Induction of β-Lactamase Activity and Decreased β-Lactam Susceptibility by CO₂ in Clinical Bacterial Isolates

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ABSTRACT Antimicrobial susceptibility testing of clinical isolates is a crucial step toward appropriate treatment of infectious diseases. The clinical isolate Francisella philomiragia 14IUHPL001, recently isolated from a 63-year-old woman with atypical pneumonia, featured decreased susceptibility to β-lactam antibiotics when cultivated in 5% CO₂. Quantitative β-lactamase assays demonstrated a significant (P < 0.0001) increase in enzymatic activity between bacteria cultivated in 5% CO₂ over those incubated in ambient air. The presence of β-lactamase genes bla TEM and bla SHV was detected in the clinical isolate F. philomiragia 14IUHPL001 by PCR, and the genes were positively identified by nucleotide sequencing. Expression of bla TEM and bla SHV was detected by reverse transcription-PCR during growth at 5% CO₂ but not during growth in ambient air. A statistically significant alkaline shift was observed following cultivation of F. philomiragia 14IUHPL001 in both ambient air and 5% CO₂, allowing desegregation of the previously reported effects of acidic pH from the currently reported effect of 5% CO₂ on bla TEM and bla SHV β-lactamases. To ensure that the observed phenomenon was not unique to F. philomiragia, we evaluated a clinical isolate of bla TEM-carrying Haemophilus influenzae and found parallel induction of bla TEM gene expression and β-lactamase activity at 5% CO₂ relative to ambient air.

IMPORTANCE β-Lactamase induction and concurrent β-lactam resistance in respiratory tract pathogens as a consequence of growth in a physiologically relevant level of CO₂ are of clinical significance, particularly given the ubiquity of TEM and SHV β-lactamase genes in diverse bacterial pathogens. This is the first report of β-lactamase induction by 5% CO₂.

KEYWORDS BLA, Francisella, Haemophilus, SHV, TEM, antimicrobial resistance, β-lactamases, carbon dioxide

Members of the genus Francisella are aerobic Gram-negative coccobacilli, and many species are known to be professional pathogens or opportunists of many animal species (1–4). Francisella philomiragia is an uncommon pathogen of humans, having primarily been reported in near-drowning victims and individuals with chronic granulomatous disease (3). F. philomiragia typically infects the lower respiratory tract but has also been reported to cause septicemia and meningitis (3). A recent case involving a 63-year-old woman who presented with shortness of breath, nonproductive cough, and bilateral peripheral edema yielded the isolate F. philomiragia strain 14IUHPL001 (1). Antimicrobial susceptibility testing of this isolate was conducted in ambient air and 5% CO₂ atmospheres. Interestingly, this bacterium became less susceptible to β-lactam antibiotics when incubated in an atmosphere enriched with 5% CO₂.
TABLE 1 Antimicrobial susceptibility of Francisella philomiragia under different atmospheric conditions

| Antimicrobial agent(s) | MIC (μg/ml) for: | 14IUHPL001 | FSC144T | 14IUHPL001 | FSC144T |
|------------------------|-----------------|------------|---------|------------|---------|
|                        | Ambient air | CO₂ | Ambient air | CO₂ |
| Amikacin               | ≤0.5        | ≤0.5 | ≤0.5        | ≤0.5 |
| Amoxicillin-clavulanic acid | 1      | 16 | 1          | 4   |
| Ampicillin             | 32          | >32 | 32         | >32 |
| Aztreonam              | 4           | 32  | 2          | 8   |
| Cefepime               | 4           | 32  | 1          | 8   |
| Cefazidime             | ≤0.5        | ≤0.5 | ≤0.5        | ≤0.5 |
| Ceftriaxone            | ≤0.5        | ≤0.5 | ≤0.5        | ≤0.5 |
| Cefazolin              | 2           | >32 | 16         | >32 |
| Ciprofloxacin          | ≤0.25       | ≤0.25 | ≤0.25       | ≤0.25 |
| Colistin               | >8          | >8  | >8         | >8   |
| Doripenem              | ≤0.25       | 1   | ≤0.25       | 0.5  |
| Doxycycline            | ≤1          | 1   | ≤1         | ≤1   |
| Ertapenem              | ≤0.25       | 1   | ≤0.25       | ≤0.25 |
| Gentamicin             | ≤0.5        | ≤0.5 | ≤0.5        | ≤0.5 |
| Imipenem               | ≤0.25       | 0.5  | ≤0.25       | ≤0.25 |
| Levofloxacin           | ≥2          | ≥2  | ≥2         | ≥2   |
| Meropenem              | ≤0.25       | 0.5  | ≤0.25       | ≤0.25 |
| Moxifloxacin           | ≤0.25       | ≤0.25 | ≤0.25       | ≤0.25 |
| Oxacillin              | ≤0.25       | >16  | >16        | >16   |
| Polymyxin B            | >4          | >4  | >4         | >4   |
| Ticarcillin-clavulanic acid | ≤4     | ≤4 | ≤4         | ≤4   |
| Tigecycline            | ≤0.25       | ≤0.25 | ≤0.25       | ≤0.25 |
| Tobramycin             | ≤0.5        | ≤0.5 | ≤0.5        | ≤0.5 |
| Trimethoprim-sulfamethoxazole | >4     | >4  | >4         | >4   |

Known mechanisms of reduced susceptibility to β-lactams include lack of a cell wall, alterations in penicillin-binding proteins, or production of β-lactamase (5). β-Lactamases hydrolyze the β-lactam ring that is present in all β-lactam antibiotics and rapidly degrade the molecule (6). A small number of extended-spectrum β-lactamases, including TEM-1, TEM-6, TEM-10, and SHV, have been suggested previously to be regulated by CO₂ or pH (7–9). These enzymes’ genes are typically found on plasmids but can also be integrated into the bacterial genome. In order to determine the mechanism of CO₂-derived susceptibility changes in F. philomiragia 14IUHPL001, we sought to characterize β-lactamase activity phenotypically, genotypically, and transcriptionally in ambient air and 5% CO₂.

RESULTS

Antimicrobial susceptibility. F. philomiragia strain 14IUHPL001 generally became less susceptible to β-lactams when incubated in 5% CO₂ (Table 1). F. philomiragia FSC144T was 8-fold more susceptible to cefepime when incubated in ambient air than CO₂. F. philomiragia 14IUHPL001, most notably, was 64-fold more susceptible to oxacillin (Table 1). In both cases, the susceptibility of F. philomiragia strains 14IUHPL001 and FSC144T demonstrated a trend toward β-lactam resistance when incubated with 5% CO₂, which warranted further investigation.

β-Lactamase activity. Nitrocefin disk testing confirmed the presence of β-lactamase activity. F. philomiragia FSC144T incubated in 5% CO₂ had a 1.5-fold increase in β-lactamase activity compared to atmospheric air (P < 0.01). In comparison, F. philomiragia strain 14IUHPL001 incubated with CO₂ had a 2.4-fold increase in β-lactamase activity compared to atmospheric air (P < 0.0001). When comparing both strains of F. philomiragia that were incubated with CO₂, F. philomiragia 14IUHPL001 had a 1.5-fold increase in β-lactamase activity over F. philomiragia FSC144T (P < 0.01). There was no significant difference between F. philomiragia FSC144T and 14IUHPL001 incubated in atmospheric air (Fig. 1A). Haemophilus influenzae IUH9 incubated in 5% CO₂ was significantly increased compared to H. influenzae IUH9 incubated in ambient air and
H. influenzae 8143T incubated in 5% CO₂ (P < 0.0001). No significant differences were observed between H. influenzae 8143T incubated in ambient air versus 5% CO₂ or 8143T versus IUH9 incubated in ambient air (Fig. 1B).

blaTEM and blaSHV amplification. Primers designed to amplify blaTEM generated the predicted product of 850 bp in F. philomiragia 14IUHPL001 and in the type strain F. philomiragia FSC144 but failed to detect the gene in either strain of H. influenzae (Fig. 2). Primers designed to amplify blaSHV generated the predicted 768-bp product in F. philomiragia 14IUHPL001 and H. influenzae IUH9 but not in either type strain (Fig. 2). F. philomiragia 14IUHPL001 and FSC144T produced blaTEM transcript in 5% CO₂ but not in ambient air. Similarly, F. philomiragia 14IUHPL001 and H. influenzae IUH9 produced blaSHV transcript in 5% CO₂ but not in ambient air. As expected, no blaTEM transcript was detected in H. influenzae IUH9 or 8143T, and no blaSHV transcript was detected in H. influenzae 8143T or F. philomiragia FSC144T, regardless of atmospheric conditions (Table 2).

Nucleotide sequencing and phylogenetic analysis. Sequencing of the F. philomiragia-derived blaTEM amplicons indicated that strains 14IUHPL001 and FSC144T are carrying an identical copy of the gene. The derived sequence identified it as a member of the TEM family of β-lactamase genes (GenBank accession no. KT781076). Sequencing of the 768-bp blaSHV product from both F. philomiragia 14IUHPL001 and H. influenzae IUH9 showed 100% identity with that of Klebsiella pneumoniae KPNIH27. While the F. philomiragia-derived blaTEM had a unique nucleotide sequence, phylogenetic analysis indicates that it clearly still belongs to the TEM family of β-lactamase genes (Fig. 3).

pH measurements. Following incubation, a significant alkaline shift in pH was observed for both F. philomiragia 14IUHPL001 and the type strain, FSC144, when incubated in CO₂ or ambient air (Fig. 4) (P < 0.0001). Differences in pH between the CO₂ and atmospheric air incubation methods were not significant for F. philomiragia FSC144T and F. philomiragia 14IUHPL001. In situ measurements of pH indicated that rapid changes did not occur. Our analysis of this phenomenon with F. philomiragia naturally decouples the effects of atmospheric CO₂ and acidic pH on blaTEM activity levels and thus β-lactam susceptibility.
Changes in susceptibility to β-lactam antibiotics were observed for F. philomiragia strain 14IUHPL001 when grown under different atmospheric conditions. Growth in 5% CO₂ resulted in significant (P < 0.05) deviations from the MIC in ambient air of β-lactam antibiotics (Table 1). There are no established interpretative criteria for any of the antimicrobials tested versus F. philomiragia, limiting our ability to discuss clinically relevant breakpoints; however, the magnitude of susceptibility decrease is substantial and in all likelihood meaningful during disease. The MICs of other antimicrobial classes did not change regardless of incubation conditions, indicating a CO₂-inducible change uniquely impacting β-lactam–F. philomiragia interactions.

The presence of β-lactamase was qualitatively detected in both F. philomiragia strain 14IUHPL001 and the type strain, FSC144, grown in either ambient air or 5% CO₂ with nitrocefin disks. Quantitative, colorimetric β-lactamase activity assays for both strains of F. philomiragia resulted in a significant increase in β-lactamase expression of blaTEM and blaSHV under different atmospheric conditions.

### Table 2

| Gene and condition | Expression of gene under condition showna |
|-------------------|------------------------------------------|
|                   | F. philomiragia | H. influenzae |
|                   | 14IUHPL0001 | FSC144T | IUH9 | 8143T |
| 5% CO₂            |               |         |      |      |
| blaTEM            | +             | +       | –     | –     |
| blaSHV            | +             | –       | +     | –     |
| Ambient air       |               |         |      |      |
| blaTEM            | –             | –       | –     | –     |
| blaSHV            | –             | –       | –     | –     |

aThe symbols “+” and “–” indicate the presence or absence, respectively, of RNA/cDNA amplification by reverse transcription-PCR.
activity level when incubated in 5% CO₂. *F. philomiragia* strain 14IUHPL001 had a 2.4-fold increase in β-lactamase activity when incubated in 5% CO₂ compared to atmospheric air (P < 0.0001). The type strain, FSC144, also had increased β-lactamase activity in 5% CO₂, but the effect was less striking (1.5-fold change; P <
These findings are consistent with the decrease in β-lactam susceptibility that was observed for both strains of F. philomiragia in the presence of 5% CO₂ (Table 1) and indicate that the organisms are expressing β-lactamase genes that are inducible by CO₂. Previous reports have described changes in penicillin and piperacillin susceptibility of Escherichia coli isolates carrying the β-lactamases TEM-1, TEM-2, and SHV at elevated CO₂ levels or acidic pH (7). Examination of blaTEM family sequences in public databases indicates a very high level of nucleotide identity across diverse Gram-negative and Gram-positive species, indicating that it is horizontally transferred and strictly conserved. Identity searches using the BLAST algorithm (10) further confirmed that the nomenclature of many TEM family β-lactamases is highly redundant (>98% identity across all TEM identifiers), and the TEM identifiers 1 and 2 (100% identity) are not indicative of distinct alleles. However, point mutations within blaTEM have been used epidemiologically to track both outbreak strains and horizontal transfer to new species (11). Regardless of the specific allele, phylogenetic analysis clearly indicates that one of the β-lactamase genes detected in F. philomiragia 14IUHPL001 belongs to the blaTEM family (Fig. 3).

The presence of a blaTEM β-lactamase gene in F. philomiragia 14IUHPL001 was confirmed by PCR with blaTEM-specific primers and sequencing of the resulting product (Fig. 2 [GenBank accession no. KT781076]). The same gene was amplified from the type strain of F. philomiragia. While blaTEM was not annotated in the FSC144T genome (12), the gene was detected from extracted plasmid DNA and therefore not represented on the chromosome. Unrelated chromosomal β-lactamase genes are annotated, however. Although F. philomiragia is reported to carry a cryptic plasmid, pFPHlo1, no β-lactamase genes are carried on this plasmid (12). Although blaTEM expression likely explains increases in β-lactamase activity in F. philomiragia 14IUHPL001 and FSC144T relative to growth in ambient air, it fails to explain the significant difference in β-lactamase activities between the two strains at 5% CO₂ (Fig. 1A). We then interrogated 14IUHPL001 and FSC144 for blaSHV, another β-lactamase gene that has been reported to be affected by CO₂ (7–9). The blaSHV gene was detected in 14IUHPL001 but not FSC144T (Fig. 2). The presence of an additional CO₂-regulated β-lactamase gene is likely responsible for the significant difference in activities found.

To ensure that the observed effect was not unique to F. philomiragia, quantitative β-lactamase activity was measured for the blaSHV-bearing H. influenzae strain IUH9 as well. As observed for F. philomiragia, H. influenzae IUH9 produced significantly more β-lactamase activity in 5% CO₂ relative to (i) growth in ambient air and (ii) the blaSHV-deficient type strain, 8143, under either atmospheric condition. Taken together, these data indicate that at least blaSHV is associated with elevated β-lactamase activity at 5% CO₂ regardless of the bacterial species producing the protein.

Given the promiscuity of TEM and SHV β-lactamases and the reported differential β-lactam susceptibility of Escherichia coli strains harboring TEM β-lactamases in elevated CO₂, F. philomiragia strain 14IUHPL001 was positively interrogated for blaTEM. Livermore and Corkill also reported decreased β-lactam susceptibility in acidic pH (9). In order to distinguish the effects of atmospheric CO₂ from drops in pH during incubation, we measured the change in pH (ΔpH) generated by 14IUHPL001 and FSC144T following incubation in either ambient air or 5% CO₂. Both 14IUHPL001 and FSC144T generated a net positive ΔpH (alkaline shift) relative to uninoculated, contemporarily incubated media in both atmospheric conditions. There was no significant difference in pH values between uninoculated growth media incubated in 5% CO₂ and atmospheric air (Fig. 4). β-Lactamase induction at 5% CO₂ in the absence of an acid shift is a critical finding given the inherent clinical significance of this activity in a respiratory pathogen as a consequence of growth in 5% CO₂ directly. The atmospheric conditions at the alveolar surface are not directly comparable to ambient air (partial CO₂ pressure [pCO₂] of 0). When the atmospheric gases equilibrate with the blood in the alveoli, the alveolar pCO₂ elevates to 40 mm Hg, or approximately 5.3%. If induction of blaTEM was secondary to acid production during laboratory incubation, the clinical relevance of this
finding would still remain only partially defined. Our findings indicate a direct effect of a physiologically relevant level of atmospheric CO₂ independent of acidic pH on the blaTEM-derived β-lactamase activity level, and thus β-lactam susceptibility during infection. Antimicrobial susceptibility testing (AST) performed under standard conditions (i.e., ambient air) would indicate that bacterial pathogens carrying blaTEM are susceptible to β-lactam treatment. If such isolates are isolated from the lower respiratory tract, β-lactam treatment failures that would not be consistent with the reported AST results are predictable.

Antimicrobial susceptibility testing is essential for appropriate treatment decisions during bacterial infection. Previous reports have questioned the clinical significance of changes induced by CO₂ on β-lactamase activity (13). Our results clearly demonstrate the clinical relevance of CO₂ regulation of β-lactamase during lower respiratory tract infections. TEM and SHV family β-lactamases have been detected in numerous pathogens associated with infection of the lung, including Acinetobacter baumannii, Burkholderia cepacia, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Stenotrophomonas maltophilia, and Yersinia pestis. Our findings therefore suggest that standard best practices for antimicrobial susceptibility testing could include physiologically relevant conditions in the future.

**Materials and Methods**

**Strains and culture conditions.** Francisella philomiragia strains FSC144ᵀ (ATCC 25015) (4, 14) and 14IUHPL001 (1) were cultured on chocolate agar (Remel, Lenexa, KS) at 37°C in either ambient air or 5% CO₂. Bacteria were harvested from plates and resuspended in 1× phosphate-buffered saline (PBS). Following one PBS wash, the bacteria were pelleted, and the mass of each pellet was measured using an AG285 balance (Mettler Toledo, Columbus, OH). Haemophilus influenzae strains 8143 (ATCC 33391) and IUH9 were cultured on chocolate agar in either ambient air or 5% CO₂. Strain IUH9 was selected from a panel of clinical isolates because growth in ambient air was tolerated and molecular screening indicated that it carried blaTEM.

**Antimicrobial susceptibility testing.** F. philomiragia strains FSC144ᵀ and 14IUHPL001 were tested by broth microdilution using panels prepared in house with cation-adjusted Mueller-Hinton broth (CAMHB [Difco, BD Sparks, MD]), according to CLSI standards. Isolates were subcultured twice from frozen stocks on chocolate agar plates, and 5 colonies were picked and suspended in saline to achieve a concentration equivalent to a 0.5 McFarland standard (CLSI M07-A10; January 2015). Panels were inoculated in duplicate; one panel was incubated at 35°C for 24 h in ambient air, and the other panel was incubated at 35°C for 24 h in an atmosphere enriched with 5% CO₂. Antimicrobial susceptibility testing (AST) in both atmospheres was repeated once to gauge reproducibility.

**pH testing.** Quantitative measurement of growth medium pH postincubation was performed as described by Livermore (9) using a model 12S pH meter (Corning, Corning, NY). Measurements were taken for FSC144ᵀ, 14IUHPL001, and uninoculated chocolate agar incubated at 37°C after 24 h in ambient air and 5% CO₂. To assess whether rapid pH shift was occurring when removing growth medium from the 5% CO₂ incubator, a sterile chocolate agar plate was acclimated in 5% CO₂ for 3 h. The pH was measured in situ and compared to the pH of uninoculated medium in ambient air.

**β-Lactamase activity.** Qualitative assessment of β-lactamase activity was made for F. philomiragia FSC144ᵀ and 14IUHPL001 using nitrocefin disks according to the manufacturer’s instructions (Remel, San Diego, CA). Quantitative β-lactamase activity was measured using β-lactamase activity colorimetric assay reagents according to the manufacturer’s specifications (Bio-Vision, Milpitas, CA). Preweighed bacterial pellets (F. philomiragia FSC144ᵀ and 14IUHPL001 or H. influenzae 8143ᵀ or IUH9, grown in ambient air or 5% CO₂) were suspended in 5 μl of β-lactamase assay buffer per mg of bacteria. Bacterial cell suspensions were then sonicated in an ice bath using a Sonifer cell disruptor 200 (Branson Ultrasonic Corp., Danbury, CT) for 10 s continuously with a 30-s cool down time, for a total of 6 cycles. Samples were then centrifuged at 16,000 × g at 4°C for 20 min. The supernatant for each sample was transferred to a new 1.5-ml microcentrifuge tube and stored on ice. To ensure enzymatic activity fell within the linear range of the nitrocefin standard, samples were diluted 2-fold, 5-fold, and 10-fold. Sample blanks consisted of substrate-free assay buffer. The absorbance (λ = 490) was measured kinetically for 45 min at room temperature using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). β-Lactamase activity was calculated using the equation β-lactamase activity = (A/ΔT × V) × D, where B (nanomoles) represents the amount of nitrocefin hydrolyzed during the change in time (ΔT [minutes]), V (milliliter) represents the amount of sample added to the reaction vessel, and D represents the dilution factor. Enzymatic activity was normalized to milligrams of bacteria.

**Nucleic acid extraction.** Bacterial DNA was extracted using a QIAprep mini-spin kit (Qiagen, Valencia, CA) following the manufacturer’s protocol specifications. Purified DNA was quantified by measuring absorbance at an optical density of 260 nm (OD₂₆₀) and OD₂₈₀. Bacterial RNA was extracted following cultivation in either ambient air or 5% CO₂ using TRIzol reagent followed by RNase-free DNase I treatment (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s specifications.
Nucleic acid amplification. One hundred nanograms of purified DNA was used as the template for each PCR. Amplification of bla_{TEM} was performed by initial denaturation at 94°C followed by 45 cycles at 94°C (30 s), 50°C (30 s), and 72°C (10 s) using the following primers: 5′ ATG AGT ATT CAA CAT TTT CGT GTC G 3′ (forward) and 5′ TAC CAA TGC TTA ATC AGT GA 3′ (reverse). Amplification of bla_{SHV} was performed by initial denaturation at 94°C followed by 45 cycles at 94°C (30 s), 58°C (30 s), and 72°C (70 s) using the following primers: 5′ TTA ACT CCC TGT TAG CCA 3′ (forward) and 5′ GAT TTG CTG ATT TCG CCC 3′ (reverse) (15). A 5-min final extension was performed at 72°C for each reaction. Products were amplified with GoTaq G2 colorless master mix reagents (Promega, Madison, WI) and purified using the PureLink PCR purification system (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer’s instructions. Positive and negative controls included amplification of a portion of the 16S rRNA gene using universal bacterial primers (19) and reagents without DNA template, respectively. One hundred nanograms of purified RNA was amplified using SuperScript IV reverse transcriptase PCR reagents (Life Technologies, Inc.) according to the manufacturer’s instructions. Reagents without RNA template, respectively. Amplification of bla_{TEM} and bla_{SHV} transcript using the aforementioned primer sets as follows: (i) reverse transcription at 55°C for 10 min followed by enzyme inactivation at 80°C for 10 min; (ii) cDNA amplification via 35 cycles at 94°C (30 s), 50°C (30 s), and 72°C (70 s); (iii) a 5-min final extension at 72°C. Positive and negative controls included amplification of a portion of 16S rRNA (19), PCR (reverse transcriptase free) with RNA templates, and reagents without RNA template, respectively.

Nucleotide sequencing and phylogenetic analysis. All DNA amplicons were sequenced using four-dye fluorescent deoxyxide labeling methods at the University of Florida Interdisciplinary Center for Biotechnology Research. Sequence reads were assembled using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). A phylogenetic tree featuring multiple β-lactamase gene families was generated using Clustal Omega (16) and visualized using iTOL 2.0 (17). Reference sequences were obtained from GenBank (18) with the following accession numbers: KJU60142.1, YP_009061958.1, https://doi.org/10.1016/j.tim.2006.07.008 KIN80010.1, ABN49114.1, AAB39956.1, ADD96657.1, YP_006959642.1, https://doi.org/10.7326/0003-4819-110-11-888 CAA38428.1, NP_052173.1, https://doi.org/10.1021/bi00516a004 BAO51997.1, ACV20891.1, EDR30442.1, ACZ37308.1, EWD96542.1, YP_009062986.1, 8 BAP75641.1, AAQ73497.1, YP_009090730.1, 50x591 bla gatedfor MI). A phylogenetic tree featuring multiple BiotechnologyResearch.SequencereadswereassembledusingSequencher4.7(GeneCodes,AnnArbor,MI) Phylogenetic analysis of the beta-lactamase gene has been submitted to the GenBank database under accession no. 8

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