Natural insertion of the bro-1 β-lactamase gene into the gatCAB operon affects Moraxella catarrhalis aspartyl-tRNA<sub>Asn</sub> amidotransferase activity

Pierre-Marie Akochy,1,2,3 Jacques Lapointe1,4 and Paul H. Roy1,3

*Correspondence*  
Paul H. Roy  
paul.roy@crchul.ulaval.ca

1Département de Biochimie, de Microbiologie et de Bio-informatique, Université Laval, QC G1V 0A6, Canada  
2Institut Pasteur de Côte d’Ivoire, 01 BP 490 Abidjan, Côte d’Ivoire  
3Centre de Recherche en Infectiologie, CHUQ Pavillon CHUL, 2705 boul. Laurier, RC-709, QC G1V 4G2, Canada  
4Institut de biologie intégrative et des systèmes (IBIS), Pavillon Charles-Eugène-Marchand, G1V 0A6, Canada

Only about half of bacterial species use an asparaginyl-tRNA synthetase (AsnRS) to attach Asn to its cognate tRNA<sup>Asn</sup>. Other bacteria, including the human pathogen *Moraxella catarrhalis*, a causative agent of otitis media, lack a gene encoding AsnRS, and form Asn-tRNA<sup>Asn</sup> by an indirect pathway catalysed by two enzymes: first, a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) catalyses the formation of aspartyl-tRNA<sup>Asn</sup> (Asp-tRNA<sup>Asn</sup>); then, a tRNA-dependent amidotransferase (GatCAB) transamidates this ‘incorrect’ product into Asn-tRNA<sup>Asn</sup>. As *M. catarrhalis* has a Gln-tRNA synthetase, its GatCAB functions as an Asp-tRNA<sup>Asn</sup> 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 GatA<sub>wt</sub> (residues 479–491) and of GatA<sub>BRO-1</sub> (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)<sub>wt</sub>(479–492)<sub>BRO-1</sub> and truncated GatCAB enzymes of *M. catarrhalis* showed that the substitution in GatA<sub>wt</sub> of residues 479–492 of GatA<sub>BRO-1</sub> causes increased specificity for glutamine, and decreased specificity for Asp-tRNA<sup>Asn</sup> in the transamidation reaction. We conclude that the bro gene insertion has altered the kinetic parameters of Asp-tRNA<sup>Asn</sup> 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-tRNA<sup>Gln</sup> (Gln-tRNA<sup>Gln</sup>) and asparaginyl-tRNA<sup>Asn</sup> (Asn-tRNA<sup>Asn</sup>) via an indirect pathway by the GatCAB-mediated transamidation of glutamyl-tRNA<sup>Gln</sup> (Glu-tRNA<sup>Gln</sup>) or aspartyl-tRNA<sup>Asn</sup> (Asp-tRNA<sup>Asn</sup>) formed by a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) or a non-discriminating aspartyl-tRNA synthetase (ND-AspRS), respectively (Becker & Kern, 1998; Curnow *et al.*, 1996; Lapointe *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<sup>Asn</sup> and Glu-tRNA<sup>Gln</sup> exist in vivo (Becker *et al.*, 2000; Curnow *et al.*, 1997; Raczniak *et al.*, 2001; Tumbula *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 *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-tRNAGln (Curnow et al., 1997; Horiuuchi et al., 2001; Sheppard et al., 2007, 2008; reviewed by Huot et al., 2010).

Bacterial GatCABs catalyse three distinct reactions during the transamination process: they activate Asp-tRNAAsn by phosphorylating the beta-carboxyl group of the aspartyl residue linked to tRNAAsn; they use NH₄⁺ directly or hydrolyse the amide group of Gln or Asn to release ammonia, and finally they amidate the activated Asp-tRNAAsn intermediate into Asn-tRNAAsn (Decicco et al., 2001; Feng et al., 2005; Horiuuchi 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 (Bootsm a 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 et al., 1990); less frequently, the resistance is altered the kinetic parameters of this Asp-tRNAAsn 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 GatAwt by the corresponding GatABRO-1 residues 479–492 causes an increased specificity for Gln, and a decreased specificity for Asp-tRNAAsn in the transamination reaction. We also found that GatCABBRO-1 shows a reduced rate of Asp-tRNAAsn transamination compared with GatCABwt (kcat 0.27 vs 1.3 s⁻¹). We conclude that the *bro-1* gene insertion has altered the kinetic parameters of this Asp-tRNAAsn 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)BRO-2 sequences, eight identical GatA(479–492)BRO-1 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)BRO-1 is 36% (5 residues of 14); the identity of GatA(478–491)wt versus GatA(478–492)BRO-2 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). [14C]Asp (207 mCi mmol⁻¹; 7.66 GBq mmol⁻¹), [14C]Gln (258 mCi mmol⁻¹; 9.55 GBq mmol⁻¹) and [α-32P]ATP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) were purchased from PerkinElmer Life and Analytical Sciences. [14C]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 cellulose, poly(ethyleneimine) (PEI) matrix (Sigma-Aldrich) and chitin agarose beads were from New England BioLabs. Bio-Spin 30 columns were from GE Health Care; high-purity L-Glu, L-Asp and L-chitin agarose beads were from New England BioLabs. Bio-Spin 30 cellulose, poly(ethyleneimine) (PEI) matrix (Sigma-Aldrich) and chitin agarose beads were from New England BioLabs.

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 fresh colonies in LB medium at 37°C (ampicillin-sensitive) and ATCC53279 (ampicillin-resistant) were M. catarrhalis. Cultures of the GlnRS and AsnRS activity measurements in a crude extract of M. catarrhalis were grown from single fresh colonies in LB medium at 37°C. Gln RS and Asn RS were from Sigma Aldrich. Phenol was from LabMat. The CCA-adding enzyme of Escherichia coli was from Invitrogen. 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).

The tRNA aminoacylation activities were measured at 37°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’-AAATTCCGGGTATAGCGCAATTTGTTGGAATGGCTCATGGTATCC-CTGACATTGGGTACGATCAGCTACCTCGGCACTGCA- A-3’ and 5’-GTGGCAGAGGTAGCTGGACTCGAACCAACGGATGT- GCCAGATCAAAACCTGATGCCTTACCAACTTGGCGATACCC- CG-3’, and the gene was inserted into the sites EcoRI/PstI of pGFB to create the pGFB/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 its N-terminal. On the other hand, because we had to use a partly different strategy to express gatCABs (see below), GatAsn has the additional residues EFLEGSS at its C-terminal. GatA BRO (see below), GatAsn has the additional residues EFLEGSS at its C-terminal, and GatA BRO because of its affinity for these proteins.

gatCABwt. 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’-GGGAATTCATGACCATGAC-3’ and 5’-TAATCCGGCTCATGGTATCCCTG-3’, and the operon was inserted into the EcoRI site of pTwin2 to generate pTwin2/gatCABwt (Fig. 2, line 1).

![Fig. 2. Cloning of various M. catarrhalis gatCAB oporons, and overproduction and purification of the three subunits. The M. catarrhalis gatCAB operons represented in this figure (1, gatCABwt; 2, gatCABh; 3, gatCA1Bwt; 4a, gatCA1BRO; 4b, gatCA1BRO; 5, gatCA1BRO) 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 OD₆₀₅ 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 NaHEPES, 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[^10] (see above) was [³²P]-labelled on its 3'-terminal phosphodiester link using the *E. coli* CCA-adding enzyme and [⁴⁻³²P]ATP as previously described (Sheppard et al., 2007, 2008), with some modifications as follows: briefly, 172 µM RNA[^10] 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 µl⁻¹ (43 nM) pure CCA-adding enzyme from *E. coli* (Cudny & Deutscher, 1986) and 1 µCi µl⁻¹ (37 kBq) [⁴⁻³²P]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 [⁴⁻³²P]ATP.

**Preparation of Asp-tRNA[^10].** Aspartylation of *M. catarrhalis* tRNA[^10] was carried out for 30 min at 37 °C in 50 mM NaHEPES, 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[^10], and 1.33 µM [⁴⁻³²P]-labelled *E. coli* CCA-adding enzyme (Cudny & Deutscher, 1986) and 1 µCi µl⁻¹ (37 kBq) [⁴⁻³²P]ATP were incubated at 37 °C, in LB medium containing about 20% of tRNA[^10], and 1 mM DTT, with nearly saturating concentrations of ATP (4 mM), and excess ATP was removed as described previously (Sheppard et al., 2007). Samples with only unlabelled tRNA[^10] were aminocylated in parallel and were used in the glutaminase assays. In place of checking the levels of aminocylisation as described elsewhere (Bullock et al., 2003), we checked the level of Asn-tRNA[^10] formation by measuring the amount of Asn formed in the transamidation reaction (see below) in the presence of a high concentration (4 nM) of GatCAB[^10].

**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[^10] (12.4 µM), and 7.5–240 µM [⁴⁻¹⁴C]Gln (232 mCi mmol⁻¹, 8.88 GBq mmol⁻¹). The concentrations of GatCAB[^10], GatCAB[^10], GatCAB[^10] and GatCAB[^10] were 2, 0.5, 1 and 20 nM, respectively. After various reaction times, 5 µl aliquots were transferred into 0.3 M sodium acetate, pH 5.0, to quench the reaction, and the tRNA was ethanol-precipitated. The supernatants containing [⁴⁻¹⁴C]Gln and [⁴⁻¹⁴C]Glu were dried and resuspended in water (3 µl). One microlitre 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.

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[^10]: *M. catarrhalis* strain ATCC25240, consisting of the full-length of gatC (nucleotides 1–1470) and gatA carrying a deletion of its 3' end (nucleotides 1435–1476). The fragment was amplified with olignucleotides 5'-GGAATTCATGACCATCACACCCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI site of pTwin2 to generate pTwin2/gatA.

[^10]: The truncated gatCA[^10] of *M. catarrhalis* strain ATCC25279 was amplified with oligonucleotides 5'-GGAATTCATGACCATCACACCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI site of pTwin2 and into the EcoRI site of pPBR322, which was inserted in the EcoRI/NheI sites of pTwin2 to form pTwin2/gatCA[^10].

[^10]: Attempts to clone gatCA[^10] as an operon failed several times, and we decided to clone gatCA[^10] and gatB[^10] separately. An active GatCA[^10] homozygous enzyme was easily formed when Gatto[^10] and Gatto[^10] were pooled together to yield a single band observed by non-denaturing PAGE. The full-length of gatC from the resistant *M. catarrhalis* strain ATCC35279 (bro-1-positive strain) was amplified with oligonucleotides 5'-GGAATTCATGACCATCACACCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI site of pTwin2 and into the EcoRI sites of pTwin2 to form pTwin2/gatCA[^10].

[^10]: Separately, the gatB gene from the resistant *M. catarrhalis* strain ATCC35279 was amplified with oligonucleotides 5'-GGAATTCATGACCATCACACCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI site of pTwin2 and into the EcoRI sites of pTwin2 to form pTwin2/gatB[^10], which was used for over-production of Gatto[^10].

[^10]: The truncated gatCA[^10] of *M. catarrhalis* strain ATCC35279 consisted of full-length gatCA[^10], and gatA carrying a deletion of its 3' end (nucleotides 1435–1476). The operon of the two fragments was amplified with oligonucleotides 5'-GGAATTCATGACCATCACACCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI/NheI sites of pTwin2 to generate pTwin2/gatCA[^10].

[^10]: The truncated gatCA[^10] of *M. catarrhalis* strain ATCC35279 consisted of full-length gatCA[^10], and gatA carrying a deletion of its 3' end (nucleotides 1435–1476). The operon of the two fragments was amplified with oligonucleotides 5'-GGAATTCATGACCATCACACCGGAG-3' and 5'-GGCTAGCTCATCATGTTGTAATG-3', and was inserted into the EcoRI/NheI sites of pTwin2 to generate pTwin2/gatCA[^10].

[^10]: 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[^10] (12.4 µM), and 7.5–240 µM [⁴⁻¹⁴C]Gln (232 mCi mmol⁻¹, 8.88 GBq mmol⁻¹). The concentrations of GatCAB[^10], GatCAB[^10], GatCAB[^10] and GatCAB[^10] were 2, 0.5, 1 and 20 nM, respectively. After various reaction times, 5 µl aliquots were transferred into 0.3 M sodium acetate, pH 5.0, to quench the reaction, and the tRNA was ethanol-precipitated. The supernatants containing [⁴⁻¹⁴C]Gln and [⁴⁻¹⁴C]Glu were dried and resuspended in water (3 µl). One microlitre 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 \(^{14}C\)Gln and \(^{14}C\)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 MgCl\(_2\), 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-tRNA\(^{\text{Asn}}\), initial velocities were measured in triplicate, while varying the concentration of Asp-tRNA\(^{\text{Asn}}\). Reactions were carried out over 45 s using purified GatCAB\(_{\text{wt}}\), GatCAB\(_{\text{h}}\), GatCA\(_B\), GatCA\(_B\)\(_{\text{BRO}}\) and GatCA\(_B\)\(_{\text{BRO}}\) 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 x 20 cm TLC plates. To separate asparaginyl-AMP (Asn-AMP) from Asp-AMP and AMP, 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-tRNA\(^{\text{Asn}}\) 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, \(\text{bro}\)-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 \(\text{bro}\) and has disseminated the \(\text{bro}\) 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...
of GatCAB and GlnRS coupled with the lack of AsnRS suggests an in vivo function of GatCAB as an Asp-tRNA<sub>Asn</sub> 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<sub>Gln</sub>, and of the indirect formation of Asn-tRNA<sub>Asn</sub>, 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<sub>Gln</sub> is formed by the direct pathway, and Asn-tRNA<sub>Asn</sub> by the indirect pathway, indicating that the GatCAB AdTs of bro-positive and bro-negative <i>M. catarrhalis</i> function as Asp-tRNA<sub>Asn</sub>-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 (<i>GatA<sub>wt</sub></i>) and the GatA of the resistant strain (<i>GatA<sub>BRO</sub></i>). The C-terminal sequences of <i>GatA<sub>479–491</sub></i><sub>wt</sub> and <i>GatA<sub>479–492</sub></i><sub>BRO</sub> are only 36 % identical, suggesting that the DNA sequence encoding the <i>GatA<sub>479–492</sub></i><sub>BRO</sub> 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>gatA</i> operons carrying the various versions of <i>gatA</i> were made successfully except for <i>gatCA<sub>BRO</sub></i> and <i>gatB<sub>BRO</sub></i>. Attempts to clone <i>gatCA<sub>BRO</sub></i> as a unique fragment into the pTwin2 vector failed. However, clones of <i>gatCA<sub>BRO</sub></i> and of <i>gatB<sub>BRO</sub></i> were made separately (Fig. 2, lines 4a and 4b), and GatCA<sub>BRO:-1</sub> and GatB<sub>BRO:-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 GatCA<sub>wt</sub> and GatCA<sub>h</sub> have the same levels of glutaminase and of Asp-tRNA<sub>Asn</sub> transamidation activities**

To test the transamidination activity of <i>M. catarrhalis</i> GatCAB variants, we used a recently developed [32P]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<sub>Asn</sub> overproduced in <i>E. coli</i> (see Methods) was used to prepare Asp-tRNA<sub>Asn</sub> 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<sub>Asn</sub> to Asn-tRNA<sub>Asn</sub>. We constructed a GatCAB hybrid (GatCA<sub>BRO</sub>) that consists of a GatCA<sub>wt</sub> in which GatA(479–491)<sub>wt</sub> is replaced by GatA(479–492)<sub>BRO</sub>. We found that GatCA<sub>wt</sub> was as active as GatCA<sub>BRO</sub> in glutaminase activity (<i>k<sub>cat</sub></i>=11.3 and 14.7 s<sup>-1</sup>, respectively) (Fig. 3, Table 1), and in transamidation of Asp-tRNA<sub>Asn</sub> (<i>k<sub>cat</sub></i>=1.3 and 1.05 s<sup>-1</sup>, respectively) (Fig. 4, Table 1). These two <i>k<sub>cat</sub></i> values are similar to that of <i>H. pylori</i> GatCAB, 1.3 s<sup>-1</sup> (Sheppard et al., 2007). The <i>K<sub>m</sub></i> value of GatCA<sub>wt</sub> for Asp-tRNA<sub>Asn</sub> (1.67 μM) resembles that of <i>H. pylori</i> GatCAB (1.12 μM) for Asp-tRNA<sub>Asn</sub> (Fig. 4, Table 1) (Fischer et al., 2012).

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

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

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

GatCA<sub>BRO</sub> is as active as GatCA<sub>h</sub> and GatCA<sub>wt</sub> in glutaminase activity (<i>k<sub>cat</sub></i>=13.1, 14.7 and 11.3 s<sup>-1</sup>, respectively) (Fig. 3, Table 1). In contrast, the GatCA<sub>BRO</sub> transamidation of Asp-tRNA<sub>Asn</sub> (<i>k<sub>cat</sub></i>=0.27 s<sup>-1</sup>) is lower than those of the wild-type and hybrid enzymes (<i>k<sub>cat</sub></i>=1.3 and 1.05 s<sup>-1</sup>, respectively) (Fig. 4, Table 1). The low <i>k<sub>cat</sub></i> value of GatCA<sub>BRO</sub> is similar to that of <i>H. pylori</i> GatCAB, at 0.25 s<sup>-1</sup> (Fischer et al., 2012).
**DISCUSSION**

This study aimed to determine the impact of the amino acid substitutions in the C-terminal part (residues 479–492) of GatABRO caused by the lateral insertion, has a 5.6-fold higher specificity constant for Gln (1256 s⁻¹ M⁻¹) than that of GatCABwt (223 s⁻¹ M⁻¹) representing the ancestral enzyme. In contrast, truncation of the C-terminal sequence results in an increased $K_m$ for Gln (74.4 μM) and a reduced specificity constant for Gln of 80 s⁻¹ M⁻¹, demonstrating that GatA(479–492)BRO-1 is important for Gln specificity. However, the few amino acid differences present in the rest of GatA(479–492)BRO-1 in the whole GatCAB BRO-1 (555 s⁻¹ M⁻¹) are likely to lower this specificity for Gln without affecting its $k_{cat}$ 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 GatCABwt.

No GatCAB structure from a mesophilic Gram-negative species has been reported to date. Only three structures of GatCAB are available: for a Gram-positive bacterium and two hyperthermophilic bacteria (Nakamura et al., 2006; Wu et al., 2009; Ito & Yokoyama, 2010). According to the structure of GatCAB of *Staphylococcus aureus*, which was established without its Asp-tRNA⁺⁻⁻⁻⁻⁻ substrates, 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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**Table 1.** Kinetic constants for Gln and Asp-tRNA⁺⁻⁻⁻⁻⁻ of GatCAB AdTs from *M. catarrhalis* strains ATCC25240 and ATCC53279, and from several variants

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glutaminase activity</th>
<th>Transamidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM ± SD)</td>
<td>$k_{cat}$ (s⁻¹ ± SD)</td>
</tr>
<tr>
<td>GatCABwt*</td>
<td>50.6 ± 23.4</td>
<td>11.3 ± 2.48</td>
</tr>
<tr>
<td>GatCABb†</td>
<td>11.7 ± 8.13</td>
<td>14.7 ± 2.68</td>
</tr>
<tr>
<td>GatCABBRO‡</td>
<td>74.4 ± 30.4</td>
<td>6.02 ± 0.9</td>
</tr>
<tr>
<td>GatCABBRO-1§</td>
<td>23.6 ± 9.48</td>
<td>13.1 ± 1.99</td>
</tr>
<tr>
<td>GatCABBRO-II‖</td>
<td>32.7 ± 3.05</td>
<td>6.9 ± 0.17</td>
</tr>
</tbody>
</table>

*GatCABwt* is from the *bro*-negative strain *M. catarrhalis* ATCC25240, which is sensitive to ampicillin.
†GatCABb is the variant corresponding to GatCABwt in which amino acid residues GatA(479–491)wt have been replaced by the homologous segment GatA(479–492)bro.
nGatCABBRO is the variant from GatCABwt in which GatA(479–491)wt was deleted.
§GatCABBRO-1 is from the *bro*-positive strain *M. catarrhalis* ATCC53279, which is resistant to ampicillin.
‖GatA(479–492)BRO-1 is the variant from GatCABbro in which the protein was replaced by the homologous segment GatA(479–492)bro sequence was deleted.

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Removing residues 479–491 of GatAwt and 479–492 of GatABRO results in lower glutaminase activity of GatCA_Bwt and of GatCA_BRO

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

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Substitution of GatA(479–492)BRO-1 for the homologous sequence in *M. catarrhalis* GatCABwt increases its specificity for Gln

The remarkable role of the GatA(479–492)BRO-1 sequence was shown when we compared the kinetic parameters of the glutaminase activity of the GatCABwt and GatCAb enzymes. The GatCAb enzyme, which represents the presumed initial state after bro insertion, has a 5.6-fold higher specificity constant for Gln (1256 s⁻¹ M⁻¹) than that of GatCABwt (223 s⁻¹ M⁻¹) representing the ancestral enzyme. In contrast, truncation of the C-terminal sequence results in an increased $K_m$ for Gln (74.4 μM) and a reduced specificity constant for Gln of 80 s⁻¹ M⁻¹, demonstrating that GatA(479–492)BRO-1 is important for Gln specificity. However, the few amino acid differences present in the rest of GatA BRO-1 in the whole GatCAB BRO-1 (555 s⁻¹ M⁻¹) are likely to lower this specificity for Gln without affecting its $k_{cat}$ 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 GatCABwt.
interaction of GatCAB and its Asp-tRNA\textsuperscript{Asn} 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)\textsuperscript{BRO} for the homologous sequence in \textit{M. catarrhalis} GatA(479–491)\textsubscript{wt} decreases its specificity for Asp-tRNA\textsuperscript{Asn}

Another remarkable role of the GatA(479–492)\textsuperscript{BRO} sequence was shown when we compared the kinetic parameters of the transmigration activity of GatCAB\textsubscript{wt} and GatCAB\textsubscript{BRO}. We showed that the GatCAB\textsubscript{BRO} enzyme (as the initial state of GatCAB, right after the insertion of \textit{bro} into the catGAB operon) has a higher $K_m$ for Asp-tRNA\textsuperscript{Asn} ($K_m$ of 5.22 \textmu M) compared with GatCAB\textsubscript{wt}, considered as the ancestral enzyme ($K_m$ of 1.67 \textmu M). The GatA(479–492)\textsuperscript{BRO} sequence may be involved in increasing the $K_m$ for Asp-tRNA\textsuperscript{Asn}. Truncating this sequence reduced the $K_m$. Interestingly, a comparison of the kinetic parameters between GatCAB\textsubscript{BRO} and GatCAB\textsubscript{BRO} showed an intermediate $K_m$ value of 2.08 \textmu M for Asp-tRNA\textsuperscript{Asn} for the latter, lower than that of GatCAB\textsubscript{BRO}, but higher than that of GatCAB\textsubscript{wt}, suggesting that amino acid differences in the rest of GatA may reduce the $K_m$ for Asp-tRNA\textsuperscript{Asn}, however, the significant reduction of the $k_{cat}$ of GatCABBRO results in a significant decrease in the specificity constant. We conclude that the C terminal of GatA is important for the $k_{cat}$ of Asp-tRNA\textsuperscript{Asn} transamidation (Table 1).

A proposed model for GatA evolution with the \textit{bro} insertion into the \textit{gatCAB} operon

We analysed the 12 new genome sequences of geographically and phenotypically diverse clinical isolates of \textit{M. catarrhalis} (Davie et al., 2011), and found that the \textit{bro} gene insertion into the gatCAB operon has affected the gatA C-terminal sequences of GatA(479–492)\textsuperscript{BRO}, with several amino acid substitutions in the GatA\textsubscript{wt} sequence. Two closely related types of \textit{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 \textit{BRO-1} and Gly in \textit{BRO-2} (Bootma et al., 1996). Eight of the 12 \textit{M. catarrhalis} genome sequences had \textit{bro-1} sequences and three genomes had \textit{bro-2} sequences; one genome was wild-type (sensitive). All eight \textit{bro-1} strains had identical GatA C-terminal sequences (479–492); the three \textit{bro-2} strains had a sequence different from the \textit{bro-1} strains but were identical to each other. The GatA\textsubscript{BRO-2} C-terminal ends were closer to GatA\textsubscript{wt} C-terminal ends (43 % identity) than the GatA\textsubscript{BRO-1} C-terminal ends (36 % identity) (Fig. 1). This supports the hypothesis of Bootma et al. (2000) that \textit{bro-2} arrived first and evolved into \textit{bro-1} by a point mutation. Strains carrying \textit{bro-1} then obtained a selective advantage by other mutations involving the \textit{bro} promoter, and were disseminated by lateral transfer by transformation rather than by clonal expansion (Bootma et al., 2000).

The events surrounding the ‘hijacking’ of what turns out to be the Asp-tRNA\textsuperscript{Asn} AdT (Asp-AdT) operon by \textit{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\textsuperscript{Asn} in the transamidation 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 \beta-lactam resistance conferred by \textit{bro}, resulting in the current predominance of resistant strains.

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