Natural insertion of the bro-1 β-lactamase gene into the gatCAB operon affects Moraxella catarrhalis aspartyl-tRNAAsn amidotransferase activity

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Only about half of bacterial species use an asparaginyl-tRNA synthetase (AsnRS) to attach Asn to its cognate tRNAAsn. Other bacteria, including the human pathogen Moraxella catarrhalis, a causative agent of otitis media, lack a gene encoding AsnRS, and form Asn-tRNAAsn by an indirect pathway catalysed by two enzymes: first, a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) catalyses the formation of aspartyl-tRNAAsn (Asp-tRNAAsn), then, a tRNA-dependent amidotransferase (GatCAB) transamidates this ‘incorrect’ product into Asn-tRNAAsn. As M. catarrhalis has a Gln-tRNA synthetase, its GatCAB functions as an Asp-tRNAAsn amidotransferase. This pathogen rapidly evolved to about 90 % ampicillin resistance worldwide by insertion of a bro-1 β-lactamase gene within the gatCAB operon. Comparison of the GatCAB subunits from bro-1 β-lactamase-positive and bro-negative strains showed that the laterally transferred bro-1 gene, inserted into the gatCAB operon, affected the C-terminal sequence of GatA. The identity between the C-terminal sequences of GatAwt (residues 479–491) and of GatABRO-1 (residues 479–492) was about 36 %, whereas the rest of the GatA sequence was relatively conserved. The characterization of these two distinct GatCABs as well as the hybrid GatCAB containing GatA(1–478)wt(479–492)BRO-1 and truncated GatCAB enzymes of M. catarrhalis showed that the substitution in GatAwt of residues 479–492 of GatABRO-1 causes increased specificity for glutamine, and decreased specificity for Asp-tRNAAsn in the transamidation reaction. We conclude that the bro gene insertion has altered the kinetic parameters of Asp-tRNAAsn amidotransferase, and we propose a model for gatA evolution after the insertion of bro-1 at the carboxyl end of gatA.

INTRODUCTION

Faithful translation of genetic information into growing polypeptides relies on the formation of correctly charged aminoacyl-tRNAs. In eukaryotes, these reactions are catalysed via a direct pathway by a set of 20 aminoacyl-tRNA synthetases, each specific for a given amino acid and for one or several cognate tRNAs (Schimmel & Soll, 1979). However, many bacterial genomes lack genes encoding an asparaginyl-tRNA synthetase (AsnRS) and/or a glutaminyl-tRNA synthetase (GlnRS), and form glutaminyl-tRNAGln (Gln-tRNA\textsubscript{Gln}) and asparaginyl-tRNAAsn (Asn-tRNA\textsubscript{Asn}) via an indirect pathway by the GatCAB-mediated transamidation of glutamyl-tRNA\textsubscript{Gln} (Glu-tRNA\textsubscript{Gln}) or aspartyl-tRNA\textsubscript{Asn} (Asp-tRNA\textsubscript{Asn}) formed by a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) or a non-discriminating aspartyl-tRNA synthetase (ND-AspRS), respectively (Becker & Kern, 1998; Curnow \textit{et al.}, 1996; Lapointe \textit{et al.}, 1986; Wilcox & Nirenberg, 1968). In nature, there is one type of bacterial amidotransferase (AdT), corresponding to GatCAB, with a dual specificity when both substrates Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln} exist in vivo (Becker \textit{et al.}, 2000; Curnow \textit{et al.}, 1997; Raczniak \textit{et al.}, 2001; Tumbula \textit{et al.}, 2000), and two types of archaeal AdT, namely GatDE and GatCAB, which act only as a Glu-AdT or Asp-AdT, respectively (Sheppard \textit{et al.}, 2008).
Similarly, most bacteria and all archaea lack GlnRS and use an ND-GluRS and GatCAB or GatDE as a Glu-tRNA AdT (Glu-AdT) to form Gln-tRNA Gln (Curnow et al., 1997; Horiiuchi et al., 2001; Sheppard et al., 2007, 2008; reviewed by Huot et al., 2010).

Bacterial GatCABs catalyse three distinct reactions during the transamidation process: they activate Asp-tRNA Asn by phosphorylating the beta-carboxyl group of the aspartyl residue linked to tRNA Asn, they use NH₄⁺ directly or hydrolyse the amide group of Gln or Asn to release ammonia, and finally they amidate the activated Asp-tRNA Asn intermediate into Asn-tRNA Asn (Decicco et al., 2001; Feng et al., 2005; Horiiuchi et al., 2001; Sheppard et al., 2007). The GatA subunit belongs to the amidase family and uses Gln or Asn as amide donor (Nakamura et al., 2006; Strauch et al., 1988; Wu et al., 2009).

Moraxella catarrhalis is a human-restricted pathogen, and is the third most common isolate after Streptococcus pneumoniae and nontypable Haemophilus influenzae as a causative agent of otitis media (Peng et al., 2005). M. catarrhalis underwent a rapid evolution from <10 to >90% ampicillin resistance in the space of a few years (Doern et al., 1996; Wallace et al., 1989). Most of this resistance is due to the insertion of the bro-1 β-lactamase gene into the chromosome (Wallace et al., 1990); less frequently, the resistance is due to the BRO-2 β-lactamase, which differs by one amino acid (Bootsma et al., 1996). Surprisingly, the upstream and downstream neighbours of the bro-1 gene were identified in the characterized bro-1 M. catarrhalis strains as the gatA and gatB genes encoding AdT subunits (D. Beaulieu and P. H. Roy; GenBank file U49269). Comparison of resistant and sensitive strains showed that the C-terminal-coding region of gatA was disrupted by the insertion of bro-1; only 36% identity was observed between GatA(479–491) wt and GatA(479–492) BRO1 (Fig. 1). Several other nucleotide substitutions resulting in amino acid changes were found in the part of the gatA gene upstream of the insertion. Analysis of the partial genomic sequence from an ampicillin-resistant strain allowed identification of genes encoding for glutamyl-tRNA synthetase (GluRS), for aspartyl-tRNA synthetase (AspRS) and for GlnRS, but no gene encoding AsnRS was found. In addition, GlnRS activity was detected biochemically (see Results below), but no AsnRS activity was found in M. catarrhalis extracts. This suggests that GatCAB in M. catarrhalis functions as an aspartyl-tRNA Asn AdT, as it does in other Pseudomonadales whose genomes have been completely sequenced and which lack an AsnRS. We report herein the characterization of two distinct GatCABs, of a hybrid and of truncated GatCAB enzymes of M. catarrhalis. Our data show that substitution of residues 479–491 in GatA wt by the corresponding GatA BRO1 residues 479–492 causes an increased specificity for Gln, and a decreased specificity for Asp-tRNA Asn in the transamidation reaction. We also found that GatCAB BRO1 shows a reduced rate of Asp-tRNA Asn transamidation compared with GatCAB wt (kcat 0.27 vs 1.3 s⁻¹). We conclude that the bro-1 gene insertion has altered the kinetic parameters of this Asp-tRNA Asn AdT, and we propose a model, supported by these results, for gatA evolution following the insertion of bro-1 at the carboxyl end of M. catarrhalis gatA.

Fig. 1. Schematic representation of the gatCAB operons of the M. catarrhalis strains ATCC25240 and ATCC53279, and comparison of the amino acid sequences of the GatA C-terminal ends. (a) M. catarrhalis bro-negative strain ATCC25240 carries a gatCAB operon. (b) M. catarrhalis bro-positive strain ATCC53279 contains a gatCAB operon with a bro-1 gene insertion between gatA and gatB. (c) Alignment of the C-terminal sequences of GatAs from these strains and 12 new genome sequences from geographically and phenotypically diverse clinical isolates of M. catarrhalis. There are three identical GatA(479–492) BRO1 sequences, eight identical GatA(479–492) BRO2 sequences and two identical GatA(479–491) wt sequences (de Vries et al., 2010; Davie et al., 2011). Amino acids in lower-case type are not conserved in the three sequences. The identity of GatA(478–491) wt versus GatA(478–492) BRO1 is 36% (5 residues of 14); the identity of GatA(478–491) wt versus GatA(478–492) BRO2 is 43% (6 residues of 14).
METHODS

Nucleotides, reagents and amino acids. Oligonucleotide synthesis and DNA sequencing were carried out by the sequencing service of the CHUL, at Université Laval and by Integrated DNA Technologies (IDT). [35S]Gln (258 mCi mmol−1; 9.55 GBq mmol−1) and [2-14C]Asp (207 mCi mmol−1; 7.66 GBq mmol−1), [14C]Glu (111 TBq mmol−1) were purchased from PerkinElmer Life and Analytical Sciences. [35S]Asn (210 mCi mmol−1; 7.77 GBq mmol−1) was purchased from Amersham Biosciences. TLC plates, cellulose, poly(ethyleneimine) (PEI) matrix (Sigma-Aldrich) and chitin agarose beads were from New England BioLabs. Bio-Spin 30 (200 μl) was from Bio-Rad Laboratories and chitin agarose beads were from New England BioLabs. Bio-Spin 30 (200 μl) was from Bio-Rad Laboratories.

GlnRS and AsnRS activity measurements in a crude extract of M. catarrhalis. Cultures of the M. catarrhalis strains ATCC25240 (ampicillin-sensitive) and ATCC53279 (ampicillin-resistant) were grown from single colonies in LB medium at 37 °C, in the presence of 40 μg ampicillin ml−1 for the latter. Cells were harvested by centrifugation at early stationary phase, suspended in 20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 0.1 mM EDTA, 0.1 % Triton 100, and lysed by sonication after treatment with lysozyme. The lysate was centrifuged at 14 000 g for 2 h, and the supernatant was dialysed against 20 mM Tris/ HCl, pH 8.0, 10 mM MgCl2, 1 mM DTT, 10 % (v/v) glycerol and 0.2 M NaCl at 4 °C for 18 h, to remove the amino acids that would interfere with the measurement of tRNA aminoacylation using 14C-labelled amino acids. A second dialysis was used to bring this crude extract to 25 mM Na HEPES, pH 7.2, 3 mM 2-mercaptoethanol, 0.2 mM EDTA, 0.2 M NaCl and 50 % (v/v) glycerol, in which it was stored at −20 °C.

The tRNA aminoacylation activities were measured at 37 °C in 50 mM HEPES-KOH, pH 7.2, 16 mM MgCl2, 2 mM ATP, 3 mM DTT, 330 μM unfraccionated tRNA from E. coli, and 200 μM [14C]glutamine or [14C]asparagine, for GlnRS and AsnRS activity measurements, respectively, using the filter paper assay described by Lapointe et al. (1985). As a control, the same measurements were done with E. coli DH5α crude extracts prepared by a similar procedure. One unit of GlnRS or AsnRS catalyses the formation of one nanomole of aminoacyl-tRNA in 10 min at 37 °C.

Cloning and preparation of M. catarrhalis tRNAAsn. In this study, the gene corresponding to M. catarrhalis tRNAAsn was artificially formed by hybridizing two phosphorylated oligonucleotides, 5′-GATGTTGACCGCGATCCTCGGACGATGCCTTACCTCCTGCACCTGC-3′ and 5′-GTGCGAGGATAGCTGCTGACAAACGTAGATGTGAGACGAACTAGC-3′, and the gene was inserted into the sites EcoRI/PstI of pGFIB to create the pGFIB/MctRNAAsn clone. M. catarrhalis tRNAAsn was prepared according to Sheppard et al. (2007). Analysis by electrophoresis on a 12 % polyacrylamide gel containing 8 M urea revealed that more than 99 % of the material was tRNA; its acceptor activity for Asn varied from 11 to 25 %.

Cloning M. catarrhalis gatCAB and its variants With the cloning and affinity purification strategy described below, all the homologous GatC and GatB subunits of the various GatCAB proteins studied here have the additional residues GRAMGGR at the N-terminal of GatC, and EFLEGSS at the C-terminal ends. On the other hand, because we had to use a partly different strategy to express gatCAB BRO (see below), GatAsn has the additional residues EFLEGSS at its C terminal, and GatB BRO has the additional residues GRAMGGR at its N terminal. These short tags are very flexible because of the presence of glycine residues, and thus are unlikely to alter the properties of these enzymes.

gatCAB wt. The full-length of the gatCAB operon (3.2 kb) of the sensitive M. catarrhalis strain ATCC25240 (bro-negative strain) was amplified with the oligonucleotides 5′-GCGAATTCGCCCATCATTGACGATGCCTTACCTCCTGCACCTGC-3′ and 5′-GTGCGAGGATAGCTGCTGACAAACGTAGATGTGAGACGAACTAGC-3′, and the operaon was inserted into the EcoRI site of pTwin2 to generate pTwin2/gatCAB wt (Fig. 2, line 1).

![Fig. 2. Cloning of various M. catarrhalis gatCAB operons, and overproduction and purification of the three subunits. The M. catarrhalis gatCAB operons represented in this figure (1, gatCAB wt; 2, gatCAB Δh; 3, gatCA D wt; 4a, gatCAB Δh; 4b, gatCAB Δh) were amplified by PCR and inserted into the EcoRI site of pTwin2 between two genes encoding an intein fused to a CBD (Evans et al., 1999). Following the overexpression of these constructs, the overproduced proteins (CBD-intein1-GatC, GatA and GatB-intein2-CBD) were purified by affinity chromatography on a column of chitin resin, and the tags on GatC and GatB were removed (see Methods and Evans et al., 1999). GatA has no tag, but co-purified with GatC and GatB because of its affinity for these proteins (Nakamura et al., 2006).]
**M. catarrhalis GatCAB purification.** *M. catarrhalis* gatCAB clones were used to transform *E. coli* BL21(DE3) strains, and were selected on LB agar plates with 120 μg ampicillin ml⁻¹. In each case, a single fresh colony was grown for 18 h at 37 °C, in LB medium supplemented by 120 μg ampicillin ml⁻¹. The culture was used to inoculate 750 ml LB supplemented with 120 μg ampicillin ml⁻¹, for 3.5 h at 28 °C, shaking at 250 r.p.m. to reach OD₆₀₀ 0.4. Induction was then initiated with 0.3 mM IPTG for 18 h at 14 °C. All GatCABs of *M. catarrhalis* were purified by affinity chromatography with intein chitin-binding domain (CBD) tags, which bind to chitin resin. The tags were removed as described elsewhere (Evans et al., 1999).

The purity of GatCAB was checked by SDS-PAGE, and pure fractions were dialysed against 20 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT, 10 % (v/v) glycerol and 0.2 M NaCl at 4 °C for 18 h. This pure GatCAB solution was then concentrated and its buffer changed to 50 mM Na HEPES, pH 7.2, 6 mM 2-mercaptoethanol, 0.4 mM EDTA and 0.4 M NaCl using an Ultra Amicon 30K centrifugal filter device (Millipore). An equal volume of glycerol was added, and the pure GatCAB in 50 % glycerol was stored at −20 °C.

**Pseudomonas aeruginosa AspRS purification.** *P. aeruginosa* AspRS was overexpressed and purified as previously described by Akochy et al. (2004).

**Preparation of *M. catarrhalis* labelled tRNA.** *E. coli* total tRNA containing about 20 % *M. catarrhalis* tRNA<sub>wt</sub> (see above) was 32P-labelled on its 3′-terminal phosphodiester link using the *E. coli* CCA-adding enzyme and [α-32P]ATP as previously described (Sheppard et al., 2007, 2008), with some modifications as follows: briefly, 172 μM tRNA<sub>wt</sub> in 50 mM Tris/HCl (pH 8.0), 20 mM MgCl₂, 5 mM DTT and 0.02 μM NaPPi was incubated for 35 min at room temperature in the presence of 2 μg ml⁻¹ (43 mM) pure CCA-adding enzyme from *E. coli* (Cudny & Deutscher, 1986) and 1 μCi ml⁻¹ (37 kBq) [α-32P]ATP (PerkinElmer and Analytical Sciences). Protein in 50 μl samples were phenol (Tris-buffered pH 7.9)/chloroform extracted, and the aqueous phase was filtered through Bio-Spin 30 columns to remove excess [α-32P]ATP.

**Preparation of Asp-tRNA<sub>wt</sub>.** Aspartylation of *M. catarrhalis* tRNA<sub>wt</sub> was carried out for 30 min at 37 °C in 50 mM Na HEPES, pH 7.2, 25 mM KCl, 15 mM MgCl₂, 4 mM ATP, 5 mM DTT, 0.5 mg ml⁻¹ *P. aeruginosa* ND-AspRS, 1 mM L-Asp, 140 μM unlabelled *M. catarrhalis* tRNA<sub>wt</sub> and 1.33 μM 32P-labelled *M. catarrhalis* tRNA<sub>wt</sub>. The samples were phenol/chloroform-extracted, and excess ATP was removed as described previously (Sheppard et al., 2007). Samples with only unlabelled tRNA<sub>wt</sub> were aminoacylated in parallel and were used in the glutaminase assays. In place of checking the levels of aminoacylation as described elsewhere (Bulllock et al., 2003), we checked the level of Asn-tRNA<sub>Asn</sub> formation by measuring the amount of Asn formed in the transamidation reaction (see below) in the presence of a high concentration (4 mM) of GatCAB<sub>wt</sub>.

**Glutaminase and Adt assays.** The kinetic parameters of the glutaminase activity of *M. catarrhalis* GatCAB were measured at 37 °C in 50 mM HEPES-KOH, pH 7.2, 15 mM MgCl₂, 25 mM KCl and 1 mM DTT with nearly saturating concentrations of ATP (4 mM) and unlabelled Asp-tRNA<sub>Asn</sub> (12.4 μM), and 7.5–240 μM [14C]Gln (232 mCi mmol⁻¹; 8.85 GBq mmol⁻¹). The concentrations of GatCAB<sub>wt</sub>, GatCAB<sub>mm</sub>, GatCAB<sub>BRO</sub>, GatCAB<sub>BRO</sub> and GatCAB<sub>RI</sub> were 2, 0.5, 5, 1 and 20 nM, respectively. After various reaction times, 5 μl aliquots were transferred into 0.3 mM sodium acetate, pH 5.0, to quench the reaction, and the tRNA was ethanol-precipitated. The supernatant containing [14C]Gln and [14C]Glu were dried and resuspended in water (3 μl). One micromolar aliquots of the resuspended reaction mixture were spotted onto 20 × 20 cm cellulose TLC plates with plastic backs (Sigma), and developed for about 5 h under acidic conditions.
(isopropyl) alcohol/formic acid/water, 20:1:5) to separate [14C]Gln and [14C]Glu. The plates were exposed on an imaging plate for 18 h and quantified as described previously (Sheppard et al., 2007). The kinetic parameters were calculated using non-linear regression plots of the initial velocity versus Gln concentration, and SDs were determined from duplicate experiments using Kaleidagraph v. 4.01 (Synergy Software) (Fig. 3).

For this study, Gln was used as the amide donor. These assays were carried out at 37 °C in 50 mM HEPES-KOH, pH 7.2, 15 mM MgCl2, 25 mM KCl and 1 mM DTT. Unless otherwise noted, nearly saturating concentrations of ATP (4 mM) and Gln (2 mM) were added. For determination of the kinetic parameters of the enzyme for Asp-tRNAAsn, initial velocities were measured in triplicate, while varying the concentration of Asp-tRNAAsn. Reactions were carried out over 45 s using purified GatCAB wt, GatCAB h, GatCAB BRO and GatCA BRO-1 concentrations of 0.53, 1.21, 0.8, 2 and 1.7 nM, respectively. Reaction mixtures containing no enzyme were preincubated at 37 °C, and the reactions were started by the addition of M. catarrhalis GatCAB. At various times, 1 μl aliquots were transferred into 3 μl of 100 mM sodium citrate, pH 4.5, and 0.66 mg ml⁻¹ nuclease P1 (Sigma) on ice to quench the reaction. The mixture was left at room temperature for 40 min for the digestion of tRNA, and aliquots (1 μl) were spotted onto PEI-cellulose 20 × 20 cm TLC plates. To separate asparaginyl-AMP (Asn-AMP) from Asp-tRNAAsn, the plates were developed in 0.5 mM ammonium acetate and 5% acetic acid for about 120 min. The plates were visualized and quantified as described previously (Sheppard et al., 2007). Kaleidagraph was used to calculate the kinetic parameters using non-linear regression plots of the initial velocity versus Asp-tRNAAsn concentration (Fig. 4).

Values of SD reported are calculated from means of two distinct experiments for glutaminase and three distinct experiments for transamidation, weighting by inverse variance (Buckland et al., 1997).

**RESULTS**

Two sorts of GatCABs encoded by M. catarrhalis ATCC25240 and ATCC53279 differ at their GatA C terminals

Within only the past three decades, bro-positive strains of the human pathogen M. catarrhalis have increased to 90% worldwide (Murphy & Parameswaran, 2009). It is known that M. catarrhalis does not contain mobile elements associated with bro and has disseminated the bro gene by natural transformation (Bootsma et al., 2000). Analysis of the complete genomic sequences of 12 M. catarrhalis isolates (Davie et al., 2011) using gapped BLAST and the Genetics Computer Group v11 software (Accelrys) revealed the presence of genes corresponding to GatCAB, GluRS, GlnRS and AspRS, but no gene for AsnRS was found. The presence

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**Fig. 3.** Determination of kinetic parameters of the glutaminase activity with respect to Gln of various GatCABs of M. catarrhalis. (a) The amount of the product [14C]Glu formed from [14C]Gln was measured by phosphorimaging of Gln and Glu after their separation by chromatography on a cellulose thin layer. Glutaminase assays were carried out at 37 °C in the presence of nearly saturating concentrations of ATP (4 mM) and of unlabelled Asp-tRNAAsn (12.4 μM), with [14C]Gln varying from 7.5 to 240 μM. (b) Initial velocities of Glu formation versus Gln concentrations in the presence of 1 nM of each of the following M. catarrhalis GatCABs: (●) GatCAB wt; (◆) GatCAB h; (▲) GatCA BRO; (■) GatCA BRO-1; (▲) GatCA BRO-1. Each curve is the average of duplicate experiments. These results were analysed according to the Michaelis–Menten model with Kaleidagraph v. 4.01 (Synergy Software), using non-linear regression to calculate the kinetic parameters K_m and k_cat (see Table 1).

**Fig. 4.** Determination of kinetic parameters of the transamidation activity with respect to Asp-tRNAAsn of various GatCABs of M. catarrhalis. The amount of the product Asn-tRNAAsn formed from Asp-tRNAAsn was measured by phosphorimaging of Asn-AMP after digestion of the aminoacyl-tRNAs with nuclease P1 (see Methods). (a) Separation of Asn-AMP from Asp-AMP and AMP by chromatography on a PEI thin layer. The transamidation reaction was conducted in the presence of nearly saturating concentrations of Gln (2 mM) and ATP (4 mM), and of 162 nM to 12.4 μM Asp-tRNAAsn. (b) Initial velocities of Asn-tRNAAsn formation versus Asp-tRNAAsn concentrations with 1 nM of the M. catarrhalis GatCAB variants according to the Michaelis–Menten model. (●) GatCA BRO; (◆) GatCA h; (▲) GatCA h; (■) GatCA BRO-1; (▲) GatCA BRO-1. Each curve is the mean of triplicate experiments. These results were analysed by Kaleidagraph using non-linear regression to calculate the kinetic parameters K_m and k_cat (see Table 1).
of GatCAB and GlnRS coupled with the lack of AsnRS suggests an in vivo function of GatCAB as an Asp-tRNA<sup>Asn</sup> AdT. Our previous study of <i>P. aeruginosa</i>, which has the same set of genes, has demonstrated the presence of the direct formation of Gln-tRNA<sup>Gln</sup>, and of the indirect formation of Asn-tRNA<sup>Asn</sup>, which involves an ND-AspRS and a GatCAB as an Asp-AdT (Akochy et al., 2004). Crude extracts of bro-negative and bro-positive <i>M. catarrhalis</i> strains contain GlnRS activity levels of 7.0 and 2.9 units (mg protein)<sup>-1</sup>, respectively, which are comparable with those found in crude extracts of <i>E. coli</i> [5.2 units (mg protein)<sup>-1</sup>]. On the other hand, no AsnRS activity was detected in crude extracts of these <i>M. catarrhalis</i> strains, compared with a level of 0.83 unit (mg protein)<sup>-1</sup> in crude extracts of <i>E. coli</i> (results not shown). These results suggest that in <i>M. catarrhalis</i>, Gln-tRNA<sup>Gln</sup> is formed by the direct pathway, and Asn-tRNA<sup>Asn</sup> by the indirect pathway, indicating that the GatCAB AdTs of bro-positive and bro-negative <i>M. catarrhalis</i> function as Asp-tRNA<sup>Asn</sup>-dependent AdTs.

We also found a remarkable organization of the <i>gatCAB</i> operon, with a bro-β-lactamase gene inserted between the <i>gatA</i> and <i>gatB</i> genes. The G + C content is about 31 and 42 % for the bro gene and the <i>M. catarrhalis</i> genome, respectively, suggesting that the bro gene was recently laterally transferred from another organism (Bootsma et al., 2000). The two GatCs are well conserved, as well as the two GatBs, but there is a significant difference between the GatA of the sensitive strain (GatA<sub>wt</sub>) and the GatA of the resistant strain (GatA<sub>BRO</sub>). The C-terminal sequences of GatA<sub>479–491</sub><i>wt</i> and GatA<sub>479–492</sub><i>BRO</i> are only 36 % identical, suggesting that the DNA sequence encoding the GatA<sub>479–492</sub><i>BRO</i> carboxyl end was imported with the β-lactamase gene (Fig. 1). There is not a clear boundary between the inserted and conserved sequence; from sequence comparison it appears that a 997 nt insert containing bro-1 replaced a 110 nt chromosomal sequence containing the original 3’ end of <i>gatA</i>. There are several amino acid changes in the rest of GatA in the resistant strain; our subsequent analysis of <i>gatA</i> sequences from multiple genomes (Davie et al., 2011) showed that they are not conserved. However, we initially hypothesized that they were compensatory mutations that were selected after the disruption of the <i>gatA</i> 3’ end. To test this hypothesis, we prepared a hybrid GatA with the carboxyl end from the resistant strain and the rest from the sensitive strain. We also prepared C-terminal-truncated GatA from both sensitive and resistant strains. We cloned, purified to homogeneity and characterized these <i>M. catarrhalis</i> GatCABs (Fig. 2).

**Overproduction and purification of <i>M. catarrhalis</i> GatCABs**

All clones of complete <i>gatCAB</i> operons carrying the various versions of <i>gatA</i> were made successfully except for <i>gatCBRO</i><sub>-1</sub>-1. Attempts to clone <i>gatCBRO</i><sub>-1</sub> as a unique fragment into the pTwin2 vector failed. However, clones of <i>gatCBRO</i><sub>-1</sub> and of <i>gatBRO</i><sub>-1</sub> were made separately (Fig. 2, lines 4a and 4b), and GatC<sub>BRO</sub><sub>-1</sub> and GatB<sub>BRO</sub><sub>-1</sub> were purified individually. Pure fractions were pooled and migrated in non-denaturing PAGE as a single band corresponding to the holoenzyme (data not shown).

**Both GatCAB<sub>wt</sub> and GatCAB<sub>h</sub> have the same levels of glutaminase and of Asp-tRNA<sup>Asn</sup> transamidation activities**

To test the transamidation activity of <i>M. catarrhalis</i> GatCAB variants, we used a recently developed [<sup>32</sup>P]tRNA/nuclease P1 assay (Sheppard et al., 2007). We used Gln, reported to be a better amide donor than Asn for bacterial GatCAB (Nakamura et al., 2006; Sheppard et al., 2007, 2008). <i>M. catarrhalis</i> tRNA<sup>Asn</sup> overproduced in <i>E. coli</i> (see Methods) was used to prepare Asp-tRNA<sup>Asn</sup> with <i>P. aeruginosa</i> AspRS as a substrate to test the efficiency of <i>M. catarrhalis</i> GatCAB variants in amidation of Asp-tRNA<sup>Asn</sup> to Asn-tRNA<sup>Asn</sup>. We constructed a GatCAB hybrid (GatCAB<sub>h</sub>) that consists of a GatCAB<sub>wt</sub> in which GatA<sub>479–491</sub><i>wt</i> is replaced by GatA<sub>479–492</sub><i>BRO</i>. We found that GatCAB<sub>wt</sub> was as active as GatCAB<sub>h</sub> in glutaminase activity (<i>k</i><sub>cat</sub>=11.3 and 14.7 s<sup>-1</sup>, respectively) (Fig. 3, Table 1), and in transamidation of Asp-tRNA<sup>Asn</sup> (<i>k</i><sub>cat</sub>=1.3 and 1.05 s<sup>-1</sup>, respectively) (Fig. 4, Table 1). These two <i>k</i><sub>cat</sub> values are similar to that of <i>Helicobacter pylori</i> GatCAB, 1.3 s<sup>-1</sup> (Sheppard et al., 2007). The <i>K</i><sub>m</sub> value of GatCAB<sub>wt</sub> for Asp-tRNA<sup>Asn</sup> (1.67 μM) resembles that of <i>H. pylori</i> GatCAB (1.12 μM) for Asp-tRNA<sup>Asn</sup> (Fig. 4, Table 1) (Fischer et al., 2012).

**The substitution of GatA<sub>479–491</sub><i>wt</i> by GatA<sub>479–492</sub><i>BRO</i> affects the <i>K</i><sub>m</sub> of this enzyme for Gin and Asp-tRNA**

This substitution generates the hybrid enzyme GatCAB<sub>h</sub>. Its <i>K</i><sub>m</sub> for Gln was one quarter that of GatCAB<sub>wt</sub>, and its <i>K</i><sub>m</sub> for Asp-tRNA<sup>Asn</sup> was threefold higher. Considering that the <i>k</i><sub>cat</sub> values of these enzymes are very similar, these differences in <i>K</i><sub>m</sub> result in a fourfold increase of specificity (<i>k</i><sub>cat</sub>/<i>K</i><sub>m</sub>) for glutamine to 1256 s<sup>-1</sup> mM<sup>-1</sup> in the glutaminase reaction, and in a fivefold decrease of its specificity for Asp-tRNA<sup>Asn</sup> to 201 s<sup>-1</sup> mM<sup>-1</sup> in the transamidase reaction (Figs 3 and 4, Table 1).

**GatCAB<sub>BRO</sub> glutaminase activity is similar to that of GatCAB<sub>wt</sub>, but GatCAB<sub>BRO</sub> displays a lower transamidation activity for Asp-tRNA<sup>Asn</sup> than that of GatCAB<sub>wt</sub>**

GatCAB<sub>BRO</sub> is as active as GatCAB<sub>h</sub> and GatCAB<sub>wt</sub> in glutaminase activity (<i>k</i><sub>cat</sub>=13.1, 14.7 and 11.3 s<sup>-1</sup>, respectively) (Fig. 3, Table 1). In contrast, the GatCAB<sub>BRO</sub> transamidation of Asp-tRNA<sup>Asn</sup> (<i>k</i><sub>cat</sub>=0.27 s<sup>-1</sup>) is lower than those of the wild-type and hybrid enzymes (<i>k</i><sub>cat</sub>=1.3 and 1.05 s<sup>-1</sup>, respectively) (Fig. 4, Table 1). The low <i>k</i><sub>cat</sub> value of GatCAB<sub>BRO</sub> is similar to that of <i>H. pylori</i> GatCAB, at 0.25 s<sup>-1</sup> (Fischer et al., 2012).
Table 1. Kinetic constants for Gln and Asp-tRNA\(^{\text{Asn}}\) of GatCAB AdTs from \(M.\) \textit{catarrhalis} strains ATCC25240 and ATCC53279, and from several variants

Glutaminase and transamidation activities were determined and statistically analysed as described in Methods.

*\text{GatCABwt} is from the \(\text{bro-negative strain} M.\) \textit{catarrhalis} ATCC25240, which is sensitive to ampicillin.

†\text{GatCAB} is the variant corresponding to \text{GatCABwt} in which amino acid residues GatA(479–491)\text{wt} have been replaced by the homologous segment GatA(479–492)\text{BRO}.

‡\text{GatCABwt} is the variant from \text{GatCABwt} in which \text{GatA(479–491)wt} was deleted.

§\text{GatCABRO} is from \text{GatCABwt} in which \text{GatA(479–492)BRO} sequence was deleted.

‖\text{GatA}\text{BRO} is the variant from \text{GatCABRO} in which \text{GatA(479–492)BRO} sequence was deleted.

\begin{tabular}{|c|c|c|c|}
\hline
Enzyme & Glutaminase activity & Transamidase activity & \\
& \begin{tabular}{c} \(K_m\) \text{Gln} \text{(mM) \pm SD} \end{tabular} & \begin{tabular}{c} \(k_{\text{cat}}\) \text{(s}\(^{-1}\)) \pm SD \end{tabular} & \begin{tabular}{c} \(k_{\text{cat}}/K_m\) \text{Gln} \text{(s}\(^{-1}\) \text{mM}^{-1}) \end{tabular} & \begin{tabular}{c} \(K_m\) \text{Asp-tRNA}^{\text{Asn}} \text{(mM) \pm SD} \end{tabular} & \begin{tabular}{c} \(k_{\text{cat}}\) \text{(s}\(^{-1}\)) \pm SD \end{tabular} & \begin{tabular}{c} \(k_{\text{cat}}/K_m\) \text{Asp-tRNA}^{\text{Asn}} \text{(s}\(^{-1}\) \text{mM}^{-1}) \end{tabular} \\
\hline
\text{GatCABwt} & 50.6 \pm 23.4 & 11.3 \pm 2.48 & 223 & 1.67 \pm 0.88 & 1.3 \pm 0.20 & 778 \\
\text{GatCAB} & 11.7 \pm 8.13 & 14.7 \pm 2.68 & 1256 & 5.22 \pm 1.53 & 1.05 \pm 0.12 & 201 \\
\text{GatCA}\text{BROwt} & 74.4 \pm 30.4 & 6.02 \pm 0.9 & 80 & 1.35 \pm 0.35 & 0.45 \pm 0.03 & 333 \\
\text{GatCABRO} & 23.6 \pm 9.48 & 13.1 \pm 1.09 & 555 & 2.08 \pm 0.41 & 0.27 \pm 0.16 & 129 \\
\text{GatCA}\text{BRO-II} & 32.7 \pm 3.05 & 6.9 \pm 0.17 & 211 & 2.07 \pm 0.41 & 0.54 \pm 0.033 & 260 \\
\hline
\end{tabular}


Removing residues 479–491 of \text{GatA}\text{wt} and 479–492 of \text{GatA}\text{BRO} results in lower glutaminase activity of \text{GatCA}\text{ wt} and of \text{GatCA}\text{ BRO}

To further test the role of the carboxyl end of GatA, we constructed \text{GatCA}\text{ A BRO wt} and \text{GatCA}\text{ A BRO}. The specificity constant of \text{GatCA}\text{ A BRO} for Gln (211 s\(^{-1}\) mM\(^{-1}\)) is comparable with that of \text{GatCA}\text{ BRO wt} (223 s\(^{-1}\) mM\(^{-1}\)) and lower than that of \text{GatCA}\text{ BRO} (555 s\(^{-1}\) mM\(^{-1}\)), suggesting that amino acid differences other than those in the carboxyl end are involved in the preference of \text{GatCAB BRO} for Gln. We noted that in the transamidation reaction, the two truncated enzymes have similar \(K_m\) values for Asp-tRNA\(^{\text{Asn}}\). Surprisingly, the \(k_{\text{cat}}\) of \text{GatCA}\text{ wt} in the transamidase activity decreased threefold to 0.45 s\(^{-1}\) compared with that of \text{GatCA}\text{ wt} (Table 1), whereas that of \text{GatCA}\text{ BRO} increased twofold to 0.54 s\(^{-1}\) compared with that of \text{GatCA}\text{ BRO}. This suggests that the amino acid differences between \text{GatCA}\text{ wt} and \text{GatCA}\text{ BRO} in the region 1–478 of \text{GatA BRO} are also involved in the specificity for Asp-tRNA\(^{\text{Asn}}\) in the transamidation reaction (Fig. 4, Table 1).

DISCUSSION

This study aimed to determine the impact of the amino acid substitutions in the C-terminal part (residues 479–492) of \text{GatCAB BRO} caused by the lateral \text{bro-L insertion} into the \text{gatCAB} operon, by comparing several natural and artificial forms of \(M.\) \textit{catarrhalis} GatCAB with respect to the following kinetic constants: \(k_{\text{cat}}\) of the glutaminase activity and \(K_m\) for Gln in that reaction, and \(k_{\text{cat}}\) of the transamidase activity and \(K_m\) for Asp-tRNA\(^{\text{Asn}}\) in that reaction. Given that the gene encoding GlnRS was identified from \(M.\) \textit{catarrhalis} sequences and that crude extracts from \text{bro-negative} and \text{bro-positive strains} showed GlnRS activity but no AsnRS activity, we assumed that \text{GatCAB} of \(M.\) \textit{catarrhalis} functions as an Asp-AdT. We used \(M.\) \textit{catarrhalis} tRNA\(^{\text{Asn}}\) and \text{GatCAB} purified to homogeneity in both the glutaminase and the transamidase assays, and used a sensitive\([\text{\textsuperscript{32P}}\text{]}\text{tRNA}/\text{nuclease P1} assay.\]

Substitution of \text{GatA(479–492)BRO-1}, for the homologous sequence in \(M.\) \textit{catarrhalis} \text{GatCABwt}

increases its specificity for Gln

The remarkable role of the \text{GatA(479–492)BRO-1} sequence was shown when we compared the kinetic parameters of the glutaminase activity of the \text{GatCABwt} and \text{GatCAB} enzymes. The \text{GatCAB} enzyme, which represents the presumed initial state after \text{bro} insertion, has a 5.6-fold higher specificity constant for Gln (1256 s\(^{-1}\) mM\(^{-1}\)) than that of \text{GatCAB wt} (223 s\(^{-1}\) mM\(^{-1}\)) representing the ancestral enzyme. In contrast, truncation of the C-terminal sequence results in an increased \(K_m\) for Gln (74.4 \text{\mu M}) and a reduced specificity constant for Gln of 80 s\(^{-1}\) mM\(^{-1}\), demonstrating that \text{GatA(479–492)BRO-1} is important for Gln specificity. However, the few amino acid differences present in the rest of \text{GatA BRO-1} in the whole \text{GatCAB BRO-1} (555 s\(^{-1}\) mM\(^{-1}\)) are likely to lower this specificity for Gln without affecting its \(k_{\text{cat}}\) of about 13.1 s\(^{-1}\). These differences may play a role in increasing the \(K_m\) for Gln, so that its kinetic parameters approach those of \text{GatCAB wt}. No \text{GatCAB structure} from a mesophilic Gram-negative species has been reported to date. Only three structures of \text{GatCAB} are available: for a Gram-positive bacterium and two hyperthermophilic bacteria (Nakamura \textit{et al}., 2006; Wu \textit{et al}., 2009; Ito \& Yokoyama, 2010). According to the structure of \text{GatCAB} of \textit{Staphylococcus aureus}, which was established without its Asp-tRNA\(^{\text{Asn}}\) substrate, \text{GatA(479–492)BRO-1} should be located about 40 Å from the ammonia channel entrance and far from the suggested point of

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interaction of GatCAB and its Asp-tRNA<sub>Asn</sub> substrate. It would be interesting to understand, by a closer look at a crystal structure of a mesophilic Gram-negative bacterial GatCAB, how the C terminal of GatA structurally affects the preference for Gln.

The substitution of GatA(479–492)<sub>BRO</sub> for the homologous sequence in <i>M. catarrhalis</i> GatA(479–491)<sub>wt</sub> decreases its specificity for Asp-tRNA<sub>Asn</sub>

Another remarkable role of the GatA(479–492)<sub>BRO</sub> sequence was shown when we compared the kinetic parameters of the transmigration activity of GatCAB<sub>wt</sub> and GatCAB<sub>BRO</sub>. We showed that the GatCAB<sub>BRO</sub> enzyme (as the initial state of GatCAB, right after the insertion of bro into the gatCAB operon) has a higher <i>K</i><sub>m</sub> for Asp-tRNA<sub>Asn</sub> (2.82 μM) compared with GatCAB<sub>wt</sub>, considered as the ancestral enzyme (<i>K</i><sub>m</sub> of 1.67 μM). The GatA(479–492)<sub>BRO</sub> sequence may be involved in increasing the <i>K</i><sub>m</sub> for Asp-tRNA<sub>Asn</sub>. Truncating this sequence reduced the <i>K</i><sub>m</sub>. Interestingly, a comparison of the kinetic parameters between GatCAB<sub>BRO</sub> and GatCAB<sub>wt</sub> showed an intermediate <i>K</i><sub>m</sub> value of 2.08 μM for Asp-tRNA<sub>Asn</sub> for the latter, lower than that of GatCAB<sub>BRO</sub>, but higher than that of GatCAB<sub>wt</sub>, suggesting that amino acid differences in the rest of GatA may reduce the <i>K</i><sub>m</sub> for Asp-tRNA<sub>Asn</sub>; however, the significant reduction of the <i>k</i><sub>cat</sub> of GatCAB<sub>BRO</sub> results in a significant decrease in the specificity constant. We conclude that the C terminal of GatA is important for the <i>k</i><sub>cat</sub> of Asp-tRNA<sub>Asn</sub> transamination (Table 1).

A proposed model for GatA evolution with the bro insertion into the gatCAB operon

We analysed the 12 new genome sequences of geographically and phenotypically diverse clinical isolates of <i>M. catarrhalis</i> (Davie et al., 2011), and found that the bro gene insertion into the gatCAB operon has affected the gatA C-terminal sequences of GatA(479–492)<sub>BRO</sub>, with several amino acid substitutions in the GatA<sub>wt</sub> sequence. Two closely related types of bro genes have been described based on a difference in their isoelectric focusing patterns attributable to a single amino acid residue difference at position 293, Asp in BRO-1 and Gly in BRO-2 (Bootsma et al., 1996). Eight of the 12 <i>M. catarrhalis</i> genome sequences had bro-1 sequences, and three genomes had bro-2 sequences; one genome was wild-type (sensitive). All eight bro-1 strains had identical GatA<sub>wt</sub> C-terminal sequences (479–492); the three bro-2 strains had a sequence different from the bro-1 strains but were identical to each other. The GatA<sub>BRO-2</sub> C-terminal ends were closer to GatA<sub>wt</sub> C-terminal ends (43 % identity) than the GatA<sub>BRO-1</sub> C-terminal ends (36 % identity) (Fig. 1). This supports the hypothesis of Bootsma et al. (2000) that bro-2 arrived first and evolved into bro-1 by a point mutation. Strains carrying bro-1 then obtained a selective advantage by other mutations involving the bro promoter, and were disseminated by lateral transfer by transformation rather than by clonal expansion (Bootsma et al., 2000). The events surrounding the ‘hijacking’ of what turns out to be the Asp-tRNA<sub>Asn</sub> AdT (Asp-AdT) operon by bro altered the C terminal of the GatA subunit, and thus the kinetic parameters of AdT, resulting in an increased specificity for glutamine and a decreased specificity for Asp-tRNA<sub>Asn</sub> in the transamination reaction. While these changes in the AdT may have slightly decreased overall fitness, the effect would be minor relative to the advantage, in clinical settings, of the β-lactam resistance conferred by bro, resulting in the current predominance of resistant strains.

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