise distance of ampicillin genes in *Haemophilus* spp. calculated using MEGA

<table>
<thead>
<tr>
<th></th>
<th>ICEHin1056</th>
<th>ICEHin299</th>
<th>ICEHpa8F</th>
<th>ICEHin2866</th>
<th>E. coli V00613</th>
<th>E. coli pIGAL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>tnpR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICEHin1056</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ICEHin299</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICEHpa8F</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICEHin2866</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.058</td>
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</tr>
<tr>
<td>E. coli V00613</td>
<td>0.056</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
<td>0.000</td>
</tr>
<tr>
<td>E. coli pIGAL1</td>
<td>0.056</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
<td>0.000</td>
</tr>
<tr>
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</tr>
<tr>
<td>ICEHin1056</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICEHin299</td>
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<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICEHpa8F</td>
<td>0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>ICEHin2866</td>
<td>0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<td>0.000</td>
</tr>
<tr>
<td>E. coli V00613</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.000</td>
</tr>
<tr>
<td>E. coli pIGAL1</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*nd: not done*
## APPENDIX D

### GENOTYPIC CHARACTERISTICS OF GIs

Table 1. Detailed functions of accessory genes in GIs of various bacteria

<table>
<thead>
<tr>
<th>Pathogens/strain</th>
<th>Island</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em> 1056</td>
<td>ICEHin1056</td>
<td>YbeB, YbeA, YbdD, tetC, tetD, tnp</td>
<td>bla, tnpR</td>
<td></td>
</tr>
</tbody>
</table>
| *H. somnus* 129PT |        | Antirestriction protein 
<sup>ardC</sup>, ATP binding protein for 
<sup>virulence</sup>, histone 
<sup>acetyltransferase</sup> |          |          |
|                   |        |          |          |          |
| *H. somnus* 2336 |        | Integrase/recombinase, NADPH quinone 
<sup>reductase</sup>, transcriptional regulator, 
<sup>Co/Zn/Cd efflux system component</sup>, 
<sup>romA</sup>, quartenary ammonium compound 
<sup>resistance protein</sup>, tetracycline resistance 
<sup>efflux protein</sup>, tetracycline resistance 
<sup>repressor protein</sup>, exporter protein, 
<sup>haloacid dehydrogenase</sup>, putative 
<sup>metal-binding protein</sup>, aspartyl-
<sup>RNA synthetase</sup> |         |          |
|                   |        |          |          |          |
| *H. ducreyi* 35000HP |        | Antirestriction protein, 
<sup>type I restriction enzyme M subunit</sup>, 
<sup>HicB protein</sup>, transposon gamma-delta resolvase, 
<sup>lstB homologue</sup>, cdh protein |          |          |
| *Pseudomonas sp.* B13 | clc | 3'-phosphoadenyl-5'-phosphosulfate 
<sup>sulfotransferase</sup> (PAPs reductase/FAD synthetase and related enzymes) |          |          |
|                   |        | Pyruvate 2-oxoglutarate dehydrogenase 
<sup>component</sup>, dihydrodipamide dehydrogenase 
<sup>component</sup>, acyl-coA synthetases, 
<sup>aminophenol repressor</sup>, ferredoxin-like 
<sup>protein</sup> |          |          |
|                   |        | Chlorocatechol 1,2, dioxygenase, 
<sup>threonine efflux protein</sup>, 
<sup>clcR, clcA</sup>, chloromuconate cycloisomerase, 
<sup>maleyacetate reductase</sup>, aromatic 
<sup>dioxygenase</sup>, oxidoreductase |          |          |
<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Island</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PA14</td>
<td>PAP1</td>
<td>Putative phage protein, pyocin, Type IV secretion: pilN, pilO, pilQ, pilV2, 70-kb heat shock protein</td>
<td><em>FimA</em>, <em>fimD</em>, kinase sensor <em>RcsC</em>, putative response regulator <em>PvrR</em></td>
<td>ATP-dependent endonuclease of the OLD family, pathogen-related protein, deoxycytidine triphosphate deaminase <em>dcd</em></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> C</td>
<td>PKLC102</td>
<td>Type IV secretion: pitl, pilN, pilO, pilP, pilQ, pilR, pilS, pilU, pilV and pilM</td>
<td>Virulence protein</td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em> SBW25</td>
<td></td>
<td><em>RpfA</em> protein, carbohydrate selective porin, permease of major facilitator superfamily/multidrug-efflux transporter, histidinol dehydrogenase, nodulation protein, glyoxalase family protein, hydrolases, succinate-semi-aldehyde dehydrogenase, ornithine cyclodeaminase</td>
<td><em>Zn</em>-dependent peptidase</td>
<td></td>
</tr>
<tr>
<td>Pathogens</td>
<td>Island</td>
<td>Attributes of accessory genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| *S. Typhi* CT18   | SPI-7  | Region 1: Type IV secretion: pilN, pilO, pilQ, pilV  
                    |        | Region 2: IbrA, DNA-binding prophage protein, type I restriction-modification, UV protection,  
                    |        | phage-related protein, Vi biosynthesis, shuflon-specific DNA recombinase  
                    |        | Region 3: Putative pathogenesis-related protein                                                  |
| *R. metallidurans* CH34 |        | Region 1: Cation transport ATPase, trpA transposase, plasmid-related protein, superfamily II DNA/RNA helicases  
                    |        | Region 2: Protein disulphide isomerase, putative DNA repair protein radC  
                    |        | Region 3: Haemolysin, haemolysin-type calcium binding protein                                     |
| *X. axonopodis* 306 |        | Region 1: Cytosine-specific DNA methyltransferase, phage related protein, permease of drug/metabolite transporter  
                    |        | Region 2: Plasmid-related protein, oxidoreductase  
                    |        | Region 3: Putative DNA-binding prophage protein, putative haemagglutinin-related protein         |
| *Y. enterocolitica* 8081 |        | Region 1: Type IV secretion: pilN, pilO, pilQ, pilR, pilS, pilV2  
                    |        | Region 2: Transposase, arsenical pump, arsenical pump-driving ATPase, arsenical resistance operon repressor  
                    |        | Region 3: Putative DNA-binding prophage protein, putative haemagglutinin-related protein         |
APPENDIX E

PHYLOGENETIC RELATIONSHIP BETWEEN GENES IN 12 GIs

Fig. 1. Virtual core genes

A SplitsTree (Fig. 2 - 5) representation of the ClustalX analysis of each of the 4 genes: 1, 6, 18 and 27 (numbered according to the coherent virtual element), and common to all the GIs. The amino acid sequences of each of the predicted genes alone were aligned using ClustalX. There are only minor differences in the topology of the SplitTrees between each of the genes or the concatenated sequences of the 15 shared genes indicating congruent structure.
Fig. 2. Gene 1 (soj/parA)

Fig. 3. Gene 6
Fig. 4. Gene 18

Fig. 5. Gene 27