**D-methionine interferes with non-typeable *Haemophilus influenzae* peptidoglycan synthesis during growth and biofilm formation**

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**Abstract**

Non-typeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen that plays a major role in a number of respiratory tract infections including otitis media, cystic fibrosis and chronic obstructive pulmonary disease. Biofilm formation has been implicated in both NTHi colonization and disease, and is responsible for the increased tolerance of this pathogen towards antibiotic treatment. Targeting metabolic pathways that are important in NTHi biofilm formation represents a potential strategy to combat this antibiotic recalcitrance. A previous investigation demonstrated increased expression of a putative D-methionine uptake protein following exposure of NTHi biofilms to the ubiquitous signaling molecule nitric oxide. We therefore hypothesized treatment with exogenous D-methionine would impact NTHi biofilm formation and increase antibiotic sensitivity. Treatment of NTHi during the process of biofilm formation resulted in a reduction in biofilm viability, increased biomass, changes in the overall biofilm architecture, and the adoption of an amorphous cellular morphology. Quantitative proteomic analyses identified 124 proteins that were differentially expressed following D-methionine treatment, of which 51 (41%) were involved in metabolic and transport processes. Nine proteins involved in peptidoglycan synthesis and cell division showed significantly increased expression. Furthermore, D-methionine treatment augmented the efficacy of azithromycin treatment and highlights the potential of D-methionine as an adjunctive therapeutic approach for NTHi biofilm-associated infections.

**Introduction**

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative coccobacillus that asymptomatically colonizes the human nasopharynx, and is also a significant opportunistic pathogen in a variety of human respiratory tract diseases including chronic obstructive pulmonary disease, cystic fibrosis and otitis media(1-3). The persistence and recurrence of NTHi infection despite antibiotic treatment is an important problem in clinical settings and is likely associated with intracellular invasion of airway epithelial cells and biofilm formation(4, 5). Biofilms are aggregated bacterial cells surrounded by a self-produced extracellular polymeric substance (EPS) comprising extracellular DNA, polysaccharides and protein. The EPS matrix, which provides a physical barrier to attenuate the ingress of antimicrobials, and dormant bacterial populations within the biofilm both contribute to the increased antibiotic tolerance associated with biofilms(6, 7). The close proximity of bacteria within the biofilm can also facilitate genetic exchange and lead to the development of antibacterial resistance. New therapeutic strategies that effectively target NTHi biofilms and reduce the risk of antibiotic tolerance and resistance are therefore needed.

One strategy to combat biofilm-associated infections uses metabolic triggers such as carbohydrates or amino acids which may stimulate a metabolically active and replicative state, thereby rendering bacteria within biofilms sensitive to antibiotic treatment. Alternatively, these substrates may serve as signals that trigger endogenous pathways to break down established biofilms. The utilization of amino acids in particular has garnered considerable interest. For example, the introduction of exogenous L-arginine has been shown to impact the regulation of biofilms formed by oral Streptococci(8-10). At high concentrations (>5 mM) L-arginine reduced biomass and altered the architecture of single-species biofilms formed by *Streptococcus gordonii*(8). In addition, L-arginine has also proven successful in the treatment of multispecies oral biofilms formed by *S. gordonii*, *Streptococcus mutans*, and *Actinomyces naeslundii* on hydroxyapatite discs coated with human whole saliva to mimic cariogenic conditions(9). In this model the addition of L-arginine repressed genes involved in the production of EPS (*gftB*) and bacteriocin (*SMU.150*) in *S. mutans*, and increased the expression of *spxB* (involved in hydrogen peroxide production) in *S. gordonii*. These effects resulted in the suppression of *S. mutans* outgrowth and significant changes in EPS. Treatment with high concentrations of L-arginine (500 mM) also altered the species composition of polymicrobial oral biofilms formed in saliva, reduced overall biovolume, and increased the efficacy of the antimicrobial agent cetylpyridinium chloride when administered together(10).

Another amino acid, L-methionine (0.5 μM), has shown efficacy in both the inhibition of biofilm formation and the disassembly of established *Pseudomonas aeruginosa* biofilms (11). These responses were specific to the L-isomeric form and were associated with up-regulation of four *DNase* genes (*sbcB*, *endA*, *eddB*, *recJ*), resulting in the degradation of eDNA in the EPS matrix and inhibition of *P. aeruginosa* swarming and twitching motility. Furthermore, the intranasal administration of L-methionine as an adjuvant with ciprofloxacin for the treatment of murine chronic *P. aeruginosa* lung infection resulted in both the increased sensitivity to antibiotic treatment, and enhanced survival of treated mice(11). No adverse effects towards D or DL methionine have been reported in adults or children, with a single dose of 100 mg/kg body weight considered safe, however, there is evidence that if administered in excess a resultant increase in homocysteine can result in vascular damage and cardiovascular disease(12).

We previously reported that treatment with the ubiquitous signaling molecule nitric oxide (NO) correlated with the increased expression of a putative D-methionine-binding lipoprotein (MetQ) in NTHi biofilms(13). The increased expression of this protein, in addition to other proteins involved in metabolic or transcriptional/translational processes, enhanced the antibiotic sensitivity of NTHi biofilms formed on ciliated primary respiratory epithelial cells(13). We therefore hypothesized that treatment of established NTHi biofilms with exogenous D-methionine would impact upon NTHi biofilm development and potentially reduce biofilm-associated antibiotic tolerance. Quantitative proteomic analyses were performed to characterise any responsive protein adaptations to the presence of exogenous D-methionine in NTHi biofilms.

**Methods**

**Bacterial strain and growth conditions.** *In vitro* biofilm experiments were performed using a clinical NTHi strain isolated from sputum of a primary ciliary dyskinesia patient (obtained under Southampton and South West Hampshire Research Ethics approval 07/Q1702/109). The strain was subcultured onto Colombia agar with chocolated horse blood (CBA; Oxoid, U.K.) from a frozen stock and incubated for 18 hours at 37oC/5% CO2. Colonies were re-suspended in Brain Heart Infusion (BHI) broth supplemented with 10 μg/mL hemin and 2 μg/mL nicotinamide adenine dinucleotide, and incubated at 37oC/5% CO2. Mid-exponential planktonic cultures were then used to inoculate untreated polystyrene 6-well plates with approximately 1x108 cells, with additional supplemented BHI introduced to support growth. Biofilms were grown for 24 h at 37oC/5% CO2.

**Biofilm treatment and assessment of viability.** To evaluate the effect of exogenous D- and L-methionine on viability, biofilms were grown in the presence of 100 μM - 20 mM of each enantiomer (prepared in supplemented BHI) for 24 h at 37oC/5% CO2. After 24 h, treatments were removed and the biofilms rinsed twice in Hanks’ Balanced Salt Solution (HBSS) to remove residual treatment and unattached cells. To assess antibiotic activity biofilms were subsequently treated with azithromycin (1 mg/ml) for 2 h then rinsed twice with HBSS. Biofilms were resuspended in HBSS through scraping and vortexing, serial diluted in HBSS, and then spot plated onto CBA plates. Plates were incubated for 18 h at 37 oC/5% CO2 and viability assessed through enumeration of colony-forming units (CFU). Confocal laser scanning microscopy (CLSM) was performed as previously described using a Leica SP8 confocal laser scanning microscope with a 63x oil immersion lens and LIVE/DEAD BacLight bacterial viability kit (Life Technologies, U.S.A.) (14). Maximum biofilm thickness and the number of individual live cells in confocal z-stacks were determined using COMSTAT 2.1 and ImageJ software(15).

**Scanning electron microscopy.** NTHi biofilms were grown in the presence of 20 mM D- or L-methionine for 24 h as described above, but ethanol-sterilized 13 mm glass coverslips were inserted in each well. Biofilms were processed as previously described(16), and imaged using a Quanta 250 scanning electron microscope (FEI, U.S.A.).

**Transmission electron microscopy.** NTHi biofilms were grown in the presence of 20 mM D- or L-methionine for 24 h as described above, rinsed twice with HBSS, then fixed in 3% glutaraldehyde (TAAB, U.K.) in 0.1 M cacodylate buffer (Agar Scientific, U.K.). The cells were then spun down into 5% aqueous sodium alginate (Thermo Fisher Scientific, U.K.) at 4,500 x *g*. The supernatant was mixed with an equal volume of 0.1 M calcium chloride (Thermo Fisher Scientific, U.K.) to set the alginate. The alginate cell pellet was rinsed in 0.1 M cacodylate buffer and post fixed in 2% osmium tetroxide (Oxkem, U.K.) in 0.1 M cacodylate buffer. Samples were rinsed briefly in distilled water, block stained in 2% aqueous uranyl acetate (Agar Scientific, U.K.) for 30 minutes, rinsed briefly in distilled water and dehydrated through a graded ethanol series (70-100%) (Thermo Fisher Scientific, U.K.), then embedded in Spurr replacement resin (Agar Scientific, U.K.). Gold/silver sections were cut using a Leica Ultra-cut E ultramicrotome. The sections were stained with Reynolds lead stain and viewed on a Hitachi H7000 transmission electron microscope equipped with a SIS Megaview III digital camera.

**Sample preparation for proteomic analysis.** All chemicals were purchased from Sigma-Aldrich (Gillingham, U.K.) unless noted otherwise. NTHi biofilms were grown in the presence of 20 mM D-methionine for 24 h as described above. Biofilms were rinsed twice with 0.1 M triethylammonium bicarbonate (TEAB) buffer then resuspended in 0.1 M TEAB by vortexing and scraping. Samples were lysed in lysing matrix B (MP Bioscience, U.K.) using a TissueLyser LT (Qiagen, U.K.) in six 30 second sessions with 30 second storage on ice between sessions. The lysates were centrifuged at 855 x *g*/5 min, the supernatant retained and sodium dodecyl sulphate (SDS) added to a final concentration of 2% v/v. Samples were shaken on ice for 1 h then subjected to protein determination using Pierce™ BCA Protein Assay (Thermo Fisher Scientific, U.K.). 50 µg of total protein of each sample were reduced by addition of DL-Dithiothreitol (DTT, final concentration 100 mM), followed by trypsin digestion using the filter-aided sample preparation (FASP) method modified from(17). For this, samples were diluted four times with 8 M urea (GE Healthcare Life Science, U.K.), applied on Nanosep 30k Omega filters (Pall Life Sciences, U.K.), and washed repeatedly with 8 M urea to remove SDS. Alkylation was performed with methyl methane thiosulfonate (MMTS, final concentration 10mM) diluted in digestion buffer (1% sodium deoxycholate (SDC), 50 mM TEAB) and the filters were repeatedly washed with digestion buffer afterwards. Protein digestion using an enzyme to protein ratio of 1:100 was performed by two sequential incubations with trypsin (Pierce Trypsin Protease, MS Grade, Thermo Fisher Scientific) at 37°C (first overnight, second for 4 hours) in digestion buffer. Resulting tryptic peptides were collected by centrifugation, and each sample was labelled with the isobaric mass tagging reagent TMT® 10plex according to the manufacturer’s instructions (Thermo Fisher Scientific, U.K.). After differential labelling, peptides of all samples were combined and acidified to pH2 to enable precipitation of SDC by centrifugation. Afterwards, peptides were fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, U.K.). Eight fractions with increasing concentrations of acetonitrile were collected according to the manufacturer’s protocol, and dried by Speedvac. For LC-MS/MS analysis, the samples were resolved in 20 μL of 3% acetonitrile, with 0.1% formic acid, potential debris pelleted by centrifugation, and supernatants transferred into TopSert: TPX Snap Ring Vials (Genetec, Canada).

**LC-MS/MS analysis.** Each of the 8 TMT-labelled fractions were analysed on an Orbitrap Fusion Tribrid mass spectrometer coupled to an Easy nanoLC1000 (Thermo Fisher Scientific, U.K.). Peptides (3 µL injection volume) were separated using an analytical column (250x0.075 mm I.D.) NanoViper Acclaim Pepmap C18 particles 3µm with a precolumn NanoViper C18 particles 5 µm (20x0.0100mm I.D) (Thermo Fisher Scientific, U.K.). Solvent A was 0.2% formic acid in water and solvent B was 0.2% formic acid in acetonitrile. The following gradient was run at 300 nL/min; 5-25 % B over 45 min, 25-80% B over 5 min, with a final hold at 80% B for 10 min. Ions were injected into the mass spectrometer under a spray voltage of 2.0 kV in positive ion mode. MS scans was performed at a resolution of 120,000, m/z range 380-1,200, MS/MS analysis was performed in a data-dependent multi-notch mode, with top speed cycle of 3s for the most intense doubly or multiply charged precursor ions. Ions in each MS scan over threshold 10,000 were selected for fragmentation (MS2) by collision induced dissociation (CID) for identification at 30% and detection in the ion trap followed by multi-notch (simultaneous) isolation of the top 5 MS2 fragment ions, with m/z 400-900, selected for fragmentation (MS3) by high energy collision dissociation (HCD) at 55% and detection in the Orbitrap at a resolution of 60,000, m/z range 100-500. Precursors were isolated in the quadrupole with a 1.6 m/z window and dynamic exclusion within 20 ppm during 30 seconds was used for m/z-values already selected for fragmentation.

**Database search and TMT quantification of proteins.** MS raw data files for the TMT set were merged for relative quantification and identification using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, U.K.). A database search for each set was performed with the Mascot search engine (Matrix Science, U.S.A.) using the *Haemophilus influenzae* (strain 86-028NP) UniProt database, version February 2016. MS peptide tolerance of 5ppm and MS/MS tolerance for identification of 500 millimass units (mmu), tryptic peptides with zero missed cleavages, methionine oxidation set as variable modifications, as wells as cysteine alkylation, N-terminal TMT-label and lysine TMT-label as fixed modifications were selected. The detected peptide threshold in the software was set to a significance of False Discovery Rate 1% by searching against a reversed database and identified proteins were grouped by sharing the same sequences to minimize redundancy. For TMT quantification, the ratios of the TMT reporter ion intensities in the HCD MS/MS spectra (m/z 126-131) from raw data sets were used. Ratios were derived by Proteome Discoverer using the following criteria: fragment ion tolerance as 3 mmu for the centroid peak with smallest delta mass and minimum intensity of 2000. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. The quantification was normalised using the protein median. Inclusion criteria for quantitative analysis were set at ≥2 peptide matches, ≥50 protein score, ≥5% sequence coverage (p < 0.05). Comparative protein data with >1.3 and <0.77 ratios were classed as being differentially expressed. Proteins were analysed using the String: functional protein association networks database (version 10.0) and the Gene Ontology (GO) database (version 1.2, released 27/10/2016) with the Protein ANalysis THrough Evolutionary Relationships (PANTHER) tool.

**Statistical analysis.** Statistical analysis of CFU data was performed using Prism 6 (GraphPad, U.S.A.) with one-way analysis of variance (ANOVA) and nonparametric Kruskal-Wallis testing. P values <0.05 were considered statistically different.

**Results**

**NTHi biofilms grown in the presence of exogenous D-methionine demonstrated reduced viability.** We previously reported increased expression of a D-methionine uptake lipoprotein in NTHi biofilms following treatment with exogenous NO, which suggested a possible role for D-methionine in an amino acid adaptive response. We therefore hypothesized that the presence of exogenous D-methionine may impact NTHi biofilm development. We found that the viability of NTHi biofilms grown for 24 h in the presence of 100 μM - 15 mM D-methionine was unaffected. However, a log-fold reduction was observed at a concentration of 20 mM of exogenous D-methionine (p<0.01; Fig. 1a). To determine if this response was specific to the D-enantiomer, we also assessed the effect of equimolar concentrations of L-methionine on biofilm formation and observed no effect on viability (Fig. 1a). We also examined whether this response was biofilm-specific by assessing the effect of D-methionine on planktonic NTHi populations. Treating with 100 μM - 10 mM D-methionine had no effect on planktonic growth over seven hours, but a notable reduction in growth was observed when treating with 15 or 20 mM D-methionine (Fig. 1b) suggesting that the resultant reduction in biofilm viability could potentially be attributed to slow-growing planktonic cells. In comparison, treatment with 20 mM L-methionine had no effect on planktonic growth (Fig. 1c).

**D-methionine impacts upon NTHi biofilm architecture and cellular morphology.** In order to assess the effect of 20 mM D-methionine on biofilm architecture we performed scanning electron microscopy (SEM). SEM imaging revealed that whilst L-methionine had no obvious effect on biofilm architecture, the presence of D-methionine resulted in the compact aggregation of cells and evidence of abnormal cellular morphology (Fig. 2a-c). To further investigate this change in cellular structure we performed transmission electron microscopy (TEM). TEM demonstrated that D-methionine had caused the majority of bacteria to adopt an irregular cell morphology and increase in size, with a number of cells reaching ~4 μm in diameter, compared to the typical size of 1 x 0.3 μm in untreated biofilms (Fig. 2d-i).

**D-methionine changed the protein expression profile of NTHi biofilms.** We performedquantitative proteomic analyses to further shed light on the possible mechanisms by which D-methionine affects NTHi biofilm formation and cellular morphology. Quantitative analyses comparing untreated 24 h NTHi biofilms to those grown in the presence of 20 mM D-methionine identified a total of 960 individual proteins shared between both populations A total of 124 (13%) were differentially expressed in the presence of 20 mM D-methionine and involved in a range of biological functions (Fig. 3). Seventy three (60%) of these proteins showed decreased expression and a significant proportion were associated with metabolic (18 proteins) or transport (15 proteins) processes (Table 1). Fifty one (40%) proteins showed increased expression, with metabolic and transport processes (9 proteins each) again being well represented. Additionally, eight proteins involved in transcriptional and translational processes were increased in expression, as well as nine proteins involved in cell wall formation, peptidoglycan synthesis and cell division (Table 1). Notably, D-methionine also resulted in the increased expression of two stress response proteins, GrpE (1.54-fold) and SpoT (1.31-fold) during biofilm formation. The expression of proteins involved in nitrogen metabolism and cellular iron ion homeostasis were also reduced in NTHi biofilms exposed to D-methionine.Specifically, there was a decrease in the expression of NrfA, NrfB, and NrfC, which belong to the single *H. influenzae* nitrite reductase encoded by the *nrfABCD* operon and are involved in nitrite reduction, as well as NapA, which is also involved in nitrate reduction (Table 1).

**Exogenous D-methionine interfered with peptidoglycan synthesis during NTHi biofilm formation.** Themost striking changes observed with exogenous D-methionine involved cellular morphology within NTHi biofilms and the increase in the expression of proteins associated with cell wall formation, peptidoglycan synthesis and cell division. In total, 31 proteins (25%) were quantitatively identified, of which nine were increased in expression and only one (MurB) showed decreased expression (Fig. 4a). Seven of these differentially expressed proteins (MurB, MurC, MurD, MurE, MurF, Ddl, MraY) are involved in peptidoglycan synthesis; FtsA and FtsL in cell division, and MraZ in the transcriptional regulation of cell division and peptidoglycan synthesis genes. Notably, the nine proteins that showed increased expression are encoded by genes belonging to the *dcw* cluster, which comprises fifteen genes involved in peptidoglycan synthesis and cell division(18-20) (Fig. 4b). MurB, the only protein decreased in expression, is encoded by a gene outside the *dcw* cluster.

**D-methionine increased biofilm biomass and sensitivity towards treatment with azithromycin.** Finally, we tested if the architectural or cellular changes induced by D-methionine during NTHi biofilm formation would impacted biofilm antibiotic tolerance. Treatment of 24 h NTHi biofilms established in the absence of D-methionine with 1 mg/ml azithromycin for 2 h, and growth in the presence of 20 mM D-methionine for 24 h without antibiotic treatment both resulted in a log-fold reduction in the number of viable cells within the biofilm (*p*<0.05; Fig. 5b). In contrast, biofilms grown in the presence of D-methionine for 24 h and then subsequently treated with 1 mg/ml azithromycin for 2 h, showed a significant 2-log reduction in biofilm viability (*p*<0.05; Fig. 5b). CLSM imaging revealed that D-methionine caused an increase in biofilm biomass over 24 h, with biofilms reaching ~41 μm in maximum height compared with ~23 μm for untreated biofilms (Fig. 5a & c). COMSTAT and ImageJ analyses also indicated that the untreated and D-methionine-treated biofilms contained 8.3 x104 and 9.6x104 individual live cells in their z-stacks respectively (Fig. 5d). Treatment with 1 mg/ml azithromycin had no effect on maximum biofilm thickness but reduced the number of live cells to 5.9 x104, whereas combined treatment with 20 mM D-methionine increased the maximum biofilm thickness to ~37 μm and reduced the number of live cells to 5.5x104 (Fig. 5c & d).

**Discussion**

Targeting pathways that are differentially expressed duringbiofilm development in response to specific signals offers a potential strategy to address elevated antibiotic tolerance in bacterial biofilms. We previously investigated the role of NO and its use as an adjuvant therapy to antibiotic treatment in NTHi biofilms. This work indicated that increased sensitivity to azithromycin treatment was associated with NO-mediated modulation of metabolic activity(13). The increased expression of the putative D-methionine-binding lipoprotein (MetQ) following exposure to NO, in particular, represented a promising target that warranted further investigation. We hypothesized that exogenous D-methionine might influence NTHi biofilm development and increase susceptibility to antibiotic treatment.

Our findings show that the introduction of 20 mM D-methionine during NTHi biofilm development resulted in both reduced viability and an increase in biomass, and that equimolar concentrations of the L-enantiomer failed to elicit the same response. Visually, confocal imaging appeared to suggest an increase in the number of live cells within D-methionine-treated biofilms, however, image analyses revealed that the live population was similar to that of untreated biofilms. This disparity can be explained, in part, by the change in NTHi morphology as a result of D-methionine treatment, with the cells appearing densely aggregated, amorphous, and significantly larger in size compared to those in untreated biofilms. This does not, however, account for the reduction in viability observed through CFU enumeration. Although the number of live-stained cells present within the biofilm is similar between untreated and D-methionine-treated biofilms the reduction in CFUs could perhaps be attributed to impaired NTHi fitness and growth, resulting in a ‘viable but non-culturable’ state. This is supported by a D-methionine-mediated reduction in planktonic growth, a response that was not observed when treating with equimolar L-methionine.

Quantitative proteomic analyses were performed to elucidate the underlying mechanisms responsible for the change in biofilm architecture and cellular structure. Of the 960 individual proteins identified and quantified only 124 (13%) of these were found to be differentially expressed in response to D-methionine. Unsurprisingly, a significant proportion of these were involved in metabolic (27 proteins), transport (24 proteins), or transcriptional/translational (10 proteins) processes which are commonly associated with biofilm formation and the response to external factors. Whilst these protein undoubtedly play an important role in biofilm adaptive responses, we hypothesized that other identified proteins provided a more mechanistic insight into the response to D-methionine. Notably, two stress-related proteins were increased in expression; GrpE which is involved in the response to hyperosmotic stress and heat shock and prevents stress-denatured protein aggregation(21), and SpoT, which has been shown to mediate the stringent response in bacteria, as well as biofilm formation through regulation of the secondary messenger (p)ppGpp(22, 23). We also found that proteins involved in nitrite (NrfA, NrfB, NrfC) and nitrate (NapA) reduction, which play important roles in protecting NTHi from host nitric oxide (NO), showed significantly decreased expression. Deletion of *nrfA* has been shown to increase NTHi sensitivity towards NO(24). These observations suggest that adjunctive treatment involving D-methionine and NO-donor compounds might represent a potential treatment strategy for NTHi biofilm-associated infections.

It is the changes in expression of proteins that are involved in peptidoglycan synthesis, cell wall formation and cell division that are most intriguing in light of the abnormal cellular morphology of the NTHi inhabiting the biofilm. D-methionine induces expression of proteins that are encoded by genes in the *dcw* operon, leading to dysregulation of peptidoglycan synthesis and the enlarged, amorphous morphology of the cells. Confocal imaging also revealed that D-methionine increased biofilm formation, a response that is in concordance with previous work that demonstrates a link between interference with peptidoglycan synthesis and increased NTHi biofilm formation(25). In that study, transposon mutants in specific peptidoglycan synthesis genes (*ponA*, *ampG*, *amiB*, *mrdA*) demonstrated increased biofilm formation as a result of bacterial lysis and the release of extracellular DNA. D-methionine has also previously been shown to elicit changes in the macromolecular peptidoglycan of *Escherichia coli* with its incorporation resulting in a significant change in the proportion of all muropeptide groups, accumulation of two major modified muropeptides, and a significant reduction in cross-linked muropeptides(26). While it is possible that a similar mechanism could be involved in NTHi, no effect on *E. coli* cellular morphology was observed in that particular study. It was however suggested that D-methionine may also have a direct inhibitory effect on penicillin binding proteins and biosynthetic enzymes. D-methionine is also incorporated into the cell wall muropeptides of other bacterial species replacing D-Ala in the 4th and 5th positions(27). Incorporation into the 5th position in *Vibrio cholerae* was shown to be dependent on D-methionine utilisation by Ddl and MurF, which both showed increased expression in our study, suggesting that D-methionine is an alternative substrate for these enzymes(27).

Finally, we also demonstrated that D-methionine has the potential to increase sensitivity of NTHi towards macrolide antibiotics through this interference in peptidoglycan synthesis, highlighting its potential role as an adjuvant treatment agent.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical Statement**

This research did not involve any experimental work with human participants, and therefore required no applications for ethical approval.

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**Figure 1:** High concentrations of D-methionine reduce *in vitro* NTHi biofilm viability and planktonic growth. **a)** *In vitro* NTHi biofilms were grown in the presence of different concentrations of L- and D-methionine for 24 h then viability measured by CFU enumeration. **b)** *In vitro* NTHi planktonic growth in the presence of 100 µM - 20 mM D-methionine measured by absorbance (OD595). **c)** *In vitro* NTHi planktonic growth in the presence of 20 µM L- or D-methionine measured by absorbance (OD595). \*\*, *p* < 0.01. (n=4)

**Figure 2:** D-methionine affects *in vitro* NTHi biofilm architecture and cellular morphology. *In vitro* NTHi biofilms were grown in the presence of 20 mM L- or D-methionine for 24 hours. Biofilm structure was assessed by scanning electron microscopy **(a-c)**, and cellular morphology assessed by transmission electron microscopy **(d-i)**. (Magnifications: a-c, X 5,000; d-f, X 2,500; g-h, X 20,000).

**Figure 3:** Overview of differential protein expression in D-methionine treated *in vitro* NTHi biofilms. *In vitro* NTHi biofilms were grown in the presence of 20 mM D-methionine for 24 hours and quantitative proteomic analyses were performed. **(a)** Overall protein expression, and **(b)** functional grouping of differentially expressed proteins.

**Figure 4: a)** String analysis of *in vitro* NTHi biofilm proteins involved in cell wall formation, peptidoglycan synthesis and cell division following D-methionine treatment.Large circles represent proteins with complete or partially known protein structure whereas small circles represent proteins with unknown structure. Red circles represent proteins with reduced expression, green circles proteins with increased expression, and grey circles proteins that showed no change in expression. Connecting blue lines represent direct binding and grey lines functional links. **b)** Organisation of the *H. influenzae dcw* cluster highlighting genes that encode differentially expressed proteins following D-methionine treatment (green - increased expression; red - decreased expression; grey - no change in expression; white - not present in proteomic dataset). Connecting line between *ftsZ* and *murB* represents intervening genes.

**Figure 5:** D-methionine treatment of *in vitro* NTHi biofilms increases biofilm biomass and susceptibility to azithromycin treatment.*In vitro* NTHi biofilms were grown in the presence of 20 mM D-methionine for 24 h, subsequently treated with 1 mg/mL azithromycin for 2 h, and viability assessed by **a)** confocal microscopy and LIVE/DEAD staining, and **b)** CFU enumeration. COMSTAT analyses of confocal z-stacks were performed to determine **c)** maximum biofilm thickness, and **d)** the number of individual live cells present within biofilms. Scale bar in confocal XY pane: 40 μm. Sagittal XZ section represents biofilm thickness. \*, *P* ≤ 0.05, \*\*, *P* ≤ 0.01.

**Table 1:** Details of differentially expressed proteins in D-methionine treated *in vitro* NTHi biofilms. Inclusion criteria for quantitative analysis were set at ≥2 peptide matches, ≥50 protein score, ≥5% sequence coverage (p < 0.05). Comparative protein data with >1.3 (green) and <0.77 (red) ratios were identified as having differential expression.