The aim of the study was to investigate the association between the presence of altered penicillin-binding protein 3 (PBP3) in non-typable *Haemophilus influenzae* (NTHi) and an increased capacity to invade bronchial epithelial cells *in vitro*. A collection of 40 clinical isolates of NTHi comprised of 20 with normal PBP3 and 20 with altered PBP3 (defined by an N526K substitution) was established. The isolates were tested for the ability to invade bronchial epithelial cells *in vitro* using a 4 h gentamicin survival assay. Invasion was measured as the percentage of intracellular organisms relative to the initial inoculum. The mean invasion rate was 0.00–14.79 % in the normal PBP3 isolates and 0.02–36.69 % in the altered PBP3 isolates. The altered PBP3 isolates had a higher (\(P=0.003\)) mean invasion rate (6.86 %, \(n=20\)) than the normal PBP3 isolates (1.31 %, \(n=20\)). Subsequently, two variants of altered PBP3 (transformant 1, N526K; transformant 2, M377I, S385T, L389F and N526K) were cloned into three of the initial isolates (parents) with normal PBP3 and relatively low invasive ability, and the parents and transformants tested for invasion as above. There was no difference (\(P=0.89\)) in the mean invasion rates for the parents (0.81 %, \(n=3\)), transformants 1 (0.90 %, \(n=3\)) and transformants 2 (1.38 %, \(n=3\)). There was an association between the presence of altered PBP3 in NTHi and an increased capacity to invade BEAS-2B cells *in vitro*, but cloning experiments suggested that the altered PBP3 was not involved directly in enhanced invasion.

**INTRODUCTION**

Non-typable *Haemophilus influenzae* (NTHi) is an important opportunistic respiratory pathogen frequently associated with acute exacerbations of chronic bronchitis, community acquired pneumonia, otitis media and occasionally with conjunctivitis. The organism has multi-faceted pathogenicity, and there is increasing evidence that NTHi invades and persists within various respiratory epithelial cells (Clementi & Murphy, 2011; King, 2012). The role of this local invasive capacity in pathogenesis is unclear; however, observations that NTHi infections frequently persist and recur despite antibiotic therapy and culture-negative laboratory investigations suggested that the intracellular environment might act as a protective reservoir for persistence (Clementi & Murphy, 2011; Ketterer *et al*., 1999; King, 2012). Recently, Okabe *et al.* (2010) reported an association between the capacity of isolates to invade bronchial epithelial cells *in vitro* and the possession of altered penicillin-binding protein 3 (PBP3).

The presence of altered PBP3 is associated with \(\beta\)-lactamase-negative ampicillin-resistant (BLNAR) strains, strictly defined as an ampicillin MIC \(\geq 4\) mg l\(^{-1}\) (often termed high BLNAR), but frequently inclusive of a non-susceptible MIC \(\geq 2\) mg l\(^{-1}\) (often termed low BLNAR) (Tristram *et al*., 2007). Alternatively, a genotypic classification based on the amino acid sequence of PBP3 can be used, where organisms with an N526K substitution are termed genetically BLNAR (gBLNAR) and those with N526K and either or all of M377I, S385T or L389F are sometimes more specifically termed high gBLNAR because they tend to have higher ampicillin MICs (Hasegawa *et al*., 2003).

In the Okabe *et al.* (2010) study, 10 of 91 isolates (57 of 91 gBLNAR with 34 of 57 high gBLNAR) were considered...
highly invasive and these were all gBLNAR, and a statistically significant difference in invasion rate between high-gBLNAR isolates compared with isolates with normal PBP3 was reported. The authors postulated that altered PBP3 may act as an adhesion molecule and play a role in macropinocytosis-mediated entry of NTHi into epithelial cells (Okabe et al., 2010).

Given that gBLNAR strains are increasing worldwide and can range in prevalence from ~20 to 50% (Kishii et al., 2011; Witherden et al., 2011), any association between altered PBP3 and local invasiveness is a cause for concern. The aims of our study were to attempt to confirm the association between altered PBP3 and invasiveness, and to clarify the role of altered PBP3 by isolating it as a variable using recombinant techniques.

METHODS

Bacterial isolates. A working collection of 40 clinical isolates of NTHi was established, comprising 10 each from the following sites and clinical conditions: ear (otitis media), eye (conjunctivitis), sputum (lower respiratory infection) and oropharynx (normal flora), such that five from each site had altered PBP3. The identity of the isolates was confirmed as NTHi using a PCR algorithm for hpd, fucK, and sodC as described previously (Witherden & Tristram, 2013), and characterized as having normal or altered PBP3 (N526K) using the PCR method of Witherden et al. (2013). Three of these isolates (parents 1, 2 and 3) with normal PBP3 were transformed subsequently using PCR-amplified ftsI from previously characterized gBLNAR (N526K, donor 1) and high-gBLNAR (M377I, S385T, L389I and N526K, donor 2) organisms as described previously (Takahata et al., 2007). Successful transformation was confirmed by ftsI sequencing. The 40 clinical isolates and six transformants were then tested for ampicillin susceptibility and invasion rate.

Susceptibility testing. Ampicillin MICs were determined using Etest (AB Biodisk) on Mueller–Hinton agar supplemented with 5% defibrinated horse blood and 20 mg l<sup>−1</sup> NAD as described by EUCAST (2013).

Invasion assay. Invasion rate was determined using a gentamicin survival assay with BEAS-2B bronchial epithelial cells (Sigma-Aldrich) grown in bronchial epithelium growth medium (BEGM; Lonza) based on the method described by Okabe et al. (2010). Briefly, a chocolate agar culture incubated at 37°C in 5% CO<sub>2</sub> for 16–18 h was used to prepare a bacterial suspension (~5 x 10<sup>6</sup> bacteria ml<sup>−1</sup>) in warm BEGM which was then added to each of three wells (technical replicates) in a 24-well microtitre plate containing confluent BEAS-2B cells and incubated for 4 h at 37°C in 5% CO<sub>2</sub>. The cells were washed and reincubated for a further 2 h with BEGM containing 200 mg gentamicin l<sup>−1</sup> to kill the extracellular bacteria. The cells were washed again, then lysed and intracellular bacteria released by incubation with 1% saponin for 15 min followed by vortexing for 1 min. Bacteria in the initial inoculum and the subsequent intracellular fraction were enumerated by serial dilution and viable counts on chocolate agar, with the invasion rate calculated as the mean percentage of intracellular bacteria in each of three wells compared with the starting inoculum. The assay was performed in triplicate (biological replicates) for the clinical isolates, and with five replicates for the transformants and associated untransformed parent isolates, with the final invasion rate for each organism being the mean of the biological replicates. Using the criteria established by Okabe et al. (2010), isolates with an invasion rate of ≥1% were designated 'highly invasive'.

Statistical analysis. Statistical analysis was performed using SigmaPlot (version 12.5) and figures were drawn using GraphPad Prism software (version 6.0d). The mean invasion rates of the clinical isolates were log<sub>10</sub>-transformed to reduce the influence of a highly positively skewed dataset and allow parametric analysis. A two-way ANOVA was performed using PBP3 genotype (normal or altered) and site of isolation (sputum, ear, eye or oropharynx) as the independent variables. Analysis of the transformants and associated parents was performed on unmanipulated data as the data satisfied assumptions of normality and equal variance. A one-way ANOVA was performed using categories of parent, transformant 1 (gBLNAR) and transformant 2 (high gBLNAR) with n=3 for each group. P values <0.05 were considered statistically significant.

RESULTS

Invasion rates and ampicillin MICs for all 40 clinical isolates and parents/transformants are given in Tables S1 and S2 (available in the online Supplementary Material). The ampicillin MICs ranged from 0.047 to 0.5 mg l<sup>−1</sup> in the isolates with normal PBP3 and 0.25 to 1.0 mg l<sup>−1</sup> in the isolates with altered PBP3 (N526K), with the mean MICs being 0.3 and 0.8 mg l<sup>−1</sup> respectively. The mean invasion rate ranged from 0.00 to 14.79% in the normal PBP3 isolates and 0.02 to 36.69% in the altered PBP3 isolates, with a group mean of 1.31% (n=20) and 6.86% (n=20) respectively. Five of 20 of the normal PBP3 isolates were found to be highly invasive (≥1%) as opposed to 10 of 20 of the altered PBP3 isolates. There was no association between mean invasion rates and site of isolation (P=0.52), but the mean invasion rate of isolates with altered PBP3 was significantly higher than for those with normal PBP3 (P=0.003) (Fig. 1). The mean invasion rates for the parental isolates, gBLNAR transformants and high-gBLNAR transformants were 0.81, 0.90 and 1.38, respectively, and this was not statistically significant (P=0.89) (Fig. 2).

![Fig. 1. Box-plot of invasion and PBP3 type. Box lines represent median, 25th and 75th percentile, and entire data range.](http://jmm.sgmjournals.org)
DISCUSSION

There were a number of important differences between our study and the study of Okabe et al. (2010). The isolates in this study were identified and differentiated from Haemophilus haemolyticus by molecular tests, and also assigned evenly between the normal and altered PBP3 groups by site of isolation. This is important because it is now known that H. haemolyticus can contaminate respiratory specimens and be misidentified as NTHi (Murphy et al., 2007), but this was not apparent when the Okabe et al. (2010) isolates were collected (2001–2005). Nothing is known about the invasive capacity of H. haemolyticus, but as a non-pathogenic respiratory commensal it might be expected to be less invasive than NTHi and inclusion in the study might inappropriately influence the results. Similarly, the Okabe et al. (2010) isolates were not identified by site of isolation and although our study did not demonstrate a significant difference, it might have been more invasive than the bronchial cell line than isolates from other sites. In the Okabe et al. (2010) study, the gBLNAR group (n=57) was heavily weighted with high-gBLNAR isolates (n=33) and it was this subset where a significant difference (P=0.0075) in invasion rate was demonstrated compared with isolates with normal PBP3 (Okabe et al., 2010). In our study, we did not attempt to further separate our altered PBP3 isolates on the basis of amino acid substitutions other than N526K, particularly into gBLNAR or high gBLNAR, as the latter are generally rare outside of Japan and very uncommon in Australia, from where our isolates were sourced (Witherden et al., 2011). Our finding of an association between the presence of altered PBP3 (defined only by the presence of N526K) and invasion rate extends the more specific finding by Okabe et al. (2010) and is significant because gBLNAR strains are so prevalent worldwide (Kishii et al., 2011; Tristram et al., 2007; Witherden et al., 2011). To further investigate any actual role of altered PBP3 in enhanced invasion, we selected three isolates with normal PBP3 and relatively low invasion rates (Ci37, 0.01%; J76, 0.15%; Ci31, 0.9%) into which we cloned both N526K and high-gBLNAR-type altered PBP3. Our failure to demonstrate a statistically significant increase in invasion rates in these transformants relative to the parental isolates suggests that the altered PBP3 itself does not play a role in increased invasiveness. This conclusion is based on cloning experiments with only a small number of isolates and could be strengthened by testing additional isolates or by attempting to reduce the invasion rate of highly invasive isolates with altered PBP3 by the introduction of normal PBP3 using allelic exchange.

An alternative hypothesis to explain the association of isolates with altered PBP3 and increased invasion rate, demonstrated by both the Okabe et al. (2010) and the current study, lies in the natural transformability of NTHi. A supra-genome hypothesis has been proposed for NTHi because when the genomes of two independent isolates are compared, they typically differ by 15–20%, which is thought to represent variation from a series of past recombination events where non-essential genes are exchanged within the species (Mell et al., 2011). Transformation experiments with genomic DNA in NTHi have shown frequent transfer of multiple fragments, up to 16 kb in size with multiple ORFs, and selection of transformants with one selectable marker increased the fraction of cells with other independent markers (Mell et al., 2011). There is now strong evidence that many BLNAR isolates acquired their altered PBP3 by recombination (Takahata et al., 2007; Witherden et al., 2014) and this might provide the opportunity to concomitantly acquire the additional independent genetic material related to enhanced invasion. This is particularly relevant given that acquisition of altered PBP3 is favoured by the selective pressure of the widespread use of β-lactam antibiotics. This hypothesis would neatly explain why not all isolates with altered PBP3 are highly invasive and why many with normal PBP3 are, and is worthy of additional investigation.

In conclusion, we have confirmed that there is an association between the presence of altered PBP3 in NTHi and an increased capacity to invade BEAS-2B cells in vitro, but also have provided some evidence that the altered PBP3 is not involved directly in enhanced invasion.

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