Haemophilus influenzae Rd KW20 has virulence properties

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Haemophilus influenzae is a human-adapted commensal and pathogen that can cause mucosal infections such as sinusitis, otitis media and bronchitis. Certain strains also cause bacteremia and meningitis. Clinical isolates are genetically heterogeneous and are often recalcitrant to standard genetic manipulation. H. influenzae strain Rd KW20 has traditionally been considered avirulent, since it does not survive in the bloodstream of animals, is readily killed by normal adult human sera and cannot colonize the nasopharynx of infant rats. The purpose of this study was to determine whether Rd KW20 could be used in certain infection models. It is shown here that strain Rd KW20 can invade certain human epithelial cell lines grown either as monolayers or as differentiated epithelium at the air-liquid interface. In addition, Rd KW20 can invade a monolayer of immortalized human brain microvascular endothelial cells. Finally, this strain can replicate and survive in human bronchial xenografts for up to 3 weeks. The complete genomic sequence of Rd KW20 is available and it is readily amenable to genetic manipulation. These properties and the results reported here indicate that this strain is a viable alternative to the use of clinical isolates for the investigation of H. influenzae virulence.

INTRODUCTION

Haemophilus influenzae is an obligate human parasite found almost exclusively on the oropharyngeal mucous membranes. Strains of this organism are classified according to eight different phenotypic characteristics. The first one used was serological typing, based on six different capsular antigen types, a–f. Those strains that lack capsule are considered non-typable (NTHi). The other seven characteristics are used to classify these non-typable strains: biotyping, outer-membrane protein differentiation by molecular mass or antigenicity, lipooligosaccharide antigenicity, enzyme electrophoretic typing, restriction fragment length polymorphism and random amplification of chromosomal DNA by PCR (Murphy & Apicella, 1987). More recently, multilocus sequence typing has been applied to both typable and NTHi strains (http://www.mlst.net). Subdivisions of Haemophilus spp. are grouped into biotypes based upon biochemical reactions for indole, urease and ornithine decarboxylase. These small, Gram-negative coccobacilli grow either aerobically or as facultative anaerobes. Aerobic growth requires exogenous haem or protoporphyrin IX and NAD. As expected of an upper respiratory parasite, growth is optimal between 35 and 37°C, with little tolerance of higher temperatures.

H. influenzae can cause a number of mucosal infections, including otitis media, conjunctivitis, sinusitis and bronchitis (Murphy & Apicella, 1987). In addition, this organism is responsible for invasive infections such as bacteremia and meningitis. Prior to 1985, most invasive H. influenzae infections were due to type-b strains. During that year, a polysaccharide vaccine composed of the type-b polyribosylribitol phosphate capsule was licensed and distributed in the United States. In 1990, several protein-carbohydrate conjugate H. influenzae b (HiB) vaccines were licensed and were immunogenic in infants. Since the initiation of HiB vaccine use, the prevalence of type-b disease has been drastically curtailed. However, since all NTHi lack the capsular antigen, the vaccine is not protective against common mucosal infections. The ability of H. influenzae to evade host immune defences by efficient phase variation and random mutation results in their surface-exposed structures being poor vaccine

Abbreviations:
- ALI, air-liquid interface
- HBMEC, human brain microvascular endothelial cells
- NHBE, normal human bronchial epithelium
candidates. Another complicating factor is that, although type-b strains exhibit marked clonality, NTHI strains isolated from infections show great genetic and phenotypic diversity.

The *H. influenzae* strain Rd KW20 was the first free-living organism to have its genome (1.8 Mbp) completely sequenced by the Institute for Genomic Research (Fleischmann et al., 1995). This strain was originally an encapsulated type-d strain that lost the genes encoding its capsule (smooth, or Sd) and is now rough (Rd). Rd KW20 (Wilcox & Smith, 1975) is considered to be an avirulent laboratory strain of *H. influenzae*, as it lacks adenins commonly found in disease-causing NTHI (Martin et al., 1998; St Geme, 2002).

A number of Rd derivatives have been engineered. Most were constructed by a general protocol that consisted of nitrosoguanidine mutagenesis followed by selection for a phenotype such as antibiotic resistance, auxotrophy (amino acids and vitamins) or recombination deficiency (as measured by UV susceptibility) (Setlow et al., 1968, 1972; Kooistra et al., 1983; McCarthy, 1989). After selection, chromosomal DNA was harvested and used to transform another Rd culture. This process was repeated many times, resulting in a number of different Rd derivatives. Many investigators have used Rd derivatives to study *H. influenzae* adhesins and putative virulence factors. Accordingly, the strains displaying the desired phenotypes often have disturbances in growth rates due to the nature of their construction, although many have been reported in the literature as simply ‘Rd’.

We and others have found that the avirulence of Rd KW20 and its derivatives does not extend to certain in vitro assays (Virji et al., 1992; van Schijndel et al., 2000). Here, we examine the ability of Rd KW20 to replicate on and invade a number of human cell types and to survive for a significant period of time on human differentiated respiratory epithelium in bronchiolar xenograft.

**METHODS**

**Bacteria.** *H. influenzae* strain Rd KW20 (Martin et al., 1998) was provided by Dr H. O. Smith (Johns Hopkins University) and strain R3801, a bronchoalveolar lavage isolate from a pediatric cystic fibrosis patient, was from the collection of one of the authors (A. L. Smith). Strains Eagan and R2866 have been described previously (Smith et al., 1973; Nizet et al., 1996). These strains were grown on chocolate agar [36 g Difco GC medium, 10 g haemoglobin, 10 ml Difco Supplement B (Becton Dickinson), 5000 U bacitracin l–1 (Remel), supplemented with 10 μg ml–1 ampicillin] or supplemented brain/heart infusion broth or agar [37 g brain/heart infusion medium, solidified as appropriate with 15 g Bacto agar l–1 (Remel), supplemented with 10 μg ml–1 NAD, 10 μg haem and 10 μg l–1-histidine ml–1] (Smith et al., 1973). Escherichia coli DH5α was obtained from Gibco-BRL.

**Eukaryotic cell culture.** Primary respiratory epithelial cells used were normal human bronchial epithelial cells (NHBE 6137) from a non-smoker, obtained from BioWhittaker (catalogue no. CC-2540). Cells were thawed and plated on human placental collagen-coated tissue culture flasks in BEGM medium (BioWhittaker). Cells were recovered by trypsin digestion and were either immediately recultured or frozen for later use. C38 cells are a ‘corrected’ cystic fibrosis respiratory epithelial cell line that is a derivative of the IB3 line, stably transfected with wild-type CFTR (Zeitlin et al., 1991). C38 cells were obtained from Dr Pamela Zeitlin (Department of Pediatrics, Johns Hopkins University) and maintained in BEGM medium. AS49 are human lung epithelial carcinoma cells and were obtained from the ATCC (CCL-185) and cultured in Ham’s F12K medium (Gibco) with 2 mmol l–1-glutamine, 100 μl heat-inactivated fetal calf serum and 1.5 g sodium bicarbonate l–1. Chang human conjunctival cells were obtained from the ATCC (CCL-20.2) and maintained in minimum essential medium (Gibco) with 100 μl heat-inactivated fetal calf serum, 2 mmol l–1-glutamine and 10 μl 100× MEM non-essential amino acid solution (Gibco) l–1. Human brain microvascular endothelial cells (HBMEC) were derived by one of the authors (K. S. Kim) (Stins et al., 2001) and were maintained in HBMEC medium [760 ml RPMI 1640 containing 25 mm HEPES and 2 ml l–1 glutamine, 100 μl heat-inactivated fetal calf serum, 10 μl each of 200× MEM non-essential amino acid solution, 100× MEM vitamin solution and 100 mm MEM sodium pyruvate solution (Gibco) and 100 μl heat-inactivated Nystatin V (Becton Dickinson) l–1]. For invasion assays using 12-well plates, all lines except NHBE were grown on collagen-coated BioCoat plates (Becton Dickinson).

**Air–liquid interface (ALI) culture.** Eukaryotic cells were initially grown to confluence and then placed at the ALI on 6-well Corning Costar Transwell-COL 0.4 μm membranes (catalogue no. 3491) and treated as described previously (Gray et al., 1996). Chang cells were grown on 6-well Falcon Transwell 0.4 μm membrane inserts (BD Labware, catalogue no. 333000). Primary human bronchial epithelial cells grown at the ALI developed a transepithelial electrical resistance (TER) of 1200–2000 Ω cm–2 measured with a Millicell (Millipore) and Chang cells at the ALI developed a TER of 1000–1500 Ω cm–2.

**Invasion assays.** To inoculate human cell monolayers, *H. influenzae* colonies were harvested from overnight cultures on chocolate-agar plates and resuspended in PBS-G (PBS, pH 7.4, with 0.1 % gelatin) to an OD600 of either 0.6 (~3×108 c.f.u. ml–1) or 1.0 (~5×108 c.f.u. ml–1). Aliquots of each suspension corresponding to between 1×106 and 1×107 c.f.u. were used to inoculate tissue culture medium overlaying monolayers for in vitro invasion assays. The volume of tissue culture medium ranged from 0.2 ml, in the case of human respiratory cells at the ALI, to 1 or 2 ml, in the case of monolayers in 12-well plates or 6-well Transwells, respectively. Serial 10-fold dilutions in PBS-G of each original bacterial suspension were then plated on chocolate agar and the number of colonies was counted after 18 h growth at 37 °C to determine the actual viable inoculum in each assay. After the desired invasion time, aliquots of medium were removed for serial dilution and plating to enumerate bacteria in the supernatant, and each monolayer was then rinsed three times with a double volume of 1× D-PBS (Dulbecco’s PBS) and either harvested as below (for cell-associated numbers) or fresh medium containing 100 μg gentamicin ml–1 was added to each monolayer in a 1× 5× volume (for intracellular bacteria counts). Following a 1 h incubation in medium containing 100 μg gentamicin ml–1, the supernatant was siphoned off and discarded and each monolayer was rinsed three times with a double volume of 1× D-PBS without calcium or magnesium. Each well was then harvested by adding 1 ml sterile 1% saponin in 1× D-PBS without calcium or magnesium and incubating at 37 °C for 10 min, followed by scrubbing with a sterile gauze tip. The cells from each well were then plated in separate 1.5 ml Eppendorf tubes and vortexed vigorously for 1 min, after which serial 10-fold dilutions in PBS-G were performed. Aliquots (100 μl) of each appropriate dilution were plated in triplicate on chocolate agar and counted after 18 h growth at 37 °C to determine viable bacterial numbers. Transwells were harvested in the same fashion. The means of three replicates, performed in triplicate, were calculated and reported.

**Xenograft preparation.** Bilateral tracheal xenografts were established in male BALB/c mice using demuced rat tracheal matrix repopulated with normal human respiratory epithelium. Xenografts were prepared using techniques described previously (Cohn et al., 2001). Briefly,
tracheas harvested from adult rats were denuded of native epithelium and devitalized through repeated freeze/thawing and mechanical rinsing. A rigid plastic stent was used to stabilize the trachea. Silastic tubing with an inner diameter of 0·76 mm and an outer diameter of 1·65 mm (Dow Corning) was inserted approximately 3 mm into both ends of the tracheal segment and the entire tracheal segment was implanted subcutaneously into the flank of a female nu/nu BALB/c mouse (Charles River) using sterile technique. The tubing was allowed to exit both incisions for approximately 15 mm, leaving the xenograft open to the air at both ends. One xenograft was inserted on each flank, so that each mouse received two xenografts.

Three days post-implantation, the tracheas were seeded with ~1·0 × 10^6 normal human bronchial epithelial (NHBE) cells, expanded in vitro. All cells used in xenografts were from either a second or third passage and were obtained from BioWhittaker. Forty-eight h after seeding, xenografts were studied a minimum of 3 weeks after seeding with epithelial cells and were considered repopulated if mucin was present in the effluent (as indicated by SDS-PAGE analysis) or if, upon removal, histological examination revealed pseudostratified respiratory epithelium. Some xenografts met both criteria.

### Bacterial inoculation of xenografts.

Twenty-four h prior to bacterial inoculation, all xenografts were flushed with 200 μl sterile PBS that had been cultured on LB agar and incubated at 37 °C to assure sterility. H. influenzae strains were grown overnight on chocolate-agar plates and colonies were harvested with a cotton swab and suspended in PBS-G to an OD600 of 0·2, corresponding to ~1·0–3·0 × 10^8 c.f.u. ml^-1. Antibiotic use in fluid solution was discontinued 1 week prior to using the xenograft in microbiological experiments. Xenografts were studied a minimum of 3 weeks after seeding with epithelial cells and were considered repopulated if mucin was present in the effluent (as indicated by SDS-PAGE analysis) or if, upon removal, histological examination revealed pseudostratified respiratory epithelium. Some xenografts met both criteria.

### Xenograft harvest.

After CO2 euthanasia, xenografts were removed aseptically. The silastic tubes and chamberous stents were removed from the tracheal matrix. Tracheal xenografts and their homogenates were kept on ice during processing. The trachea was minced with scissors and homogenized with a standard-cancellation Potter-Elvahjem homogenizer and then quantitatively cultured in triplicate. Serial 100-fold dilutions of the homogenate were performed and 0·1 ml of each dilution was spread over the surface of chocolate-agar plates and incubated for 18 h at 37 °C prior to colony counting. Blood (0·2 ml) was obtained by cardiac puncture, serially diluted in PBS-G and plated on chocolate-agar plates to determine whether bacteriaemia was present. The limit of detection was 10 c.f.u. ml^-1.

### RESULTS

#### Rd KW20 invades certain epithelial cell lines in vitro

Entry of strain Rd KW20 into a monolayer of C38 human respiratory epithelial cells resulted in numbers that were not significantly different from those observed for the organism’s invasion of NHBE cells at the ALI (Table 1). A mean inoculum of either 4·2 or 4·5 × 10^8 c.f.u. ml^-1 and an invasion time of 4 h were used to compare entry into the epithelial cells. Adherence to the two monolayers was virtually identical. This indicates that either cell line can act as an acceptable in vitro model for Rd KW20 invasion into human bronchial epithelium for the purposes of studying Haemophilus pathogenesis. However, the same was not observed for two other human epithelial cell lines, Chang conjunctivial cells and A549 bronchial cells. Rd KW20 adhered to monolayers of both cell types, but did not invade either to any significant degree (Table 1). Similar results were obtained for Rd KW20 when the invasion was carried out on monolayers that had been previously fixed with glutaraldehyde, preventing any uptake by the cells. R3001, a clinical isolate, was used as a positive control and successfully invaded both the A549 and Chang monolayers, at 7·7 × 10^7 and 2·6 × 10^7 c.f.u. ml^-1, respectively. This indicated that the non-entry of Rd KW20 into these monolayers is a strain characteristic and not a deficiency in the eukaryotic cells used.

#### Table 1. Results of 4 h invasion assays of Rd KW20 into human epithelial cell monolayers

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inoculum</th>
<th>Supernatant</th>
<th>Cell-associated</th>
<th>Invaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>C38</td>
<td>4·5 × 10^8</td>
<td>4·7 × 10^7</td>
<td>1·5 × 10^8</td>
<td>2·9 × 10^7</td>
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<tr>
<td>NHBE</td>
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<td>5·2 × 10^7</td>
<td>1·5 × 10^8</td>
<td>4·2 × 10^7</td>
</tr>
<tr>
<td>A549</td>
<td>4·8 × 10^8</td>
<td>3·0 × 10^8</td>
<td>3·4 × 10^8</td>
<td>1·3 × 10^8</td>
</tr>
<tr>
<td>Chang</td>
<td>1·1 × 10^8</td>
<td>1·8 × 10^8</td>
<td>8·4 × 10^8</td>
<td>6·9 × 10^8</td>
</tr>
</tbody>
</table>

Data represent means (c.f.u. ml^-1) of three independent experiments performed in triplicate. The mean number of bacteria recovered from the medium prior to gentamicin addition. Cell-associated numbers represent adherent and invaded bacteria, whereas ‘invaded’ represents gentamicin-resistant bacteria.

**Rd KW20 invasion of endothelial cells displays inoculum effects**

Rd KW20 adhered to and invaded HBMEC monolayers. Characteristically, this strain invaded HBMECs to a maximum of approximately 1·0 × 10^8 gentamicin-resistant c.f.u. ml^-1, which was reached after 4–6 h contact with the monolayer. When uptake was blocked by glutaraldehyde fixation of the HBMEC monolayer, Rd KW20 did not survive gentamicin treatment. However, initial entry into the HBMEC monolayer was significantly affected by the number of bacteria inoculated. In 2 h invasion assays performed in 12-well plates, a mean inoculum of 2·3 × 10^7 c.f.u. ml^-1 resulted in 4·4 × 10^7 gentamicin-resistant bacteria (Fig. 1). Increasing the mean inoculum to 1·1 × 10^8 c.f.u. ml^-1 resulted in a mean of 2·1 × 10^8 c.f.u. Rd KW20 internalized by the HBMEC monolayers over the same time period. Increasing the inoculum by an order of magnitude, while maintaining a constant duration of contact, increased the number of bacteria internalized into the HBMEC monolayer to a significant degree. This suggests that there may be a mechanism by which Rd KW20 senses and responds to the number of organisms in its immediate environment as it interacts with eukaryotic cells. As a negative control, a mean
inoculum of 1·0 \times 10^7 \text{ c.f.u. ml}^{-1} \text{ of } E. coli \text{ DH5} \text{Æ} \text{ onto HBMEC monolayers resulted in the recovery of no bacteria in two separate experiments, and 2·3 \times 10^7 \text{ c.f.u. ml}^{-1} \text{ DH5} \text{Æ} \text{ recovered once, over the same time period.}

**Rd KW20 replicates on Chang cells at the ALI**

Since Rd KW20 did not invade submerged Chang conjunctival cell monolayers, we investigated the organism’s ability to invade Chang cells cultured at the ALI. Maintaining cells at the ALI allows differentiation of the epithelium into a polarized monolayer. Using a mean inoculum of 1·0 \times 10^2 \text{ c.f.u. ml}^{-1} \text{ and an incubation time of 18 h, Rd KW20 replicated and survived on Chang cells at the ALI. The mean number of cell-associated bacteria on the monolayer after 18 h was 1·9 \times 10^6 \text{ c.f.u. ml}^{-1}. Consistent with conventional tissue-culture studies, Rd KW20 did not invade Chang cells cultured at the ALI.

**H. influenzae Rd KW20 can colonize human xenografts**

Twenty-seven normal human bronchiolar xenografts were each instilled with a varying inoculum of strain R3001 and cultured 5 days later. All inocula greater than 3·0 \times 10^6 \text{ c.f.u.Æ} \text{ resulted in infection of the xenograft, characterized by production of purulent mucus containing strain R3001. When fewer than 3·0 \times 10^6 \text{ c.f.u.Æ} \text{ were inoculated, 9 of 13 xenografts (69\%) were sterile upon harvesting and culture. The bacterial density in the xenograft at harvest varied from 40 \text{ c.f.u.} \text{(initial inoculum 127 c.f.u.) to 3·2 \times 10^7 \text{ c.f.u.} \text{(initial inoculum 9·0 \times 10^6 \text{ c.f.u.)}. The mean density of strain R3001 in the colonized xenografts was 3·1 \times 10^6 \text{ c.f.u.Æ per xenograft. Two xenografts inoculated with 3·0–5·0 \times 10^7 \text{ c.f.u.Æ of strain R3001 were harvested at 7, 14 and 21 days after inoculation, homogenized and quantitatively cultured. All grew strain R3001, with a mean density of 4·2 \times 10^6 \text{ c.f.u.Æ per xenograft.}

To determine whether Rd KW20 could survive in normal human xenografts, 2·0–4·0 \times 10^6 \text{ c.f.u.Æ in a volume of 30 \text{ ml was instilled into mucin-expressing xenografts. Strain Rd KW20 was recovered from each xenograft at 7, 14 and 21 days, indicating that the organism could colonize and survive in this model for an extended period of time. The density of Rd KW20 ranged from 2·7 \times 10^3 \text{ to 3·2 \times 10^6 \text{ c.f.u.Æ per xenograft. These data indicate that Rd KW20 can be used to study persistence for at least 3 weeks on differentiated human respiratory epithelium in a xenograft.}

Blood cultures from xenograft-bearing immunodeficient mice inoculated with strain R3001 or Rd KW20 were uniformly sterile. These data indicate that respiratory epithelial cell invasion by strains R3001 and Rd KW20 in the xenograft model is not accompanied by persistence in the bloodstream. In contrast, the two strains isolated from the blood of patients, R2866 and strain Eagan, caused bacteremia. Two xenografts, both in the same animal, were inoculated with 3·0 \times 10^7 \text{ c.f.u.Æ, two with 5·0 \times 10^7 \text{ c.f.u.Æ and two with 5·0 \times 10^7 \text{ c.f.u.Æ of strain R2866. In all three animals, bacteremia was detected, with the density ranging from 4·0 \times 10^3 \text{ to 2·3 \times 10^6 \text{ c.f.u. ml}^{-1} \text{ blood. Four xenografts, two in each mouse, were inoculated with 3·4 \times 10^7 \text{ c.f.u.Æ of strain Eagan. At xenograft harvest, both animals had H. influenzae strain Eagan present in blood at a density greater than 1\times 10^6 \text{ c.f.u. ml}^{-1}. These data indicate that different bacterial components are required for persistent bacteremia after colonization and invasion of human respiratory epithelium.

As a control for the H. influenzae studies, 1·4 \times 10^7 \text{ c.f.u.Æ E. coli DH5} \text{Æ was inoculated into the lumen of four xenografts; 5 days later, the lumen flush and the xenograft homogenates did not culture any bacteria. Two additional xenografts were inoculated with 7·8 \times 10^6 \text{ c.f.u.Æ strain DH5} \text{Æ. Five days later, one xenograft homogenate contained 7·5 \times 10^6 \text{ c.f.u.Æ, while the other contained 1\times 10^7 \text{ c.f.u.Æ. The blood was sterile in all animals whose xenografts were inoculated with E. coli.}

**DISCUSSION**

H. influenzae strain Rd KW20 and closely related Rd strains are considered avirulent. They are considerably less adherent to human cell lines, such as Chang conjunctival cells and Hep2 cells (Gilsdorf et al., 1996; St Geme, 2002). This property has permitted the cloning of adhesins from wild-type disease-associated strains (St Geme, 2002). Recently, an Rd rec1 mutant was used to examine the expression of genes of a wild-type H. influenzae as they interacted with a human respiratory cell line (van Ulsen et al., 2002). In animal models, intraperitoneally inoculated Rd derivatives are cleared rapidly from the bloodstream, and intranasal inoculation of 5-day-old rats does not result in colonization (Smith et al., 1973; Morlin et al., 2002). However, there is evidence...
that the type-d strains represent the minimal \textit{H. influenzae} genome, which can become virulent with the acquisition of the type-d capsule gene cluster (Morlin et al., 2002). Invasive \textit{H. influenzae} type-d disease was very rare even in the pre-antibiotic era, a fact that probably reflects its paucity of confirmed and putative virulence factors (Sell, 1970; Fleischmann et al., 1995). Conversion of Rd strains to type-b capsulation by transformation with whole-cell DNA permits the encapsulated transformant to cause persistent bacteremia and meningitis in infant rats at inocula not too dissimilar to that seen with wild-type b strains (Roberts et al., 1981; Zwahlen et al., 1989).

Invasion of human cells may be a transient event as the bacteria pass through the epithelium to invade the submucosa, in the case of respiratory epithelium, or transcytose brain endothelial cells to infect the cerebrospinal fluid. Alternatively, \textit{H. influenzae} may reside chronically within or between the cells after invasion. Previous studies have hypothesized that intracellular residence and re-emergence from respiratory epithelium and adenoidal tissue by \textit{H. influenzae} was responsible for chronic otitis media in children (Forsgren et al., 1994).

Conclusions

Our data support the concept that virulence is a multifactorial process, dependent on a constellation of bacterial factors and the appropriate host cell. Understanding these host–pathogen interactions should be facilitated by methods such as microarray and proteomics analysis of \textit{H. influenzae} grown under a variety of conditions.

Rd KW20 has been considered a non-pathogenic, domesticated lab strain because it was ‘avirulent’ in animal models of \textit{H. influenzae} infection. Here, we have shown that this strain adheres to and invades certain cell lines in vitro, adheres to and replicates on conjunctival cells and colonizes human respiratory epithelium in a xenograft for up to 3 weeks. Rd KW20 is readily transformable and will accept plasmids by conjugation or electroporation, techniques that facilitate genetic manipulation. The ability of \textit{H. influenzae} Rd KW20 to invade and persist on eukaryotic cells, coupled with the availability of the complete genomic sequence of Rd KW20, can be exploited to provide insights into \textit{H. influenzae} virulence.

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