Mucoid morphotype variation in *Burkholderia multivorans*: Role of a Two-Component Regulatory System and a LysR Regulator

Ana Rita Guerreiro

**ABSTRACT:** Bacteria from *Burkholderia cepacia* complex (*Bcc*) can cause severe chronic infections in patients with Cystic Fibrosis (CF). During chronic infection with *Bcc*, mucoid-to-nonmucoid morphotype variation occurs with the two morphotypes exhibiting different phenotypic properties. Consistent decreased expression of *Bmul_2557* gene encoding a LysR-type transcriptional regulator (LTTR) was seen for nonmucoid variants compared with isogenic mucoid isolates. Here we developed a strategy for the complementation of the Δ*Bmul_2557::dhfR* isogenic mutant of *Burkholderia multivorans* ATCC 17616 and performed a phenotypic characterization of the wild-type, isogenic deletion mutant and complemented strain. Exopolysaccharide production showed similar levels in all strains, excluding a direct role of this regulator in EPS biosynthesis regulation. Identification of metabolites present in culture supernatants revealed the accumulation of 2-ketogluconic acid and D-lactate in wild-type culture, metabolites resulting from glucose consumption through the oxidative pathway and from the conversion of excess pyruvate into D-lactate. The relevance of an OmpR-like response regulator in *Burkholderia* morphotype variation was also studied. A plasmid containing the *ompR* gene was mobilized into nonmucoid *B. multivorans* variants and the mucoid phenotype restored, suggesting that nonmucoid phenotype is due to mutations in *ompR* gene. Overall, the functional analysis of the LysR-type and OmpR-type regulators contribute to the understanding of metabolic/virulence traits in *Bcc* and will help in directing future studies on the molecular mechanisms of morphotype variation in these bacteria.

**INTRODUCTION**

The *Burkholderia cepacia* complex (*Bcc*) is a group of Gram-negative bacteria, belonging to Proteobacteria β-subdivision, which currently comprises 18 closely related species (Coenye et al. 2001; Peeters et al., 2013). *Bcc* bacteria are ubiquitous in nature and can be found among different environments and lifestyles, including animals, plants, water and soil (Mahenthiralingam et al., 2008). *Bcc* bacteria are also opportunistic pathogens and have attracted considerable interest due to infections in immunocompromised patients and, particularly, in the airways of cystic fibrosis (CF) patients (Baldwin et al., 2007).

Infections of CF patients with *Bcc* are extremely difficult to treat since these bacteria have inherent resistance to antibiotics and capacity to spread between CF patients. During CF chronic infections, *Bcc* bacteria produce a wide range of virulence factors including LPS, EPS, flagella, cable pili, adhesins, extracellular enzymes, secretion systems, siderophores, biofilms and resistance to antibiotics and oxidative stress (reviewed in Leitão et al., 2010). One of these traits, EPS, is associated with phenotypic variation, a phenomenon that has been described in CF patient’s airways. In CF lungs, bacterial pathogens are challenged with a harsh environment that leads to the emergence of phenotypic variants (Lyczak et al., 2002). Whilst in *P. aeruginosa*, most phenotypic conversions occur from the nonmucoid to mucoid morphotype (Govan et al., 1996), in *Bcc* bacteria, phenotype transitions in isolates recovered from CF patients have also been reported. However, in *Bcc* bacteria little is known about the adaptive traits and mechanisms underlying this phenomenon. Regarding the discovery of the regulatory mechanisms by which *Bcc* bacteria undergo mucoid-to-nonmucoid variation, global transcriptomic profiling studies have been carried...
out in our laboratory. Transcriptomic profiles of mucoid B. multivorans D2095 and B. multivorans ATCC 17616 were compared with the ones from nonmucoid variants obtained in vitro for each strain (Tavares, 2012). The analysis of the transcriptomic data sets showed consistent decreased expression in the nonmucoid strains of gene Bmul_2557, from B. multivorans ATCC 17616, encoding a transcriptional regulator from the LysR family of transcriptional regulators (LTTR). Also, whole-genome sequencing of nonmucoid variants of B. multivorans D2095 obtained in vitro (Silva et al, 2013) ORF frame encoding a response regulator (RR) of a two-component system (TCS), being annotated as OmpR-like (unpublished results).

The LysR-type transcriptional regulator (LTTR) family includes a group of transcriptional regulators that play a role in the regulation of transcription of genes involved in metabolism, quorum sensing, virulence, motility, toxin production, attachment and secretion (reviewed in Maddocks & Oyston, 2008; Schell, 1993). Until now, only one LTTR regulator is reported as involved in virulence in Bcc bacteria, being responsible for colony morphotype variation - ShvR. Mutants in shiny variant regulator (shvR) gene were shiny, defective in biofilm formation and show an absence of extracellular matrix (Subramoni et al., 2011).

TCSs are signal transduction devices, typically composed by a membrane-bound histidine kinase (HK), which senses a specific environmental stimulus and a response regulator (RR) that binds DNA (Beier & Gross, 2006). Some TCSs sense and respond to changes in medium osmolarity, such as EnvZ/OmpR TCS in E. coli (Foster & Spector, 2002). Besides, the EnvZ/OmpR system is also responsible for flagellar expression, cell division, fatty acid transport and acid tolerance (Foster & Spector, 2002). Due to their versatility in sensing diverse intracellular and extracellular signals and their variable modular architecture, TCSs are convenient devices for the regulation of the expression of virulence properties. Here, we report the study of a LysR-type transcriptional regulator that showed consistent decreased expression in nonmucoid B. multivorans variants. A strategy to complement a previously constructed deletion mutant for this gene was developed and phenotypic assays of B. multivorans ATCC 17616, its isogenic deletion mutant and complemented strain were envisaged, in order to assess the influence of this regulator at carbohydrate metabolism level. As mutations in ompR were also found in nonmucoid variants obtained in vitro, we also aim to comprehend the relevance of this RR in mucoid-to-nonmucoid morphotype variation.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are described in Tables 1 and 2. E.coli was grown at 37°C in Lennox Broth (LB) supplemented with kanamycin (50 μg/ml) or chloramphenicol (25 μg/ml) when required to maintain selective pressure. Burkholderia strains were grown in LB or in S (Richau et al. 2000) or SM media (12.5g/l NaH2PO4, 2H2O, 3g/l KH2PO4, 1g/l K2SO4, 1g/l NaCl, 1g/l Yeast Extract, 1g/l Casamino acids, 20g/l of glucose (in S medium) or mannitol (in SM medium), at 37°C. The deletion mutant in Bmul_2557 gene in B. multivorans ATCC 17616 was grown in LB supplemented with trimethoprim (100 μg/ml). The deletion mutant in Bmul_2557 gene complemented with pARG015-1 was grown in LB supplemented with chloramphenicol (200 μg/ml).

DNA manipulation. Genomic DNA from Burkholderia was extracted and purified using the DNeasy blood and tissue kit (Qiagen) following the manufacturers’ recommendations. A fragment of 2.7 kb of B. multivorans ATCC 17616 genome containing the promoter region of Bmul_2557, Bmul_2557 and Bmul_2558 genes was amplified by polymerase chain reaction (PCR) using 1p and 1R primers sequences (Sup. table 1, Appendix) under the following conditions: 5 min at 95°C; 34 cycles of 30 seconds at 95°C and 1 min at 72°C; followed by an additional extension step at 72°C for 7 min. PCR reaction mixture included 1 ng/μl of template DNA, 200 μM dNTPs, 0.5 pmol/μl of each primer oligonucleotide, 1.5 μM MgSO4 and 2U of Taq DNA polymerase (Cloned). Amplification product was separated by 0.8% (w/v) agarose gel electrophoresis at 10 V/cm. For amplified DNA purification, ZymoResearch gDNA was used. Plasmid DNA was extracted and purified using ZymoResearch Miniprep kit following the manufacturers’ recommendations. Plasmid and amplified DNA were restricted with HindIII and DNA ligation of the amplified DNA frame was performed using standard protocols (Sambrook, 2001). E. coli DH5α cells were transformed by classic transformation and grown for 1 hour at 37°C before plating in selective media supplemented with 0.1 mM X-gal and 0.1 mM IPTG. The originated plasmid, pARG015-1 (pBBR1MCS containing the promoter region of Bmul_2557, Bmul_2557 and Bmul_2558 genes) was confirmed by DNA sequence determination, pARG015-1 plasmid was mobilized into B. multivorans ATCC 17616 ΔBmul_2557::dfrR mutant, by triparental conjugation using plasmid pRK2013 as helper. Transformants were selected in LB plates supplemented with 100 μg/ml ampicillin and 200 μg/ml chloramphenicol.

Phenotypic characterization of Burkholderia strains. To assess growth of Burkholderia strains, triplicate cultures were grown in 100 ml of S or SM media (initial OD660nm of 0.1) at 37°C, with orbital agitation at 250 rpm for 7 days. Aliquots of 100 μl were collected and serially diluted with NaCl 0.9%.
spread onto the surface of LB plates, and incubated at 37°C for 2 days. Plates were examined with respect to the number of colony forming units (CFU). 1-ml aliquots of cell culture were also taken at each day, centrifuged at 13,000 g for 5 min and supernatants were stored at -20°C before high pressure liquid chromatography (HPLC) analysis. HPLC analysis. Cell free supernatants of *Burkholderia* cultures were diluted 1:5 with mobile phase (H₂SO₄ 5 mM). Standard solutions of D-glucose, 2-keto gluconic acid, 5-keto gluconic acid and D-lactate were prepared, in the following concentrations: 50 mM, 25 mM, 5 mM, 2.5 mM, and 0.5 mM for 2-keto glucose and 5-keto gluconic acids; 200 mM, 100 mM, 50 mM, 10 mM, 5 mM, and 1 mM for D-glucose and 100 mM, 50 mM, 10 mM, 5 mM, and 1 mM for D-lactate. HPLC was performed using an Aminex HPX-87H column (BioRad) at 65°C using 5mM H₂SO₄ at a constant flow of 0.6 ml/min. For a qualitative analysis, the retention times of peaks obtained for each sample, in UV-Vis and RI detectors, were compared with those obtained for standard samples, to identify the compound produced by *B. multivorans* strains during growth. For quantification of carbon source consumption, 2-keto gluconic acid and D-lactate production, standard curves were drawn.

Triparental conjugation. *B. multivorans* nonmucoid variants (Table 1) were complemented with plasmid pBBR1MCS as negative control, by triparental conjugation using pRK2013 helper plasmid. Transformants were selected in EPS-producing Yeast Extract Mannitol (YEMi) plates (Zlosnik et al., 2008), supplemented with 100 µg/ml ampicillin and 200 µg/ml chloramphenicol. Construction of the *ompR* gene replacement vector with Gateway-compatible allelic exchange system. To generate *ompR* mutants in *B. multivorans* strains, the upstream fragment of *ompR* gene was amplified using the primers P3 and P4 (Sup. table 1, Appendix) and the downstream fragment of *ompR* gene was amplified using the primers P5 and P6. Both fragments were amplified using Phusion High-Fidelity DNA polymerase (Jena Bioscience) according to the manufacturer’s instructions and the following thermal cycling conditions: 98°C for 2 min; 30 cycles of 98°C for 20 sec, 57°C for 20 sec (ompRupstream region) or 54°C for 20 sec (ompR downstream region), and 72°C for 1 min; a final extension step of 72°C for 7 min. The PCR fragments were separated by 0.8% (w/v) agarose gel electrophoresis at 10 V/cm, purified with ZymoResearch gel extraction kit and their concentrations and quality were estimated using an UV spectrophotometer (ND-1000 UV-Vis, NanoDrop Technologies, USA). The up- and down-nstream fragments will be fused together and amplified using the primers P7 and P8 (Sup. table 1, Appendix) in splicing-by-overlap extension PCR to generate the *ompR* mutant allele. Equal amounts (50 ng) of each up- and down-nstream fragments and the other components of the PCR reaction except the primers P7 and P8 will be mixed. The PCR reaction will be carried out using the following thermal cycling conditions: 98°C for 2 min; 10 cycles of 98°C for 15 sec, 58°C for 30 sec and 72°C for 1 min; and a final extension step of 72°C for 1 min. The final extension step will be paused at 30 sec, the primers GW-attB1 and GW-attB2 will be added, and the thermal cycling continued with 27 cycles of 98°C for 15 sec, 64°C for 30 sec and 72°C for 2 min; and a final extension step of 72°C for 7 min. The PCR product will be separated by 0.8% (w/v) agarose gel electrophoresis at 10 V/cm.

Table 1 – Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. multivorans</em> ATCC 17616</td>
<td>Soil isolate, USA</td>
<td>(Vandamme et al, 1997)</td>
</tr>
<tr>
<td><em>B. multivorans</em> ATCC 17616 \ΔBmul_2557::dhfR</td>
<td><em>B. multivorans</em> ATCC 17616 deletion mutant in Bmul_2557 gene; Tp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Silva et al, unpublished)</td>
</tr>
<tr>
<td><em>B. multivorans</em> ATCC17616 \ΔBmul_2557::dhfR + PAR3015-1</td>
<td><em>B. multivorans</em> ATCC17616 ΔBmul_2557::dhfR complemented with Bmul_2557 gene, its promoter region, and also Bmul_2557 gene; Tp&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>B. multivorans</em> D2095</td>
<td>CF clinical isolate, Canada, EPS&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Zlosnik et al, 2008)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/121</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM&lt;sup&gt;+&lt;/sup&gt;; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/122</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/123</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/124</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/126</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/127</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/129</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/130</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/131</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/132</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>B. multivorans</em> NMV/133</td>
<td>Nonmucoid variant obtained in vitro from an original CF clinical isolate BM; EPS</td>
<td>(Silva et al, 2013)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>recA1, lacU69, F&lt;sup&gt;Φ&lt;/sup&gt;diacZΔM15</td>
<td>Gibco BRL</td>
</tr>
</tbody>
</table>
Complementation of *B. multivorans* ATCC 17616 gene *Bmul_2557* deletion mutant. To better understand the role of the LysR protein encoded by *Bmul_2557* gene, a deletion mutant was previously obtained and named Δ*Bmul_2557::dhfR* (Tavares, 2012). As *Bmul_2557* may be in an operonic structure along with *Bmul_2558*, replacing of *Bmul_2557* by the *dhfR* cassette might have affected the expression of *Bmul_2558*. This way, a strategy to obtain a plasmid for complementation of *B. multivorans* ATCC 17616 Δ*Bmul_2557::dhfR* mutant was designed (Fig. 1), and included the *Bmul_2557* gene, its promoter region, and also gene *Bmul_2558* cloned into pBBR1MCS cloning vector. A fragment of 2.7 kb was amplified by PCR from genomic DNA of *B. multivorans* ATCC 17616. The ligation between the amplified DNA product and pBBR1MCS DNA was performed and the resulting plasmid, pARG015-1, was then introduced into *E. coli* DH5α cells using classic transformation and the transformants were selected.

After that, triparental conjugation where *E. coli* DH5α was used as donor strain of the pARG015-1 plasmid to *B. multivorans* ATCC 17616 Δ*Bmul_2557::dhfR* was performed. Transconjugants were selected and mutant complementation was confirmed by phenotypic assays.

**Role of Bmul_2557 LysR regulator in cepacian biosynthesis.** Since *Bmul_2557* gene displayed decreased expression in nonmucoid isolates (Tavares, 2012), we postulated this gene could be involved in the expression of this phenotype. To test this hypothesis, the wild-type *B. multivorans* ATCC 17616, the Δ*Bmul_2557::dhfR* deletion mutant and the complemented mutant harboring pARG015-1 were grown in EPS producing medium supplemented with glucose or mannitol. In mannitol containing medium, all strains produced between 10–12 g/l of cepacian after 48 hours and that value remained approximately constant for the next two days (Fig. 2A).

![Fig. 1 - Cloning strategy to obtain pARG015-1 plasmid. Plasmid pBBR1MCS-1 serves as backbone of the expression vector pARG015-1.](image)

![Fig. 2 - EPS production by *B. multivorans* ATCC 17616 (▲), Δ*Bmul_2557::dhfR* mutant (Δ) and Δ*Bmul_2557::dhfR* + pARG015-1 (▲) in the presence of mannitol (A) and glucose (B) at 37°C.](image)
Regarding growth in glucose containing medium, only the ΔBmul_2557::dhfR deletion mutant produced cepacian (Fig. 2B). Although the same amount (~12 g/l) as in mannitol containing medium was reached, detection of this EPS in the culture supernatant was delayed by 48 hours. From this experiment we could conclude that Bmul_2557 transcriptional regulator did not have a significant influence in cepacian biosynthesis in mannitol supplemented medium as the wild-type, ΔBmul_2557 deletion mutant and the complemented mutant produced similar levels. Nevertheless, the result obtained in the presence of glucose was unexpected since, in a Bmul_2557 gene background, these strains were unable to produce any polysaccharide and we could clearly see cell lysis. Only in the absence of the Bmul_2557 gene was possible to obtain cepacian production. This observation prompts us to investigate the effect of other sugars, and glucose in particular, in growth properties of the strains under study.

Growth of the LysR mutant in the presence of glucose or mannitol. To assess growth of B. multivorans ATCC 17616, ΔBmul_2557::dhfR, and ΔBmul_2557::dhfR + pARG015-1, cultures were inoculated in S medium (glucose as carbon source) and SM medium (mannitol as carbon source) during 7 days, and the OD_{600nm}, CFUs and pH were registered (Fig. 3, 4 and 5).

![Fig. 3](image1.png) - Growth curves of B. multivorans ATCC 17616 (▲), ΔBmul_2557::dhfR mutant (Δ) and ΔBmul_2557::dhfR + pARG015-1 (▲) in SM (A) and S medium (B) at 37°C. The data are based on mean values from three independent cell cultures.

![Fig. 4](image2.png) - Number of viable cells of B. multivorans ATCC 17616 (▲), ΔBmul_2557::dhfR mutant (Δ) and ΔBmul_2557::dhfR + pARG015-1 (▲) in SM (A) and S medium (B) at 37°C. The data are based on mean values from three independent cell cultures.

![Fig. 5](image3.png) - Culture supernatant pH of B. multivorans ATCC 17616 (▲), ΔBmul_2557::dhfR mutant (Δ) and ΔBmul_2557::dhfR + pARG015-1 (▲) in SM (A) and S medium (B) at 37°C. The data are based on mean values from three independent cell cultures.
In SM medium with mannitol as carbon source, all strains presented similar growth as measured by OD$_{640\text{nm}}$, or by assessing CFUs/ml (Fig. 3A and Fig. 4A) and similar culture supernatant pH values (Fig. 5A). However, in S medium, with glucose as carbon source, wild-type \textit{B. multivorans} ATCC 17616 and complemented mutant strain, both OD$_{640\text{nm}}$ (Fig. 3B) and CFUs/ml (Fig. 4B) decreased as consequence of bacterial lysis. This effect was not seen, however, for the mutant strain, as the OD$_{640\text{nm}}$ is maintained as well as the CFU number. Also, whilst in the wild-type and in the complemented strain, the supernatant pH decreases until 3.5 and 3.6 by 48 hours of growth, respectively, in the mutant, the pH decreases until reaching a minimum of 5.1 and thereafter, occurs a progressive neutralization of the culture media until initial pH of 7.0, by the 7\textsuperscript{th} day of culture (Fig. 5B). Given these results, we conclude that the mutant complementation appears to have occurred efficiently with pARG015-1, which results in the restoration of the original phenotype in S medium. Besides, since cell lysis of the ∆Bmul\_2557::dhfr mutant in S medium does not occur it seems that Bmul\_2557-encoded LysR has a negative effect on cell growth in the presence of glucose, probably being related with glucose metabolism.

**Analysis of extracellular metabolites by HPLC.** To identify differences in metabolite composition of bacterial cultures of \textit{B. multivorans} ATCC 17616, \textit{B. multivorans} ATCC 17616 ∆Bmul\_2557::dhfr mutant, and ∆Bmul\_2557::dhfr + pARG015-1, both at qualitative and quantitative level, we performed HPLC analysis of culture supernatants. By doing this, we aimed to identify and quantify the compound(s) resulting from \textit{B. multivorans} ATCC 17616 metabolism that may be responsible for culture acidification upon growth with glucose as carbon source, as well as carbon source (glucose) consumption. During the growth in S medium, aliquots of growth supernatant of the wild-type strain, mutant strain and the complemented mutant were processed as well as appropriate standard solutions (materials & methods). The metabolites 2-keto gluconic acid (2-KG) and 5-keto gluconic acid (5-KG) were chosen because they are products of glucose metabolism through the oxidative pathway. D-lactate is the possible product of Bmul\_2558 activity and therefore, relevant in this study. For a qualitative analysis, the retention times of peaks obtained for each sample were compared with those obtained for standard samples. By analyzing the chromatograms of the wild-type, mutant and complemented mutant supernatants in S medium over time, the only differences were two peaks with retention times of 7.8 and 12.7 minutes, in the UV-vis and RI chromatogram respectively. The area of the first peak was seen to increase in the wild-type and complemented strains’ supernatant whereas in the mutant the peak area decreased after the second day of growth. Under the same conditions, the retention time of the 2-KG was 7.8 minutes, which is indicative of the production of this acid during the growth in S medium. The second peak at 12.7 minutes was present in the wild-type and complemented mutant supernatants whereas in the mutant the peak area decreased after the second day of growth.

Under the same conditions, the retention time of D-lactate used as standard was also 12.7 minutes in the RI detector. Glucose consumption was evaluated by assessing the evolution of peak area with a retention time of 9.23 minutes in the RI detector.

![Fig. 6 - 2-keto gluconic acid (A) and D-lactate (B) production by B. multivorans ATCC 17616 (∆), ∆Bmul\_2557::dhfr mutant (Δ) and ∆Bmul\_2557::dhfr + pARG015-1 (∆) in S medium, at 37°C. The data are based on mean values from the results of three independent cell cultures.](image)
It is possible to observe that *B. multivorans* ATCC 17616 reached 117 mM of 2-KG by the second day of growth, and from there on the concentration of this metabolite remains unchanged (Fig. 6A). In contrast, the Δ*Bmul_2557*::*dhfR* mutant reached 134 mM of 2-KG by the second day of growth but instead, a successive decrease in the production of 2-KG occurred, presenting in the 6th day a practically null concentration. Consumption of this acidic metabolite leads to a pH recovery by the mutant strain to physiological levels, allowing its survival. In contrast, the wild-type and complemented mutant strain, maintain 2-KG acid levels very high, which keeps pH at critical levels, inducing bacterial lysis. Regarding D-lactic acid production and accumulation in the culture supernatant, the wild-type strain showed the highest concentration already at 24 hours of growth (Fig. 6B). Interestingly, from the Δ*Bmul_2557*::*dhfR* deletion mutant supernatant was not detected D-lactic acid, while the complemented mutant showed D-lactic acid accumulation although at lower concentration than the wild-type strain. Taking into account glucose consumption (Fig. 7), it is possible to observe that the wild-type strain takes longer to metabolize the sugar, which is consistent with the lower 2-KG accumulation (Fig. 6A). In contrast, both mutant and complemented mutant strain degrade glucose faster, presenting in the 2nd day of growth very low concentration of this metabolite and higher levels of 2-KG.

**Complementation of nonmucoid *B. multivorans* variants with an ompR-containing plasmid.** Nonmucoid variants of *B. multivorans* D2095 were previously obtained in *vitro* (Silva et al., 2013). Whole-genome sequencing of those variants revealed that about 90% have mutations in an open reading frame (BMD20_11660) encoding an OmpR-like response regulator (RR) from a two-component system (TCS) (unpublished results). To assess whether mutations in this ompR-like gene were specifically occurring in the nonmucoid variants from the D2095 clinical isolates or were extended to other isolates, we have chosen four other isolates recovered from the same CF patient (Table 3). These *B. multivorans* isolates were exposed to prolonged stationary phase (21 days at 42°C in SM medium). Several nonmucoid colonies were obtained and several were kept for further analysis.

**Table 3 - Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Nonmucoid variants</th>
<th>Complementation of the mucoid morphotype with pLM014-5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. multivorans</em> BM4</td>
<td>NW121, NW122, NW123</td>
<td>Yes, Yes, Yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> BM6</td>
<td>NW124, NW126, NW127</td>
<td>Yes, Yes, Yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> BM7</td>
<td>NW129</td>
<td>Yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> BM9</td>
<td>NW130, NW131, NW132, NW133</td>
<td>Yes, Yes, Yes, Yes</td>
</tr>
</tbody>
</table>

To evaluate the relevance of this RR in the nonmucoid morphotype displayed by the variants, the *ompR* gene and its promoter region cloned into pBBR1MCS (pLM014-5) was mobilized into these variants by triparental mating. After complementation with the *ompR* containing plasmid pLM014-5, all variants restored the mucoid phenotype (Fig. 8). These results suggest that the nonmucoid phenotype is due, most likely, to mutations in *ompR* gene.

**Fig. 8 - Colony morphologies of *B. multivorans* NW121 (A) and NW124 (B) variants, in YEM medium after 72 hours at 30°C, complemented with pBBR1 vector or pLM014-5.**
Strategy to obtain the *B. multivorans* D2095 deletion mutant in *ompR* gene. To study the relevance of the OmpR-like regulator in morphotype variation, a strategy to knock-out that gene in *B. multivorans* D2095 was initiated (Fig. 9). First, a fragment upstream (985 bp) and downstream (1099 bp) regions of the *ompR*-like gene was amplified by PCR, using primers P3/P4 and P5/P6, respectively (Sup. table 1, Appendix). As non-specific amplification occurred, the two fragments were purified from the agarose gel and quantified, to obtain the desired concentration. The next step is the Gateway PCR, where by using primers P7 and P8, the upstream (L') and downstream (R') regions are fused together to generate the *ompR* mutant allele (Fig. 9). Amplification of this fragment was attempted a few times, but with no amplicon being detected. Further optimization for this step is required. When this amplicon is obtained, it will be recombined into pDONRPEX18Tp-SceI-pheS plasmid using BP clonase reaction. The product will be transferred to competent *E. coli* DH5α cells and mobilized to *B. multivorans* D2095, by triparental conjugation. The transformants will be selected in appropriate selective medium and screened by colony PCR to verify the insertion of the *ompR* gene flanking regions. In the second part of gateway strategy, a single positive merodiploid clone will be transformed with pDAI-SceI-pheS by triparental mating. The I-SceI endonuclease expressed from the plasmid induces a break in the double DNA strand, stimulating the second homologous recombination event that can happen in different locations, generating the desire gene deletion or the wild-type allele, which is easily identify by PCR.

**DISCUSSION**

*Bcc* bacteria are a group of important opportunistic pathogens that cause severe infections in cystic fibrosis patients. In *Bcc* bacteria, little is known about the adaptive traits and mechanisms underlying phenotype variation. Regarding the discovery of the regulatory mechanisms by which *Bcc* bacteria undergo mucoid-to-nonmucoid variation, global transcriptomic profiles of *B. multivorans* ATCC 17616 (mucoid isolate) and comparing variants obtained *in vitro* (Tavares, 2012) showed consistent decreased expression of *Bmul_2557* gene, encoding a transcriptional regulator from the LysR family of transcriptional regulators (LTTR).

In this work, we report the study of *Bmul_2557* gene. A strategy to complement the ∆*Bmul_2557::dhfR* mutant for this gene was developed and phenotypic assays of *B. multivorans* ATCC 17616, its isogenic deletion mutant and complemented strain were performed. Looking at cepacian biosynthesis, the strains reached the same levels, suggesting that this regulator is not directly involved in the regulation of mucoid phenotype. Discarding a direct influence in the regulation of EPS biosynthesis, we hypothesize a role for this LTTR in carbon metabolism regulation. The ability of *B. multivorans* ATCC 17616 and ∆*Bmul_2557::dhfR* mutant to metabolize different carbon sources was compared. In mannitol, all strains presented similar growth. However, in glucose containing medium, whilst in wild-type *B. multivorans* ATCC 17616 and complemented strain bacterial lysis occurred, in the mutant strain, this phenomenon did not happened, suggesting that *Bmul_2557*-encoded LysR and the downstream lactate dehydrogenase encoding gene (*Bmul_2558*) may have a deleterious effect on cell survival. The consumption of glucose occurs via two routes, the direct oxidative and the phosphorylative pathways, that converge at 6-phosphogluconate (6PGA), which is metabolized to produce in the end pyruvate (Allenza and Lessie, 1982). Thus, it is possible that when wild-type strain grows with glucose as carbon source, the favored pathway is the oxidative which leads to the formation of gluconic and 2-ketogluconic acids that causes culture medium pH lowering. Additionally, D-lactic acid is also being produced and secreted to the culture medium, further decreasing the pH value to critical levels which lead to cell death and lysis. Contrastingly, the ∆*Bmul_2557::dhfR* mutant also oxidize D-glucose to 2-keto gluconic acid with the concomitant lowering of the culture medium pH, but the values never decrease that much, and cells recover to the medium pH to neutral levels. This behavior might have to do with the absence of D-lactic acid in the mutant culture supernatant. Based on the obtained data we propose that glucose metabolism in *B. multivorans* ATCC 17616 leads to excess of pyruvate formation. Among the different routes for pyruvate utilization is the one leading to D-lactic acid production.
Fig. 9 – Gateway strategy to generate a deletion mutant in ompR encoding gene (Adapted from Fazli et al. 2015). The green balloons represent E.coli DH5α cells and the orange ones represent B. multivorans D2095.
Is then possible that production of D-lactate dehydrogenase (encoded by \textit{Bmul\_2558}) is dependent on the induction of the LysR transcriptional regulator (encoded by \textit{Bmul\_2557}) through binding of pyruvate or even the end product, D-lactate. In the absence of \textit{Bmul\_2557} gene, there is no expression of \textit{Bmul\_2558} gene and therefore no D-lactic acid is being produced. In a second part of this work, we studied the relevance of an OmpR-like regulator from a TCS, in \textit{Burkholderia} morphotype variation. Previous results of whole-genome sequencing of nonmucoid variants of \textit{B. multivorans} D2095 obtained \textit{in vitro} (Silva et al., 2013) revealed that about 90% have mutations in an ORF encoding a response regulator (RR) of a two-component system (TCS), being annotated as OmpR-like (unpublished results). Here, a plasmid containing the \textit{ompR} gene was mobilized into nonmucoid \textit{B. multivorans} variants by biparental mating. In all cases mucoid phenotype was restored, confirming that nonmucoid phenotype is due to mutations in \textit{ompR} gene. Also, a novel strategy to knock-out the \textit{ompR} gene in \textit{B. multivorans} D2095 was initiated. The aim was to obtain an unmarked deletion mutant to proceed studies envisaging the understanding of \textit{bce} genes expression and regulon determination. This strategy is being performed and, until now, the main “bottleneck” is the optimization of the Gateway PCR, when the PCRs resultants from amplification of the upstream and downstream regions of the \textit{ompR} gene are fused together, to generate the \textit{ompR} mutant allele. In conclusion, we found that the \textit{Bmul\_2557} encoding LTTR acts as a positive regulator, transcriptionally regulating gene \textit{Bmul\_2558} encoding a D-lactate dehydrogenase. In the second part of this work, results evidence that mutations in the \textit{ompR} gene are a mechanism to mucoid-to-nonmucoid switch, and occur in several isolates irrespective of genetic background. Therefore, more studies of this LysR-type regulator and OmpR will enlighten regulation of metabolic and/or virulence traits in \textit{Bcc} and may help to understand the biology of this organism.

\textbf{REFERENCES}


**APPENDIX**

Supplementary table 1 – Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 P_2557_58_Fw</td>
<td>5'–GGGAAGCTTGGCGGGGATTTGTG–3'</td>
</tr>
<tr>
<td>P2 P_2557_58_Rev</td>
<td>5'–AGGAAGCTTGGCGAGGGCAGGG–3'</td>
</tr>
<tr>
<td>P3 OmpR-UpF-GWL</td>
<td>5'–TAACAAAAAGCTGGCTGGGCTATGATGAAAGG–3'</td>
</tr>
<tr>
<td>P4 OmpR-UpR-tail</td>
<td>5'–AAACCCCTCAAGATTTCTGTGGGGCTCGGCTACGTGTTCAT–3'</td>
</tr>
<tr>
<td>P5 ompR-DnF</td>
<td>5'–CGACGAGAAATCTTGAGGGGTGTT–3'</td>
</tr>
<tr>
<td>P6 OmpR-DnR–GWR</td>
<td>5'–TAACGAAAAGCTGGGTTAAGTGGCATGCAAGAAAC–3'</td>
</tr>
<tr>
<td>P7 GW–attB1</td>
<td>5'–GGGGACAAAGTTTGTACAAAAAACGAGGCT–3'</td>
</tr>
<tr>
<td>P8 GW–attB2</td>
<td>5'–GGGGACCACCTTTGTAAGAAGCTGGGT–3'</td>
</tr>
</tbody>
</table>