The regulator LdhR and the D-lactate dehydrogenase LdhA of Burkholderia multivorans play a role in carbon overflow and in planktonic cellular aggregates formation.

Running title: Burkholderia multivorans suspended biofilm

Inês N. Silva¹, Marcelo J. Ramires¹, Lisa A. Azevedo¹, Ana R. Guerreiro¹, Andreia C. Tavares¹, Jörg D. Becker², and Leonilde M. Moreira¹,³

¹IBB- Institute for Bioengineering and Biosciences, Instituto Superior Técnico (IST), Av. Rovisco Pais, 1049-001 Lisbon, Portugal
²Instituto Gulbenkian de Ciência, Rua da Quinta Grande Nº 6, 2780-156 Oeiras, Portugal
³Department of Bioengineering, IST, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

*Correspondence: Leonilde M. Moreira
Mailing address: Instituto Superior Técnico, Torre Sul, Piso 6, Av. Rovisco Pais, 1049-001 Lisbon, Portugal. Phone: (351)218419031. Fax: (351)218419199. E-mail: lmoreira@tecnico.ulisboa.pt

Present address: Instituto de Tecnologia Química e Biológica António Xavier, ITQB NOVA, Avenida da República, 2780-157 Oeiras, Portugal
LysR-type transcriptional regulators (LTTR) are the most commonly found regulators in *Burkholderia cepacia* complex, comprising opportunistic pathogens causing chronic respiratory infections in cystic fibrosis (CF) patients. Despite LTTRs being global regulators of pathogenicity in several bacteria, few have been characterized in *Burkholderia*. Here, we showed that gene *ldhR* of *B. multivorans* encoding a LTTR is co-transcribed with *ldhA* encoding a D-lactate dehydrogenase, and evaluate their implication in virulence traits like exopolysaccharide (EPS) synthesis and biofilm formation. Comparison of wild-type (WT) and its isogenic ΔldhR mutant grown in medium with 2% D-glucose revealed a negative impact on EPS biosynthesis and on cells’ viability in the presence of LdhR. Loss of viability in WT cells was caused by intracellular acidification as consequence of cumulative organic acids secretion including D-lactate, this last one absent from the ΔldhR mutant supernatant. Furthermore, LdhR is implicated in the formation of planktonic cellular aggregates. WT cell aggregates reached 1000 μm after 24 hours in liquid cultures; in contrast to ΔldhR mutant aggregates that never grew more than 60 μm. Overexpression of D-lactate dehydrogenase LdhA in the ΔldhR mutant partially restored formed aggregates size, suggesting a role for fermentation inside aggregates. Similar results were obtained for surface-attached biofilms, with WT cells producing more biofilm. A systematic evaluation of planktonic aggregates in *Burkholderia* CF clinical isolates showed aggregates in 40 out of 74. As CF patients’ lung environment is microaerophilic and bacteria are found as free aggregates/biofilms, LdhR and LdhA might have central roles in adaptation to this environment.
IMPORTANCE

Cystic fibrosis patients often suffer from chronic respiratory infections caused by several microorganisms. Among them are the *Burkholderia cepacia* complex bacteria which cause progressive deterioration of lung function and, in some patients, might develop into fatal necrotizing pneumonias with bacteremia, known as “cepacia syndrome”. *Burkholderia* pathogenesis is multifactorial since they express several virulence factors, form biofilms, and are highly resistant to antimicrobial compounds, making their eradication from the CF patients’ airways very difficult. As *Burkholderia* is commonly found in the CF lungs in the form of cell aggregates and biofilms, the need to investigate the mechanisms of cellular aggregation is obvious. In this study we demonstrate the importance of a D-lactate dehydrogenase and a regulator, in regulating carbon overflow, cellular aggregates and surface-attached biofilm formation. This not only enhances our understanding of *Burkholderia* pathogenesis, but can also lead to the development of drugs against these proteins to circumvent biofilm formation.

Keywords: LysR-family transcriptional regulator, D-lactate dehydrogenase, *Burkholderia multivorans*, Planktonic cellular aggregates, Biofilms, Exopolysaccharide; Cystic fibrosis
INTRODUCTION

*Burkholderia cepacia* complex comprises bacteria ubiquitous in the environment, but also responsible for persistent infections in the airways of cystic fibrosis (CF) sufferers, strongly contributing to lung function deterioration (1). *Burkholderia* may grow as single cells, but they are often found in small clusters such as the ones identified in the airways of CF patients (2). To cope with the different environments, these bacteria are equipped with a wide range of metabolic functions and virulence traits, reflected in their genome size ranging from 6 to 9 Mbp (3, 4). One such trait shared by many *B. cepacia* complex strains is the expression of the mucoid phenotype due to the biosynthesis of exopolysaccharides (EPSs) (5, 6). One of those EPSs, cepacian, has been implicated in biofilm formation, inhibition of neutrophil chemotaxis, protection against phagocytosis by human neutrophils, neutralization of reactive oxygen species *in vitro*, facilitation of persistent bacterial infection in animal models, and protection against abiotic stressors (7–12). The synthesis in large amounts of this polymeric compound requires high carbon to nitrogen ratio, being mannitol or glucose commonly used carbon sources (6–8). One particular feature of glucose metabolism in *B. cepacia* complex bacteria is the presence of alternative routes for its conversion into gluconate-6P prior entry into the Entner-Doudoroff pathway: the direct oxidative pathway mediated by the activities of membrane-associated glucose and gluconate dehydrogenases, and the phosphorylative pathway mediated by glucokinase and a NAD(P)-dependent glucose 6P-dehydrogenase (Fig. 1) (13). The main utilization of one pathway or another is dependent on the glucose source and is strain specific (14). Whatever sugar is used as carbon source for EPS biosynthesis, it needs to be fed into the central metabolic pathways and the required activated sugar-nucleotide precursors.
synthesized. Then, these sugar-nucleotide precursors are assembled into repeating units that are subsequently polymerized and EPS chains secreted to the external milieu (5).

Although genes involved in cepacian biosynthesis have already been described (7, 15) the regulation of transcription of those bce-I and bce-II gene clusters remains mostly unknown. To understand the mechanisms regulating EPS production, Silva and coworkers compared the transcriptome of mucoid and nonmucoid clonal isolates of *Burkholderia multivorans*, leading to the identification of a putative LysR-type transcriptional regulator (LTTR) whose expression was downregulated in the nonmucoid isolate (16). This LTTR is located upstream a gene encoding a putative D-lactate dehydrogenase, an enzyme that reversibly converts pyruvate into D-lactate.

Besides the important role of D-lactate dehydrogenase in fermentation and energy production under oxygen-limiting conditions, this metabolic conversion has been shown as required for microcolony formation, a hallmark of biofilm architecture in *Pseudomonas aeruginosa* (17) especially associated with CF lung infections (2, 18). Petrova and coworkers, by studying the role of the two-component regulator MifR, demonstrated that inactivation of genes involved in pyruvate utilization or depletion of pyruvate from the growth medium abrogated microcolony formation, and pyruvate supplementation significantly increased microcolony formation (17).

Although that study revealed that MifR-dependent microcolony formation is associated with stressful, oxygen-limiting yet electron-rich conditions, other mechanisms seem also to be implicated in microcolony formation in *P. aeruginosa*. Indeed, the LTTR BvLR of *P. aeruginosa* PA14 was implicated in tight microcolony formation possibly mediated through repression of fimbrial-based surface attachment (19). Tight microcolonies (also named planktonic cellular aggregates) are a mode of biofilm that does not require a surface to attach to. Instead, cells self-aggregate to form free-floating suspended biofilms. In another study, the free-floating cellular
aggregates formed by \textit{P. aeruginosa} PAO1 were analyzed by microscopy, with data showing that they comprise up to 90\% of the total planktonic biomass, ranging from 10 to 400 μm in diameter, and dispersing into single cells upon carbon, nitrogen, or oxygen limitation (20). During growth, these cellular aggregates contain densely packed viable cells, but upon starvation cell death increases, and metabolites and bacteriophages are released to the supernatant.

In this work, we asked whether the \textit{B. multivorans} ATCC 17616 LTTR (Bmul\_2557) identified plays a role at the interface between metabolism and pathogenesis, namely by governing carbon overflow, EPS production, and cellular aggregates/biofilm formation. To address these questions, we made use of a Bmul\_2557 mutant and evaluated EPS production with different carbon sources, measured carbon consumption and metabolic end-products, as well as cellular aggregates and biofilm formation. This LTTR, through direct or indirect regulation of the expression of a D-lactate dehydrogenase encoding gene and possibly other genes, was found to have a significant influence on cellular aggregates and attached biofilm formation, but a negative effect on polysaccharide biosynthesis. Furthermore, it regulated the overflow products generated in excess of glucose and similar sugars. Our data support a role for Bmul\_2557 LTTR as a key regulator at the boundary between metabolic performance and virulence of \textit{B. multivorans}. 
RESULTS

Expression of Bmul_2557 gene is decreased in nonmucoid variants. Previous work on the nonmucoid B. multivorans D2214 and the clonal mucoid D2095 CF isolates revealed that the gene homolog of Bmul_2557 from B. multivorans ATCC 17676 encoding a putative LTTR had decreased expression in the nonmucoid isolate (16). To assess whether this gene could have a role in the expression of the mucoid phenotype due to cepacian production, its expression level was measured in several stress-induced nonmucoid variants derived from mucoid strains of different Burkholderia cepacia complex species (11) grown in S medium with 2% D-mannitol. In agreement with the previous finding in B. multivorans D2214/D2095 isolates, the transcription of Bmul_2557 gene homologue was decreased in all tested nonmucoid variants relative to the respective mucoid parental strain B. multivorans D2095, B. contaminans IST408, B. anthina FC0967, B. vietnamiensis PC259, and B. dolosa CEP0743 (Fig. 2A).

Bmul_2557 gene, tentatively named ldhR (lactate dehydrogenase regulator), is located on chromosome 1 of the soil isolate B. multivorans ATCC 17616. Downstream and in the same orientation is located gene Bmul_2558 encoding a putative D-lactate dehydrogenase (LdhA) (Fig. 2B). Comparison of ldhR gene upstream region with the one of the characterized homolog scmR from Burkholderia thailandensis E264, whose expression is known to be induced by quorum sensing and having a lux-box (21), shows absence of such conserved region in B. multivorans (Fig. 2B). In silico analysis predicts an operonic structure for ldhR and ldhA genes. Reverse transcription PCR experiments on wild-type (WT) cells grown for 18 hours in S medium with D-mannitol confirmed their co-transcription and that Bmul_2559 belongs to another transcriptional unit (Fig. 2C).
LdhR and LdhA display conserved domains of LTTR regulators and D-lactate dehydrogenases, respectively. *In silico* analysis indicated high conservation of the genomic location of genes *ldhR* and *ldhA* within the *Burkholderia* genus. From the 673 strains whose genome sequence is available at the Burkholderia Genome Database (Mai, 2017), only 9 lack the *ldhR* gene homolog while 18 do not have the *ldhA* gene homolog. None of these 27 strains is from the *Burkholderia cepacia* complex. Homology search at the amino acid level between LdhR and other characterized LTTRs indicated that the highest degree of similarity is within the N-terminal helix-turn-helix domain responsible for binding DNA (Fig. S1A). The best characterized homolog is ScmR of *B. thailandensis* E264, showing 65% identity (77% similarity). Some amino acid residues important for DNA binding identified by mutagenesis in proteins CrgA, CysB and OxyR (22) are also conserved in LdhR from *B. multivorans*. The less conserved C-terminal region, where the co-inducer domain is located, has some homology to sugar-binding domains present in ABC transporters and other sugar-binding proteins, suggesting that a sugar-derived metabolite might be involved in LdhR activation.

Protein LdhA is homologous to members of the superfamily of NAD-dependent D-isomer specific 2-hydroxyacid dehydrogenases. Alignment of the amino acid sequence of LdhA with the one of *B. thailandensis* E264 and other D-lactate dehydrogenases, which had their tridimensional structure determined, showed conservation of important residues in both the nucleotide binding domain and the catalytic domain, as exemplified by the conservation of R235 and E264 involved in substrate binding, and D259 and H296 involved in catalysis (Fig. S1B).

To substantiate the putative function of LdhA as a D-lactate dehydrogenase, a phylogenetic analysis of the family of D-isomer specific 2-hydroxyacid dehydrogenases was performed.
Based on PROSITE pattern_1 we aligned 185 proteins from the families of D-lactate dehydrogenase (D-LDH), D-3-phosphoglycerate dehydrogenase (SERA), erythronate-4-phosphate dehydrogenases (PDXB), formate dehydrogenase (FDH), glyoxylate/hydroxypyruvate reductases (GHPR), and C-terminal binding protein (CTBP). Phylogenetic analysis shows clustering of the proteins according to substrate specificity, with LdhA from *B. multivorans* ATCC 17616 grouping together with D-lactate dehydrogenases from *E. coli*, *Lactobacillus plantarum*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Treponema pallidum* (Fig. 3).

**LdhR regulator has a negative effect in EPS production.** To test the hypothesis of the LdhR regulator being involved in EPS production, *B. multivorans* ATCC 17616 and the ΔldhR deletion mutant were grown in S medium with different carbon sources for 3 days. In the presence of 2% D-galactose, D-fructose, D-mannitol, or D-mannose, the mutant produced approximately 32-45% more EPS than the WT strain (Fig. 4A). In medium supplemented with 2% D-glucose, the WT strain was unable to produce EPS while the ΔldhR mutant produced approximately 6 g/l.

To test whether introducing the *ldhR* gene in the mutant would decrease EPS production, a complementation experiment by expressing *ldhR* gene from pMM137-2 was performed. EPS quantification in medium with the different sugars was not significantly different from the mutant carrying the empty vector (data not shown), suggesting a possible polar effect of the trimethoprim resistance cassette replacing *ldhR* gene on the *ldhA* gene expression, as will be demonstrated in the next section. Due to this observation, genes *ldhR* and *ldhA* were expressed simultaneously from their own promoter region cloned into plasmid pARG015-1. When grown
in the presence of D-mannose or D-mannitol, there was indeed reduction in the amount of EPS produced either by overexpression of ldhRA in the mutant or in the WT (Fig. 4B). In the presence of D-glucose, the ΔldhR mutant expressing ldhRA genes produced no EPS, restoring the phenotype of the WT strain. Similarly, overexpression of both genes in the WT strain confirmed the loss of EPS production (Fig. 4B). Expression of ldhA gene in the ΔldhR mutant had a similar effect as expressing both ldhRA (data not shown). Together, these results suggest a negative effect of ldhR and ldhA gene products on the biosynthesis of EPS.

Deletion of ldhR gene has a positive effect on cell viability in glucose-rich medium. Due to impairment of EPS production by B. multivorans ATCC 17616 in medium containing 2% of D-glucose, but not by the ΔldhR mutant, we compared their growth and culture medium pH in the presence of 2% D-mannitol or of 2% D-glucose as carbon sources. No significant difference was observed in the exponential growth phase of both strains in mannitol-rich medium, although the final biomass of the ΔldhR deletion mutant was consistently higher (Fig. 5A). The pH of the culture medium of both strains remained constant for the time of the experiment. In medium with 2% D-glucose, both strains displayed similar growth rates, but when entering stationary phase, the WT culture showed a decrease in optical density (Fig. 5B) as well as cell viability as determined by CFUs count (Fig. 5C). Since it is known that glucose metabolism can lead to medium acidification, we measured the growth medium pH during the course of the experiment. Up to 48 hours, both the WT and the ΔldhR mutant caused a drop in pH, reaching pH 3.5 and pH 4.4, respectively. After that time, the medium pH of the WT culture remained at 3.5, whereas the ΔldhR mutant reproducibly increased the culture medium pH to 6.0 by day 3 (Fig. 5D). When 0.5% D-glucose was used, no significant differences in growth and viability
were observed, despite the higher biomass of the ΔldhR mutant (Fig. 5B and C). The culture medium pH also remained constant and at a neutral value (Fig. 5D).

Genetic complementation of the ΔldhR mutant by expressing gene ldhR under the control of the bce promoter did not restore either the WT impaired cell viability in the presence of 2% D-glucose or the culture medium acidification to around pH 3.5 (Fig. 6A-C). This suggests lack of ldhR expression from the plasmid or a polarity effect of the trimethoprim resistance cassette on ldhA gene expression. To test these hypotheses we performed quantitative RT-PCR. Data confirmed the expression of ldhR gene in the complemented mutant, although the level was lower than the one of the WT strain (Fig. 6D). Overexpression of ldhR gene in the WT strain also resulted in increased levels of ldhA gene expression, discarding the hypothesis of deficient expression from the bce promoter. Additionally, the expression of ldhA gene was decreased both in the ΔldhR mutant and the complemented mutant (Fig. 6D), confirming not only the polarity of the trimethoprim resistance cassette on ldhA expression, but also that ΔldhR mutant acts effectively as double ldhRA mutant. Nevertheless, ldhA gene expression was increased when ldhR gene was overexpressed in the WT strain, giving additional support of ldhR and ldhA genes being in a polycistronic operon and confirming the direct or indirect involvement of LdhR regulator in ldhA gene expression. Transcription data of B. thailandensis E264 shows 4.8-fold decreased expression of the ldhA gene when the upstream gene scmR was deleted (21), also supporting our observations in B. multivorans ATCC 17616.

Overexpression in the ΔldhR mutant of ldhA gene alone (pLM016-2) or together with the ldhR gene restored the WT phenotype with a decrease in optical density and cell viability and no recovery of the culture medium pH from 3.5 to 6 (Fig. 6A-C), confirming protein LdhA involvement in these phenotypes. Introduction of each of the three plasmids into the WT strain
slightly enhanced the growth negative effects in the presence of 2% D-glucose, being more visible for the strain expressing ldhRA genes simultaneously (Fig. S2A-C).

Taken together, our data shows that the extreme acidification of the growth medium is possibly the cause of decreased cell viability of WT cells in the presence of 2% D-glucose. This acidification is most likely due to organic acids secretion and in particular to the activity of the D-lactate dehydrogenase LdhA involved in the production of D-lactic acid from pyruvate.

The negative effect on cell viability and EPS production in glucose-rich medium is prevented by buffering the growth medium. To confirm that loss of cell viability is dependent on the acidic pH observed, we grew the WT and ΔldhR mutant cells in medium supplemented with 2% of D-glucose buffered with 0.2 M Tris.Cl with an initial pH of 7.2. Under these conditions, the WT and ΔldhR mutant showed similar growth trends and viability, although the ΔldhR mutant exhibited higher final biomass (Fig. S3A-B). The acidification of the growth medium was also observed for both strains, but the lowest pH values obtained were 4.8 for the WT strain and 5.3 for the ΔldhR mutant (Fig. S3C). After reaching this minimum, both strains recovered the culture medium pH towards neutrality. EPS production was also quantified after 96 hours of growth in buffered 2% D-glucose containing medium, with the WT strain recovering its ability to produce EPS, despite a reduction of 25% when compared to the ΔldhR mutant (Fig. S3D).

Cell survival threshold is surpassed by secretion of D-lactate. To examine whether the pH drop in glucose-rich medium was due to organic acid production, we employed reverse-phase HPLC. By comparing peak retention times with standards, we identified the peaks
corresponding to gluconate (GN), 2-ketogluconate (2KG), and D-lactate. In accordance to the pH drop by 48 hours of growth in the presence of 2% D-glucose, both the WT and ΔldhR mutant strains converted GN to 2KG although the concentration was higher for the WT strain (Fig. 7A). In the following hours, 2KG concentration decreases in the ΔldhR mutant, most likely due to its consumption while in the WT strain the levels remained high and constant due to loss of cell viability (Fig. 7A). An inspection of D-glucose consumption shows the same trend for both strains, with no detectable quantity by 48 hours (Fig. 7B). D-lactate increased in the growth medium of the WT strain up to 7 mM at 32 hours, decreased to 5 mM until 48 hours and remained the same until the end of the experiment (Fig. 7B). From the ΔldhR mutant supernatant, no D-lactate was detected.

Growth of WT and ΔldhR mutant in 0.2 M Tris-buffered medium leads to similar consumption of D-glucose and conversion into 2KG until 48 hours, but then both strains are able to consume this metabolite at similar rate (data not shown). D-lactate concentration increased in the growth medium of the WT strain up to 48 hours, but then it was reduced by consumption and at 72 hours, no D-lactate was detected. From the ΔldhR mutant supernatant, no D-lactate was detected. Overexpression of ldhR gene in the WT strain increases the concentration D-lactate in the supernatant, possibly due to a positive effect on the expression of ldhA gene (Fig. S2D). When ldhA gene is expressed in the WT strain, D-lactate concentration was even higher.

Overall, D-glucose metabolism in the WT strain leads to organic acids secretion with a concomitant pH decrease, and if that value reaches a critical level, cells loose viability. Contrastingly, the absence of D-lactate accumulation by the ΔldhR mutant prevents such stronger acidification with cells remaining viable.
Enzymatic activities confirm organic acids secretion with ΔldhR mutant displaying lower lactate dehydrogenase activity than the WT strain. Data obtained for organic acids secretion by the WT strain and the ΔldhR mutant was confirmed by measuring key enzymes from the direct oxidative and phosphorylative pathways of D-glucose dissimilation, as well as conversion of pyruvate into lactate. Cells were grown in the presence of 2% D-glucose or D-mannitol to measure activation of one or the other pathway. In general, in the presence of D-glucose the enzymes of the oxidative pathway (GDH and GNDH) have higher specific activities than in D-mannitol-rich medium (Fig. 7C). Conversely, G6PDH activity is higher in D-mannitol rich medium. Comparison of WT and ΔldhR mutant showed no difference in the enzymatic activity of the tested enzymes, except lower decreased activity in the mutant of GDH and G6PDH in medium supplemented with D-glucose and D-mannitol, respectively (Fig. 7C).

Total lactate dehydrogenase activity (LDH) in crude extracts revealed lower specific activity in the ΔldhR mutant when compared with the WT, in both D-glucose and D-mannitol-rich medium (Fig. 7C). This result confirms the involvement of D-lactate dehydrogenase LdhA in the WT cells ability to convert pyruvate into D-lactate.

Growth medium acidification depends on the metabolized sugar and is strain dependent. To test whether the involvement of LdhR in relieving carbon overflow was specific for D-glucose or also involved other sugars, we grew the WT and ΔldhR mutant in medium supplemented with other carbon sources. Growth in the presence of sugars metabolized by the phosphorylative pathway (Fig. 1) such as D-fructose and D-mannitol, resulted in no medium acidification for both tested strains while D-mannose led to a slight pH
decrease of the culture medium. No D-lactate, 2KG, and GN were detected either in the WT or the \( \Delta ldhR \) mutant (data not shown). Growth of the two strains in the presence of another sugar metabolized by the direct oxidative pathway (Fig. 1) such as D-galactose led to culture medium pH acidification to a minimum of 5.2 in the WT strain and 5.5 in the \( \Delta ldhR \) mutant at 48 hours, with both strains recovering to neutrality by 72 hours. D-lactate was identified in the supernatant of the WT, but not of the \( \Delta ldhR \) mutant (data not shown). The consumption rate of the several sugars measured at 48 hours of growth shows that while D-glucose is depleted, for the other sugars there is still considerable amount of the initial concentration and no significant differences were observed between WT and \( \Delta ldhR \) mutant (Fig. 7D). This data shows that metabolic overflow is only caused by the high D-glucose dissimilation rate.

Next, we tested whether the negative effect on cell survival registered in the presence of 2% D-glucose was unique for \( B. \) multivorans ATCC 17616 or it was a more common phenomenon in the \( B. \) cepacia complex. Eighteen additional strains were tested regarding the minimum pH value reached in the culture medium and whether they recovered to higher pH values, and if D-lactate was produced and consumed. Data shown in Table 1 indicates that only three strains (\( B. \) multivorans VC3161, D2095, and \( B. \) dolosa CEP1010) had the same behavior as \( B. \) multivorans ATCC 17616, namely culture medium pH decrease to 3.5-3.7 and no recovery, and D-lactate secretion but no consumption. A second group of ten strains (\( B. \) multivorans JTC, VC7495, VC6882, VC12539, VC8086, VC9159, VC12675, \( B. \) ambifaria CEP0996, \( B. \) stabilis LMG 14294, and \( B. \) cenocepacia ATCC 17765) showed minimum culture medium pH values ranging from 4.0-6.3, but this value increased towards neutrality. All these strains secreted D-lactate into the growth medium during the first 48 hours, but it was consumed in the following hours. From the last group of five strains (\( B. \) anthina J2552, \( B. \) cenocepacia J2315, \( B. \) cepacia...
ATCC 25416, *B. contaminans* IST408, and *B. vietnamiensis* G4) we were unable to detect D-lactate, but the culture medium pH decreased to values ranging 4.5-6.5 and then increased to neutrality (Table 1). Enzymatic activities of GDH, GNDH, G6PDH, and LDH in *B. dolosa* CEP1010 (51.7 (± 9.8), 16.0 (± 1.3), 11.2 (± 2.9), and 119.2 (± 12.5) nmoles min\(^{-1}\) mg\(^{-1}\), respectively) and *B. cenocepacia* ATCC 17765 (15.5 (± 0.8), 5.0 (± 0.4), 52.6 (± 3.7), and 97.4 (± 8.8) nmoles min\(^{-1}\) mg\(^{-1}\), respectively) grown in medium with 2% D-glucose confirmed differential use of both D-glucose dissimilation pathways. Together, this data suggests that most of the tested strains use the direct oxidative pathway for D-glucose utilization, but some might use the phosphorylative pathway only or a combination of both.

Growth in glucose-rich medium induces higher stress to WT than to \(\Delta ldhR\) mutant cells. During the first 24 hours of growth in medium containing 2% D-glucose, organic acids secretion by *B. multivorans* ATCC 17616 and the \(\Delta ldhR\) mutant differed mainly by the presence of D-lactate in the WT culture medium and absence in the mutant and consequently a lower medium pH in the former. To understand the physiological adaptations under these acidic conditions and identify genes being possibly under control of the LdhR regulator, expression profiling studies were performed. The transcriptomes of the \(\Delta ldhR\) mutant and WT strain grown in medium with 2% D-glucose were determined at 22 hours of growth with the pH of the culture medium being of 5.2 for the WT and 6.0 for the \(\Delta ldhR\) mutant. A total of 132 genes were differentially expressed, 15 with increased expression and 117 genes with decreased expression (≥1.2-fold lower confidence bound change with a false discovery rate of ≤4.6 %) (Table S1).
Among the genes with increased expression in the mutant we found genes \textit{glnL} and \textit{glnB}-1, encoding a signal transduction histidine kinase and the nitrogen regulatory protein P-II, respectively, as well as genes for the acquisition of inorganic (\textit{amt}, \textit{nark}) and organic nitrogen (\textit{urtA}) (Table 2). Genes encoding nitrogen assimilating enzymes such as \textit{nirB}, \textit{nirD} and \textit{glnA} were also upregulated in the mutant strain, suggesting higher needs of nitrogen for anabolic reactions. When compared to the WT growth, the mutant strain is in a more favorable environment since the culture medium is at higher pH. That is reflected in the downregulation of many genes related to stress response, RNA metabolism and protein synthesis (Table 2).

Regarding stress response we observed the downregulation of \textit{rpoH} gene encoding RNA polymerase factor sigma-32, as well as several genes encoding peptidases/proteases (\textit{Bmul}_0546, \textit{lon}, \textit{clpB}), heat-shock proteins (\textit{Bmul}_2055, \textit{Bmul}_2056, \textit{Bmul}_2384, \textit{hslU}, \textit{grpE}) and chaperones (\textit{groEL}, \textit{groES}, \textit{dnaK}), and \textit{katE} gene encoding catalase. Genes whose products are involved in synthesis (\textit{rpoZ} gene encoding the omega subunit of DNA-directed RNA polymerase) or degradation (\textit{dnaK}, \textit{groEL} and \textit{hfq2}) of RNA showed decreased expression in the \textit{ΔldhR} mutant. In the same line of evidence, 31 genes of ribosomal proteins, the \textit{infC} gene encoding a translation initiation factor IF-3, \textit{thrS} encoding threonyl-tRNA synthetase and \textit{map} gene encoding a methionine aminopeptidase were downregulated in the mutant (Table S1). In terms of central metabolic pathways few differentially expressed genes were found. Of relevance is the increased expression of \textit{cydA} in the WT. This gene encodes the cytochrome bd ubiquinol oxidase subunit I, an enzyme less prone to inhibition by oxidative stress, enabling aerobic metabolism to continue under adverse conditions. Regarding secondary metabolism, there is a cluster of genes (\textit{Bmul}_5943 to \textit{Bmul}_5949) whose expression is downregulated in the \textit{ΔldhR} mutant (Table 2). The products of these genes are homologous to
methyltransferases, cytochrome P450, and Rieske (2Fe-2S) domain-containing proteins and might be required for the production of an unknown metabolite. It is also of notice the decreased expression in the ΔldhR mutant of adhesin BapA which has been implicated in biofilm formation.

LdhR is required for planktonic cellular aggregates formation and adhesion to surfaces. During aerobic batch growth we observed striking differences between the WT and the ΔldhR mutant. After 72 hours of growth in the presence of 0.5% or 2% of sugars like D-glucose, D-galactose, D-fructose, D-mannitol, and D-mannose, we notice the formation of macroscopic cellular aggregates by the WT strain *B. multivorans* ATCC 17616. These cellular aggregates could reach up to 2-3 mm diameter after 3 days of growth. In contrast, the ΔldhR mutant generated a more homogeneous cell suspension with occasional small aggregates (Fig. S4A).

To determine when these planktonic cellular aggregates started to form, WT and ΔldhR mutant strains were incubated in liquid medium containing 2% D-glucose for 24 hours and samples analyzed by microscopy. Wild-type strain planktonic aggregates of different sizes were visible at 4 hours incubation (size range of 3-30 μm) and its number and size increased until the end of the experiment (size range 50-1000 μm) (Fig. 8A). Contrastingly, the ΔldhR mutant was able to form aggregates by 24 hours, but their size range was within 10-60 μm. Similar results were obtained for both strains when D-mannitol (not shown) or D-fructose were used in the growth medium (Fig. S4B).

To understand whether cellular aggregates formation is also dependent on the expression of *ldhA* gene encoding a D-lactate dehydrogenase, the WT and ΔldhR mutant were complemented
with genes ldhR or ldhA alone or simultaneously and grown in liquid medium containing D-
mannitol. Microscopy analysis of the aggregates formed by the WT overexpressing the
different genes showed dense aggregates with irregular surfaces with numerous ramifications
for all of them (Fig. 8B). Planktonic aggregates formed by the ΔldhR mutant with the empty
vector or expressing ldhR gene alone are of a small size, but when ldhA is co-expressed with
ldhR, aggregates of higher dimension and ramification are evident (Fig. 8B). Expression of
ldhA gene alone in ΔldhR mutant restored the formation of macroscopic cellular aggregates,
but in lower number than complementation with ldhRA. This result suggests that planktonic
cell aggregation also depends on ldhA gene product activity, but it is not the only determinant.

To evaluate whether adhesion to surfaces in the form of biofilms was also altered, the WT
and ΔldhR mutant complemented with the several genes were grown in 24-well plates with
agitation for 24 hours in medium supplemented with D-mannitol. Results shown in Figure 8C
indicate that biofilm formation is approximately 40% lower in the ΔldhR mutant when
compared to the WT strain (P <0.001). An image of the plastic surface before crystal violet
staining shows macroscopic WT aggregates attached to surface while the ΔldhR mutant formed
a smooth surface (Fig. 8C). Complementation of the ΔldhR mutant with ldhA gene had slight,
but statistically significant (P <0.05), increase in biofilm formation while complementation
with both ldhRA genes fully recovered biofilm formation to levels even higher than the WT
strain. Overexpression of ldhR or ldhA alone in the WT strain had a small increase in biofilm
formation, but when both genes were expressed this increase was of 45% (Fig. 8C). Together,
these results suggest that LdhR is relevant for surface attached biofilm formation, and although
the D-lactate dehydrogenase activity of LdhA protein contributes to it, is not the only factor
affecting biofilm formation.
Planktonic aggregates formation is a trait shared by several *B. cepacia* complex strains. To evaluate whether planktonic aggregates are common across the *B. cepacia* complex, we tested 74 additional strains from 9 different species, most of them isolated from CF patients’ lung infections, and we included also 4 different *P. aeruginosa* strains. Microscopy analysis revealed that 40 out of 74 *Burkholderia* strains showed cellular aggregates larger than 10 μm when grown in medium containing 2% D-mannitol for 48 hours with agitation (Fig. 9). The size and structure of the aggregates was variable and strain specific. *B. multivorans*, *B. ambifaria* and *B. contaminans* were the species with more strains being able to form planktonic cellular aggregates. The four *P. aeruginosa* strains tested, all isolated from CF patients’ infections, were also able to form cellular aggregates of different sizes (Fig. 9, panel Q and R). This analysis shows that a considerable number of CF pathogens can grow as planktonic cellular aggregates in addition to single cells.

Finally, to evaluate whether EPS plays a role in cellular aggregate formation, the mucoid *B. multivorans* VC5602 and a nonmucoid mutant derivative with a frameshift mutation in the *bceF* gene required for EPS biosynthesis were grown for 3 days in D-mannitol rich-medium. After inspection of the cultures, we found planktonic cellular aggregates in both strains, indicating that EPS was not relevant for cellular aggregate formation under the conditions tested.
DISCUSSION

This work presents the functional analysis of *B. multivorans* ATCC 17616 LdhR regulator and the D-lactate dehydrogenase LdhA. Despite the same genetic organization and high homology levels with ScmR and LdhA from *B. thailandensis* E264, there are striking differences between them. One important difference might be the dependence of quorum sensing (QS) to induce *scmR* (21), but not *ldhR* gene expression. Although we cannot exclude the possibility of *ldhR* expression being QS-dependent, the lack of a *lux*-box sequence in the *ldhR* upstream region and the detection of D-lactate during exponential growth phase give support to the non QS-dependency of *ldhR* gene expression. Other striking differences are in virulence and biofilm formation. While ScmR represses virulence in *Caenorhabditis elegans* and biofilm formation (21), we found no differences between WT and ΔldhR mutant when infecting *Galleria mellonella* larvae (data not shown) and our observations implicate LdhR as an activator of biofilm formation. Furthermore, ScmR is both a repressor and activator of secondary metabolism (regulate the synthesis of peptides, bacteriocins, acids) while our transcriptomic data suggests LdhR as being required for the expression of perhaps only one cluster of genes possibly involved in some unknown secondary metabolite production. Despite these differences between the regulatory networks controlled by the two regulators, both studies showed the upregulation of several genes involved in stress response in the WT cells. Altogether, our work and the one of Mao and coworkers shows an interesting example on how subtle changes in regulatory and/or coding sequences of otherwise similar regulatory proteins, can affect differently the same phenotypes, preventing general conclusions on their role among different (but related) species.
Although *B. multivorans* LdhR does not seem to have such strong involvement in secondary metabolism as ScmR, it still has an important role in regulating carbon overflow, especially when D-glucose is used as carbon source. The metabolism of D-glucose by *B. multivorans* ATCC 17616 (formerly *Pseudomonas cepacia* 249) was investigated previously (14). These authors estimated that the rate of dissimilation of glucose to gluconate and 2-ketogluconate via the direct oxidative pathway exceeds the rate of conversion of glucose to glucose-6P via the phosphorylative pathway by a factor of at least 12, concluding that the predominant route of glucose utilization in this strain is the direct oxidative pathway. Furthermore, they also reported the culture medium acidification in the presence of D-glucose. Our data fully confirm these observations, but we further demonstrated that medium acidification is caused by accumulation of gluconate, 2-ketogluconate, and D-lactate, and this strong pH decrease leads to loss of cell survival. Similarly to D-glucose, D-galactose utilization in *B. multivorans* ATCC 17616 also resulted in D-lactate secretion, but the culture medium pH never decreased below a critical level and cells were able to adapt and resume growth. The most likely explanation is the lower rate of D-galactose consumption when compared to D-glucose as shown in Figure 7D.

Our data on the consumption of sugars such as D-fructose and D-mannose, and the sugar-alcohol D-mannitol by *B. multivorans* ATCC 17616 showed their dissimilation through the phosphorylative pathway. These compounds are transported across the plasma membrane, converted into fructose-6P prior to gluconate-6P formation and entering the Entner-Doudoroff pathway (Fig. 1). Possibly due to transport limitations and/or enzymatic activity of the phosphorylative pathway enzymes, the consumption rate of these sugars is considerably lower when compared to D-glucose. As a consequence, no accumulation of organic acids was detected and the culture medium pH remained close to neutrality.
The role of LdhR, and especially LdhA, in carbon overflow seems to be dependent on the sugar being consumed and on the dissimilation rate. The faster catabolism of D-glucose and D-galactose (to a lesser extent) by the Entner-Doudoroff pathway leads to increased formation of glyceraldehyde-3P and pyruvate. To regenerate the NADH used in the previous reactions, pyruvate is converted into lactate (and possibly other acids) which is also secreted to the culture medium, further contributing to the extracellular acidification observed in the WT strain. In the absence of LdhR and consequently also the absence of D-lactate dehydrogenase LdhA activity (although there was still lactate dehydrogenase activity in the crude extracts), no secretion of D-lactate was observed and the drop in pH was attenuated. The absence of carbon overflow caused by the slower catabolism of sugars that use the phosphorylative pathway puts lower pressure into NADH regeneration, and LdhA might have a more limited role in regulating metabolic fluxes.

We have shown that growth of several strains from different species of the B. cepacia complex reveals a strain-dependent utilization of a pathway for D-glucose dissimilation. These differences might be caused mainly by the enzymatic activity of glucose dehydrogenase. Lessie and coworkers have shown that B. multivorans ATCC 17616 expressed the enzyme glucose dehydrogenase constitutively (23). Our enzymatic activity on the same strain and in B. dolosa CEP1010 confirmed this result. In addition, we observed that B. cenocepacia ATCC 17765 which has a much lower level of glucose dehydrogenase activity, has an increase of glucose-6P dehydrogenase activity and is most likely using the phosphorylative pathway for D-glucose dissimilation. These differences in D-glucose consumption might be relevant in an environment where several organisms compete for the same carbon sources. The fast conversion of glucose into less accessible compounds such as gluconate and 2-ketogluconate, together with the
lowering of the surrounding pH will certainly exclude some competitors. Another advantage is that protons generated from these oxidation steps contribute directly to transmembrane proton motive force and therefore to ATP synthesis (24).

Expression data showed that another side effect of organic acids secretion when cells are grown in excess of D-glucose is the induction of a stress response possibly against intracellular acidification. Since the pKₐ for D-lactate, gluconate and 2-ketogluconate is 3.86, 3.39 and 2.67, respectively, a major cause of intracellular acidification might be the entrance of D-lactic acid. Our data shows that cultures where the pH stayed above 4 (Table 1) were still able to survive and resume growth, but once the pH was below this value, cells lost viability rapidly. D-lactate is a weak acid which means that an increase of the undissociated form occurs with lowering of the pH. This undissociated form is capable of diffusing through the cell membrane, affecting its structure and causing intracellular acidification as the acid dissociates in the cytosol and releases protons. The ΔldhR mutant grown in excess of D-glucose lowers the culture medium pH to 4.4, but this value seems to be harmless because cells continue growth and eventually consume the organic acids. This different behavior is reflected in the transcriptomic data which confirms the stronger (although inefficient under our in vitro conditions) induction of stress response mechanisms, including increased expression in the WT of the gene encoding the heat shock response transcriptional regulator RpoH, as well as several genes encoding heat shock proteins, chaperones and the proteases ClpB, ClpS, Lon and HslU. An increase of these proteins in the cell would help to fold proteins and degrade the denatured ones. A study of the sigma factor RpoH1 in the regulation of Sinorhizobium meliloti genes upon pH stress also identified several RpoH1-dependent genes as differentially expressed, including the upregulation of genes encoding heat shock proteins like IbpA, GrpE, GroEL5, Hsp20, proteases
such as ClpA, ClpB, ClpS1, ClpP2, ClpX, DegP1, Lon, and HslV, as well as the
downregulation of genes involved in translation and nitrogen metabolism like NarB, NirB,
NirD, GlnK (25). The upregulation of stress response genes in the WT *B. thailandensis* E264 is
attributed as an adaptation to stationary phase (21). Yet, it cannot be excluded that this
induction of stress response gene expression might result from the presence of high
concentration of toxic secondary metabolites in the culture medium.

One of the main findings implicates LdhR and, to a certain extent, also LdhA in planktonic
cellular aggregates formation. These aggregates are free-floating biofilm-like structures not
requiring a surface to attach to. Growth of *P. aeruginosa* PAO1 in liquid batch cultures
confirmed the preferential formation of planktonic cellular aggregates during the growth phase
as opposed to free cells, but under stress conditions, such as the ones imposed by nutrient
limitation these aggregates disperse into single cells as reflected by an increase in optical
density (20). Contrastingly to *P. aeruginosa*, we did not observe the sudden increase of optical
density at the beginning of the stationary phase and the number/size of the *Burkholderia*
aggregates continued to increase with time. Planktonic cellular aggregates are particularly
relevant for bacteria infecting the CF lungs as it has been shown that *P. aeruginosa* is found in
lung tissues near the epithelial cell surface as non-surface attached microcolonies (18). In
another study that examined *P. aeruginosa* and *B. cepacia* complex infected CF lung tissues by
immunostaining, *P. aeruginosa* was found in the form of microcolonies while *B. cepacia*
complex bacteria were found both as single cells and as cellular aggregates (2). At least two
regulators have been shown to influence microcolony formation by *P. aeruginosa*. One of them
is the LTTR BvIR whose inactivation prevented microcolony formation, but the mechanism is
unknown (19). The other identified regulator is the two-component regulator MifR, with the
**mifR** mutant biofilms exhibiting thin, structure lacking microcolonies (17). This phenotype was dependent on pyruvate utilization since inactivation of genes encoding lactate dehydrogenase and aconitate hydratase abrogated microcolony formation in a manner similar to **mifR** inactivation, suggesting that fermentation of pyruvate is required for microcolony formation. An explanation is that within microcolonies *P. aeruginosa* cells experience oxygen limiting but energy rich conditions and use pyruvate fermentation as a means of redox balancing allowing microcolony formation and biofilm development (17). The contribution of D-lactate dehydrogenase for *B. multivorans* ATCC 17616 planktonic aggregates and biofilm formation might also be due to the anoxic environment in the interior of the aggregate, inducing cells to ferment pyruvate as a means of redox balancing. Nevertheless, LdhA activity is not the only factor involved in cellular aggregates and biofilm formation, as shown by the partial complementation of these phenotypes by the mutant strain. Adhesin BapA might also contribute to the formation of these planktonic cell aggregates and biofilms, since its expression was upregulated in the WT strain. This adhesin has been implicated in *B. cenocepacia* microcolony formation and its inactivation gave rise to a porous and disconnected biofilm (26). A study carried out in *B. thailandensis* showed the role of C8-homoserine lactone in cell aggregation once a sufficient population number was reached, implicating quorum-sensing in the self-aggregation phenotype (27). Further studies need to be done in *B. multivorans* to assess these possibilities.

Our data showing higher biofilm formation ability of the WT strain, with visible aggregates attached to the surface, in contrast to the smooth biofilm formed in lower amount by the ΔldhR mutant is in line with a study to determine the relative fitness of single cells and preformed aggregates during early development of *P. aeruginosa* biofilms, which showed a single cells
density-dependent fitness of the aggregates (28). These authors showed that when growth resources are abundant, aggregates have a disadvantage because the aggregate’s interior has poor access to resources. However, if competition for resources is high, aggregates have higher fitness because they can protrude above the surface and cells at the top of the aggregate have better contact to growth resources. Another possible link between biofilm formation and the LdhR regulator was observed for an experimentally evolved *B. cenocepacia* HI2424 biofilm during 1050 generations of selection (29). Mutation analysis revealed early beneficial mutations in the gene encoding the LdhR homologue (Bcen2424_0826), generating new haplotypes. Three different mutations were identified in the three ecotypes and consisted of a two-codon deletion and a SNP (Δ38A, Δ39M, L40V) which mapped into the DNA-binding domain (Fig. S1A). The effect of these mutations in the DNA-binding ability of the regulator and its effect on gene expression are unknown. These observed mutations might be beneficial for growth in the D-galactose minimal medium used in that study, but can also reflect selection for biofilm production.

The production of EPS, namely cepacian, is a widespread trait in the *B. cepacia* complex bacteria (6, 7). Although the genes encoding the proteins involved in cepacian biosynthesis are well known (5), the regulatory elements controlling the expression of this phenotype remain unknown. The exception is the regulator σ^54_ which has been shown to positively regulate EPS production in *B. cenocepacia* grown under nitrogen starvation (30). Here, we have shown a negative effect in EPS biosynthesis by LdhR and LdhA, but this might be a consequence of planktonic cellular aggregates formation. Cells of the ΔldhR mutant grow as single cells and small aggregates and most of them contribute to EPS production. Contrastingly, WT aggregates are much larger and possibly have smaller contributions to EPS biosynthesis, explaining the
lower yield in the presence of carbon sources such as D-mannitol, D-mannose, and D-fructose. Additionally to cell aggregation having an influence in EPS biosynthesis, we observed that EPS production does not seem to influence planktonic aggregate formation. Indeed, the EPS producer *B. multivorans* VC5602 and its isogenic mutant VC5602-nmv1 deficient in EPS due to a mutation in *bceF* gene, both form aggregates, although we did not assess whether they are structurally similar. The downregulation of *ldhR* gene expression in nonmucoid isolates can be explained as a genetic program for adaptation to different oxygen tensions. During growth in liquid medium, nonmucoid cells are not exposed to significant limitation to oxygen diffusion, while the presence of EPS surrounding mucoid cells would create a somewhat less aerated environment. In this last circumstance, cells might sense some degree of oxygen limitation and induce alternative ways, such as pyruvate fermentation, to obtain energy.

Although *ldhR* or *ldhA* genes were not found as mutated in serial isolates of *B. multivorans* and *B. dolosa* sampled from long-term CF lung infections (31, 32), a possible role in these persistent infections where oxygen gradients are present and fermentation is an alternative to obtain energy, cannot be excluded. The observation of *B. cepacia* complex bacteria being present in the mucus layer also as cell aggregates (2) is an indication of the relevance of this type of growth, possibly providing additional resistance against antimicrobials and the immune system. Since planktonic cellular aggregates formation was observed in almost all tested *B. cepacia* complex species and in more than 50% of the CF isolates analyzed, these are good indications on the possible role of LdhR and LdhA as persistence determinants, and more research into their function is needed.

In conclusion, we have shown that the LTTR LdhR and the D-lactate dehydrogenase LdhA are implicated in planktonic cellular aggregates and in biofilm formation, a property possibly
relevant in natural environments and within hosts. These cellular aggregates have decreased oxygen gradients towards the center and fermentation of pyruvate would allow these cells to stay viable. We also showed the role of LdhA in the production of D-lactate to decrease the overflow of metabolic intermediates caused by dissimilation of excess of sugars such as D-glucose and D-galactose. The fast catabolism of preferred carbon sources into organic acids is especially advantageous in natural environments. Overall our findings evidence the important role of LdhR regulatory circuits for cell adaptation to diverse environments.
MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 3. *E. coli* was grown at 37°C in Lennox Broth (LB) with or without agar, supplemented with kanamycin (50 µg/ml), trimethoprim (50 µg/ml), or chloramphenicol (25 µg/ml) when required to maintain selective pressure. *Burkholderia* strains were grown in LB or in S medium (12.5 g/l Na$_2$HPO$_4$.2H$_2$O, 3 g/l KH$_2$PO$_4$, 1 g/l K$_2$SO$_4$, 1 g/l NaCl, 0.2 g/l MgSO$_4$.7H$_2$O, 0.01 g/l CaCl$_2$.2H$_2$O, 0.001 g/l FeSO$_4$.7H$_2$O, 1 g/l yeast extract, 1 g/l casamino acids, pH 7.2) (33) supplemented with 2% (w/v) of one of the following carbon sources: D-glucose, D-mannitol, D-galactose, D-mannose, or D-fructose at 37°C with 200 rpm of orbital agitation. Growth medium for *B. multivorans* was supplemented with the following antibiotics: trimethoprim (100 µg/ml), ampicillin (100 µg/ml), and chloramphenicol (200 µg/ml).

DNA manipulation and cell transformation techniques. Genomic DNA from *Burkholderia* was extracted by using the DNeasy blood and tissue kit (Qiagen), following the manufacturers’ recommendations. Plasmid DNA isolation and purification, DNA restriction, agarose gel electrophoresis, DNA amplification by PCR, and *E. coli* transformation were performed using standard procedures (34). *Burkholderia* electrocompeotent cells were transformed by electroporation using a Bio-Rad Gene Pulser II system (200 Ω, 25 µF, 2.5 kV) and grown overnight before being plated on selective medium. Triparental conjugation to *B. multivorans* strains was performed using the helper plasmid pRK2013.
**Mutant construction.** The 1791-bp HindIII/XbaI upstream region of \(ldhR\) (Bmul_2557) gene was amplified by PCR from \(B.\ multivorans\) ATCC 17616 genomic DNA using the primers Bmul2557L (fwd/rev) (Table 4). After digestion with the appropriate restriction endonucleases, the fragment was cloned into pBCKS vector giving rise to pAT312. The 1800-bp XbaI/SacI downstream region of \(ldhR\) was amplified using the primers Bmul2557R and cloned into the same restriction sites of pAT312 and the resulting plasmid was named pAT812. A fragment containing the trimethoprim resistance cassette from pUC-TP was then cloned into the XbaI site of pAT812 originating pAT812-Tp. To delete the gene \(ldhR\) from \(B.\ multivorans\) ATCC 17616, pAT812-Tp was introduced into this strain by electroporation. Recombinant colonies were first selected in the presence of trimethoprim and counter selected in a medium supplemented with chloramphenicol. Gene deletion was confirmed by PCR amplification followed by DNA sequence determination.

**Complementation assays.** A 1,070-kb NdeI/XbaI fragment containing \(ldhR\) gene was amplified by PCR from \(B.\ multivorans\) ATCC 17616 genomic DNA using primers P1 (fwd/rev) (Table 4). The fragment was cloned into the NdeI/XbaI restriction sites of pLM135-5, a pUK21-derivative plasmid carrying 0.4-kb containing the bce promoter region directing the expression of the \(bce\) operon required for cepacian biosynthesis. The resulting pMM137-1 intermediate plasmid was digested with HindIII and XbaI and the 1.47-kb fragment containing the bce promoter and \(ldhR\) gene was cloned into vector pBBR1MCS, resulting in plasmid pMM137-2 (Table 3). The same strategy was used to clone \(ldhA\) gene (amplified with P2 (fwd/rev) primers) under the control of the bce promoter, resulting in plasmids pLM016-1 and the final pLM016-2. To clone the \(ldhRA\) genes under the control of their own promoter, a 2.7-
kb fragment of *B. multivorans* ATCC 17616 genome containing the upstream region of *ldhR* and the coding sequence of *ldhR* and *ldhA* genes was amplified by PCR using P3 (fwd/rev) primer sequences. The amplified DNA was restricted with HindIII and ligated to pBBR1MCS originating plasmid pARG015-1 (Table 3). After *E. coli* DH5α transformation and clone selection, the inserted genes were confirmed by DNA sequence determination. Plasmids pMM137-2, pLM016-2 and pARG015-1 were mobilized into *B. multivorans* ATCC 17616 and the Δ*ldhR* mutant by triparental conjugation. Transformants were selected in LB plates supplemented with 100 µg/ml of ampicillin and 200 µg/ml of chloramphenicol.

**Isolation of RNA samples.** For qRT-PCR and microarray analyses, cells were grown in S medium with 2% D-mannitol or D-glucose for 22 hours at 37°C. For reverse transcription-PCR cells were grown for 18 hours in S medium supplemented with 2% D-mannitol under the same conditions. Three biological replicates were obtained for each tested strain. For RNA analysis bacterial cells were resuspended in RNAprotect bacteria reagent (Qiagen), and total RNA extraction was carried out using the RNeasy MiniKit (Qiagen) by following manufacturer’s recommendation. RNA was treated with DNase (RNAse-free DNase, Qiagen) for 1 hour at room temperature following manufacturer’s protocol and total RNA concentration was assessed using NanoDrop ND-1000 spectrophotometer. RNA integrity for microarray analysis was checked on an Agilent 2100 Bioanalyzer using an RNA Nano assay.

**Quantitative real-time RT-PCR.** Total RNA was used in a reverse transcription reaction with TaqMan Reverse Transcription Reagents (Applied Biosystems). qRT-PCR amplification of genes *ldhR*, *ldhA*, and *trpB* (for primer sequence see Table 4) was performed with a model
7500 thermocycler (Applied Biosystems). The expression ratio of the target genes relative to
the reference gene trpB, which showed no variation in the transcription abundance under the
conditions tested, was determined. Relative quantification of gene expression by real-time
qRT-PCR was determined using the ∆∆Ct method (35).

**Reverse transcription-PCR.** To evaluate if ldhR and ldhA genes are co-transcribed, a
semi-quantitative RT-PCR was performed. Total RNA from *B. multivorans* ATCC 17616
grown in S medium supplemented with D-mannitol for 18 hours at 37°C 200 rpm was
extracted. A total of 200 ng of total RNA was used for reverse-transcription reaction using
Taqman reagent kit (Applied Biosystems, Roche). Synthesized cDNA was used as template for
25-µl PCR reactions with 0.2 µM primers 2557/2558_RT or 2558/2559_RT (Table 4), 0.2 mM
deoxyribonucleosides triphosphate, 1.6 mM MgSO4, 1× PCR amplification buffer, and 2.5 U of
Taq polymerase (Bioline). Amplification occurred as following: initial denaturation at 95°C for
5 minutes; 30 cycles of 30 seconds at 95°C, 45 seconds at 52°C, and 30 seconds at 72°C; final
extension at 72°C for 10 minutes.

**Processing of RNA samples for transcriptomic analysis.** Total RNA was processed for
use on Affymetrix custom *Burkholderia* arrays, according to the manufacturer’s Prokaryotic
Target Preparation Assay as described previously (16). Biological triplicates of RNA from
each bacterial culture were processed and analyzed.

**Microarray analysis.** Scanned arrays were analyzed with Affymetrix Expression Console
software to guarantee that all quality parameters were in the recommended range. Subsequent
analysis was performed with DNA-Chip Analyzer 2008. Since the used custom arrays represents two different *Burkholderia* species (16), a digital mask leaving for analysis only the 9610 probe sets representing *B. multivorans* ATCC 17616 transcripts was applied. Then the 6 arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (36). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a Perfect Match (PM)-only model (37). Replicate data (triplicates) for each bacterial isolate was weighted gene-wise by using inverse squared standard error as weights. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change (LCB) between experiment and baseline was above 1.2. The lower confidence bound criterion meant that we could be 90% confident that the fold change was a value between the lower confidence bound and a variable upper confidence bound. Li and Wong have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression (36).

**Exopolysaccharide quantification.** The amount of EPS was assessed based on dry weight of the ethanol-precipitated polysaccharide recovered from triplicates of 100 ml culture samples of the different strains grown in liquid S medium supplemented with 2% (w/v) of different sugars over 3 days at 37°C with 200 rpm of orbital agitation as previously described (33).

**Determination of carbon source and organic acids concentration in the culture medium.** The supernatant of each culture (2 ml) was obtained by centrifugation at 14,000 g for 10 minutes followed by filtration through 0.2 μm Whatman membrane filter. Carbon source
consumption and organic acids production were monitored by high pressure liquid chromatography (HPLC) using an Aminex HPX-87H (BioRad) ion exchange chromatography column, eluted at 65°C with 5 mM H$_2$SO$_4$ at a flow rate of 0.6 ml/min for 30 min and detected by UV-Visible (UV-Vis) and refractive index (RI) detectors. Linearity of the method was tested and concentrations were estimated based on calibration curves.

**Preparation of bacterial extracts and enzyme assays.** Bacteria were grown in 50 ml of S medium supplemented with 2% D-glucose or D-mannitol at 37°C for 20 hours and cells collected by centrifugation. After being washed, pellets were stored at -80°C until used. Thawed cells were then suspended in 20 mM phosphate buffer, pH 6.8, and disrupted by sonication for 8 cycles of 30 seconds. For assays of glucose or gluconate dehydrogenases, lysates were supplemented with 1% (v/v) Triton-X100. Lysates were then centrifuged for 30 minutes at 12,000 g, and supernatant fractions were immediately assayed for enzyme activities.

*In vitro* activities of D-glucose or D-gluconate dehydrogenases (E.C. 1.1.5.2 and E.C. 1.1.99.3, respectively) were measured at 37°C by monitoring the glucose or gluconate-dependent reduction of 0.3 mM 2,6-dichlorophenol indophenol (DCPIP) by following the decrease in absorbance of the assay mixtures at 600 nm with a spectrophotometer (UV-1700 PharmaSpec, Shimadzu). Reaction mixtures contained 150 mM phosphate buffer pH 6.8, 10 mM D-glucose (or sodium D-gluconate), 0.3 mM phenazine methosulfate (PMS), and bacterial protein extract as described previously (13). The enzyme activity was calculated using and extinction coefficient of 20600 M$^{-1}$cm$^{-1}$ for DCPIP. Activity of glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49) was determined at 37°C by monitoring substrate-dependent formation of reduced nicotinamide adenine dinucleotide in reaction mixtures at 340
nm. Reaction mixtures contained 200 mM Tris·HCl pH 8.5, 0.5 mM NAD\(^+\), 10 mM glucose-6-phosphate, and bacterial protein extract. The enzyme activity was calculated using and extinction coefficient of 6220 M\(^{-1}\)cm\(^{-1}\) for NADH. Total activity of lactate dehydrogenase was determined at 37°C by monitoring oxidation of NADH at 340 nm in the presence of sodium pyruvate. Reaction mixtures contained 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) pH 7.0, 0.12 mM NADH, 20 mM sodium pyruvate, and bacterial protein extract. One unit was defined as the amount of enzyme converting 1 nmol of substrate/minute. Protein concentrations were determined by using the Bradford method (BioRad reagent), using bovine serum albumin as a standard protein. Enzymatic activities were performed from two biological replicates and each reaction was performed five times.

Microscopy analysis. B. multivorans ATCC 17616 and the \(\Delta ldhR\) deletion mutant with/without complementation were grown up to 72 hours in S medium supplemented with D-glucose or D-mannitol. Images were acquired on a commercial Leica High Content Screening microscope, based on Leica DMI6000 equipped with a Hamamatsu Flash 4.0 LT sCMOS camera, using a 10x 0.3 NA objective, and controlled with the Leica LAS X software (Fig. 8A, Fig. S4B), or a Zeiss Axioplan equipped with a Photometrics CoolSNAP fx camera, using a 10x 0.3 NA objective and controlled with software MetaMorph version 4.6r9 (Fig. 8B, Fig. 9) or a Pentax *ist DS digital camera (Fig. 8C, Fig. S4A). Cellular aggregate size was estimated with a ruler incorporated in analysis software (Adobe Photoshop CS5) and result from at least 50 measures. Due to the weight of the coverslip the aggregates are flattened.
**Adhesion to surfaces and biofilm formation.** To elucidate the role of *ldhR* gene in biofilm formation, cells were grown in a 24-well plate system modified from the procedure of Caiazza and O’Toole (38). Briefly, overnight cultures were adjusted to an OD 600 nm of 0.1 in fresh S medium supplemented with 2% D-mannitol and 100 µg/ml chloramphenicol and grown at 37°C, 200 rpm in 24-well microtiter plates (Orange Scientific) at 45° angle, ensuring that the air-liquid interface crossed the center of the flat-bottomed well. After 24 hours, cell cultures were removed, wells washed three times with saline buffer and stained for 15 minutes with crystal violet (1% solution). After washing, the extent of biofilm formation was quantified as previously described (33).

**In silico analysis of nucleotide and amino acid sequences.** The algorithm BLAST (39) was used to compare sequences of the deduced LdhR and LdhA amino acids to database sequences available at the National Center for Biotechnology Information (NCBI) or the Burkholderia Genome Database (http://www.burkholderia.com/bgd). Alignments were performed using the program ClustalW (40). Molecular evolutionary relationships between LdhA and its homologues was performed using full-length protein sequences of all 185 members of the d-isomer specific 2-hydroxyacid dehydrogenase family (PROSITE documentation entry PDOC00063; last updated April 2006). Analyses were conducted using the software package MEGA version 6.0 (41) which uses ClustalW for alignment and a neighbor joining method of tree construction.

**Statistical analysis.** All quantitative data were obtained from at least three independent assays with two biological replicates. Error propagation was used to calculate standard errors.
and one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were performed to assess statistical significance. Differences were considered statistically significant if the $P$ value was lower than 0.05.

**Microarray data accession number.** Microarray data were deposited in the Gene Expression Omnibus (GEO) repository at NCBI under accession numbers: GSE97006.

**Ethics statement.** The bacterial clinical isolates are from an anonymous patients and none of them was taken specifically for this study.
ACKNOWLEDGMENTS

Mário R. Santos from Instituto Gulbenkian de Ciência, Oeiras (IGC), Portugal, is acknowledged for technical assistance with microscopy analysis. Microarrays were processed at the IGC’s Gene Expression Unit. This work was supported by Programa Operacional Regional de Lisboa 2020 (LISBOA-01-0145-FEDER-007317) and Fundação para a Ciência e a Tecnologia, Portugal (PTDC/QUI-BIQ/118260/2010, UID/BIO/04565/2013) and a post-doctoral grant (SFRH/BPD/86475/2012) to I.N.S.
REFERENCES


44. Ferreira AS, Silva IN, Fernandes F, Pilkington R, Callaghan M, McClean S, Moreira LM.


Fig. 1. Alternative pathways of gluconate-6P formation by dissimilation of glucose in *Burkholderia*. Glucose utilization can follow the direct oxidative pathway (orange arrows) or the phosphorylative pathway (blue arrows). Catabolism of other monomeric carbon sources (marked with brown boxes) is also indicated. Central metabolic pathways are: glycolysis (G), Entner-Doudoroff pathway (ED), pentose-phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, and gluconeogenesis (GN). Other abbreviations: 2KG, 2-ketogluconate; KGP, 2-ketogluconate-6P; KDPG, 2-keto-3-deoxy-gluconate-6P; gcd, glucose dehydrogenase; gad, gluconate dehydrogenase; gntK, glucono kinase; kgk, 2-ketoglucono kinase; kgr, KGP reductase; zwf, glucose-6P dehydrogenase; glk, glucokinase; edd, glucose-6P dehydratase; eda, KDPG aldolase; tpi, triose isomerase; fba, fructose-1,6P aldolase; fbp, fructose-1,6P phosphatase; pgi, phosphogluco isomerase; mdh, mannitol dehydrogenase; aceA, isocitrate lyase; frk, fructokinase; ldhA, D-lactate dehydrogenase; phbA, β-ketothiolase; phbB, acetoacetyl-CoA reductase; phbC, poly-β-hydroxybutyrate synthase; gltA, citrate synthase; acn, aconitate hydratase; icdA, isocitrate dehydrogenase; suc, succinate-CoA transferase; sdh, succinate dehydrogenase/fumarate reductase; fum, fumarate hydratase; mdh, malate dehydrogenase; gap, glyceraldehyde-3P dehydrogenase; pkg, phosphoglycerate kinase; pgm, phosphoglycerate mutase; eno, phosphopyruvate hydratase; pta, phosphate acetyltransferase; ack, acetate kinase; pps, phosphoenolpyruvate synthase; pyk, pyruvate kinase; PHB, poly-β-hydroxybutyrate; IM, inner membrane; and OM, outer membrane. This simplified catabolic pathway was based on reactions available for *B. multivorans* ATCC 17616 at Kyoto Encyclopedia of Genes and Genomes (KEGG) database.
Fig. 2. The LdhR regulator shows decreased expression in nonmucoid variants derived from mucoid *Burkholderia* strains and is co-transcribed with gene *ldhA*. (A) Expression by qRT-PCR of gene *ldhR* in nonmucoid variants when compared with the respective mucoid parental strains of *B. multivorans* D2095, *B. contaminans* IST408, *B. anthina* FC0967, *B. vietnamiensis* PC259, and *B. dolosa* CEP0743. (B) In *B. multivorans* ATCC 17616 the genomic region containing gene *ldhR* and the flanking regions are located in chromosome 1 between the nucleotides position indicated in the figure. The new NCBI locus tags for the indicated genes are BMUL_RS12975 (*Bmul_2556*), BMUL_RS12980 (*Bmul_2557*), BMUL_RS12985 (*Bmul_2558*), and BMUL_RS12990 (*Bmul_2559*). Nucleotide regions upstream genes *ldhR* and *scmR* showing a lux-box (conserved residues in red) preceding *scmR* gene, but absent from *ldhR* upstream region. Asterisks denote conserved nucleotides between the two regions. The putative start codon is underlined. (C) Reverse transcription PCR showing the co-transcription of genes *ldhR* and *ldhA* in *B. multivorans* ATCC 17616 grown for 18 hours in S medium with 2% D-mannitol. The image shows the amplification from genomic DNA (1), cDNA (2), or total RNA (3) of the 319-bp region comprising the end of *ldhR* and beginning of *ldhA* genes; amplification from genomic DNA (4), cDNA (5), or total RNA (6) of the 305-bp region comprising the end of *ldhA* and beginning of *Bmul_2559* genes; M, DNA marker; nt, nucleotides; aa, amino acids.

Fig. 3. Unrooted neighbor-joining tree of the D-isomer specific 2-hydroxyacid dehydrogenase superfamily (PROSITE entry PDOC00063_pattern_1. The evolutionary history was inferred using the Neighbor-Joining method. The analysis involved 185 amino acid sequences. All positions containing gaps and missing data were eliminated. Sequences
clustering together and representing separate enzymatic subgroups are shaded. D-LDH, D-lactate dehydrogenase; SERA, D-3-phosphoglycerate dehydrogenase; PDXB, erythronate-4-phosphate dehydrogenases; FDH, formate dehydrogenase; GHPR, glyoxylate/hydroxyxypyruvate reductases; and CTBP, C-terminal binding protein. B. multivorans LdhA is in red color.

Fig. 4. LdhR decreases exopolysaccharide production. (A) Production of EPS by the WT B. multivorans ATCC 17616 (WT) and the ΔldhR mutant in the presence of different sugars as main carbon source for 3 days at 37°C. EPS production is expressed as ethanol precipitate dry weight (g/l). Significantly greater amount of EPS was produced by the ΔldhR mutant (*, P<0.05; **, P<0.01; ***, P<0.001), by Tukey’s HSD multiple comparison test. (B) Effect on EPS production by the complementation of the wild-type strain or the ΔldhR mutant by expressing in trans the ldhRA genes from pARG015-1 or the empty vector pBBR1MCS. Cells were incubated in the presence of the indicated sugars for 3 days at 37°C followed by EPS quantification. Significantly lower amount of EPS was produced when the ldhRA genes were overexpressed in both WT and mutant strain (*, P<0.05; **, P<0.01; ***, P<0.001), by Tukey’s HSD multiple comparison test.

Fig. 5. Loss of cell viability and medium acidification is dependent on the glucose concentration. Culture growth as measured by turbidity (OD₆₄₀nm) and colony forming units (CFU) plating of B. multivorans ATCC 17616 and the ΔldhR mutant grown for 72 hours in the presence of 2% of D-mannitol (A) and 0.5 or 2% D-glucose (B, C). Culture medium pH in the presence of D-glucose is shown in panel D. Error bars indicate the standard deviation.
Fig. 6. Complementation of the ΔldhR mutant only rescues the wild-type growth properties in glucose-rich medium if gene ldhA is expressed alone or together with ldhR.

Culture growth as measured by turbidity (OD₆₀₀nm) (A) and colony forming units (B) of ΔldhR mutant complemented with empty vector pBBR1MCS, pMM137-2 expressing ldhR gene from the bce promoter, pLM016-2 expressing ldhA gene from the bce promoter, and pARG015-1 expressing ldhRA genes from their own promoter. (C) Culture medium pH measured for the indicated strains. Genotype symbols are consistent for each panel. Cells were grown in medium supplemented with 2% D-glucose. (D) Quantitative RT-PCR analysis of transcript levels of genes ldhR and ldhA in the WT B. multivorans ATCC 17616, ΔldhR mutant, and ΔldhR mutant expressing ldhR gene from pMM137-2. Cells were grown in medium supplemented with D-glucose for 22 hours at 37°C. Error bars indicate standard deviation.

Fig. 7. D-lactate accumulation in the culture medium was only observed for the wild-type strain. Concentration of the organic acids gluconate (GN) and 2-ketogluconate (2KG) (A), and D-lactate (B, right scale) in the supernatants of B. multivorans ATCC 17616 wild-type cells and the ΔldhR mutant in medium supplemented with 2% D-glucose as measured by HPLC. Glucose consumption is shown in panel B (left scale). (C) Levels of the enzymes glucose dehydrogenase (GDH), gluconate dehydrogenase (GNDH), glucose-6P dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) in total extracts of the indicated strains grown for 20 hours in S medium supplemented with 2% D-glucose, or 2% D-mannitol. Significantly lower enzymatic activity was found for the ldhRA mutant when compared to the WT strain (**, P<0.01; ***, P<0.001), by Tukey’s HSD multiple comparison test. (D) Sugar consumption at 48 hours of growth by B. multivorans ATCC 17616 cells and the ΔldhR mutant in medium.
supplemented with 2% (111 mM) D-sugar as measured by HPLC. Glc, D-glucose; Gal, D-galactose; Fru, D-fructose; Man, D-mannose. Error bars indicate the standard deviation.

Fig. 8. Planktonic cellular aggregates development and biofilm formation is dependent on LdhR and LdhA activities. (A) Images of planktonic aggregates formed during growth of *B. multivorans* ATCC 17616 wild-type cells and the ∆ldhR deletion mutant in medium supplemented with 2% D-mannitol for the indicated times. (B) Images of the planktonic cellular aggregates that sediment after 5 minutes static incubation of the indicated cultures grown for 72 hours under constant agitation in medium containing 2% D-mannitol. The bar scale is shown. (C) Images of biofilm cells attached to the solid surface of 24-well microtiter plates after 24 hours of incubation with agitation in medium containing 2% D-mannitol and biofilm quantification by the crystal violet staining method. The bar scale of images from panel C measures 2.5 mm.

Fig. 9. Planktonic growth of several strains from the *B. cepacia* complex leads to cellular aggregate formation. Phase contrast microscopy images of planktonic cellular aggregates obtained after 3 days of liquid batch cultures grown in medium supplemented with 2% D-mannitol (left panel) for the indicated strains. Bar scale shown in panel Q is the same for all other images. Right panel bottom shows the number of isolates of each *B. cepacia* complex species analyzed and their ability to form planktonic cellular aggregates. UTI, urinary tract infection; CF, cystic fibrosis infection; and CGD, chronic granulomatous disease.
Table 1 Acidification of the culture medium and secretion and consumption of D-lactate as a consequence of 2% D-glucose dissimilation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Medium acidification/maximal recovery (pH)</th>
<th>D-lactate secretion/consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. multivorans</em> JTC</td>
<td>Chronic granulomatous disease</td>
<td>4.0/6.3</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC3161</td>
<td>Cystic fibrosis isolate</td>
<td>3.6/3.6</td>
<td>yes/no</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC7495</td>
<td>Cystic fibrosis isolate</td>
<td>4.0/6.5</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC6882</td>
<td>Cystic fibrosis isolate</td>
<td>4.9/6.3</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC12539</td>
<td>Cystic fibrosis isolate</td>
<td>4.8/6.6</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC8086</td>
<td>Cystic fibrosis isolate</td>
<td>4.8/6.6</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC9159</td>
<td>Cystic fibrosis isolate</td>
<td>4.0/6.4</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> VC12675</td>
<td>Cystic fibrosis isolate</td>
<td>4.9/6.2</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. multivorans</em> D2095</td>
<td>Cystic fibrosis isolate</td>
<td>3.5/3.5</td>
<td>yes/no</td>
</tr>
<tr>
<td><em>B. ambifaria</em> CEP0996</td>
<td>Cystic fibrosis isolate</td>
<td>5.1/6.7</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. stabilis</em> LMG 14294</td>
<td>Cystic fibrosis isolate</td>
<td>6.1/6.7</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. anthina</em> J2552</td>
<td>Rhizosphere</td>
<td>5.2/6.9</td>
<td>n.d./n.a.</td>
</tr>
<tr>
<td><em>B. dolosa</em> CEP1010</td>
<td>Cystic fibrosis isolate</td>
<td>3.7/3.7</td>
<td>yes/no</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> ATCC 17765</td>
<td>Urinary tract infection</td>
<td>6.3/7.1</td>
<td>yes/yes</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> J2315</td>
<td>Cystic fibrosis isolate</td>
<td>6.5/6.5</td>
<td>n.d./n.a.</td>
</tr>
<tr>
<td><em>B. cepacia</em> ATCC 25416</td>
<td><em>Allium cepa</em></td>
<td>5.7/7.0</td>
<td>n.d./n.a.</td>
</tr>
<tr>
<td><em>B. contaminans</em> IST408</td>
<td>Cystic fibrosis isolate</td>
<td>4.5/5.9</td>
<td>n.d./n.a.</td>
</tr>
<tr>
<td><em>B. vietnamiensis</em> G4</td>
<td>Industrial waste treatment facility</td>
<td>5.5/6.3</td>
<td>n.d./n.a.</td>
</tr>
</tbody>
</table>

n.d., not detected; n.a., not applicable.

Table 2 Selection of a set of differentially expressed genes, separated by functional groups, when the ΔldhR mutant transcriptome was compared with the one of *B. multivorans* ATCC 17616, obtained in medium supplemented with 2% D-glucose.

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Gene identifier</th>
<th>LB-FCa</th>
<th>Gene name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulatory genes</td>
<td><em>Bmul</em>0486</td>
<td>-1.3</td>
<td><em>rpoH</em></td>
<td>RNA polymerase factor sigma-32</td>
</tr>
<tr>
<td></td>
<td><em>Bmul</em>1123</td>
<td>1.3</td>
<td><em>glnL</em></td>
<td>signal transduction histidine kinase, N₂ specific, NtroB</td>
</tr>
<tr>
<td></td>
<td><em>Bmul</em>1722</td>
<td>-1.3</td>
<td><em>hfq2</em></td>
<td>RNA chaperone Hfq</td>
</tr>
<tr>
<td></td>
<td><em>Bmul</em>2393</td>
<td>-1.3</td>
<td>-</td>
<td>cold-shock DNA-binding domain-containing protein</td>
</tr>
<tr>
<td></td>
<td><em>Bmul</em>2400</td>
<td>-1.2</td>
<td><em>rpoZ</em></td>
<td>DNA-directed RNA polymerase, omega subunit</td>
</tr>
<tr>
<td>Nitrogen acquisition and assimilation</td>
<td><em>Bmul</em>0437</td>
<td>2.4</td>
<td><em>glnB</em></td>
<td>nitrogen regulatory protein P-II</td>
</tr>
<tr>
<td>Bmul_0438</td>
<td>1.4</td>
<td>amt</td>
<td>ammonium transporter</td>
<td></td>
</tr>
<tr>
<td>Bmul_1122</td>
<td>1.8</td>
<td>glnA</td>
<td>glutamine synthetase, type I</td>
<td></td>
</tr>
<tr>
<td>Bmul_1636</td>
<td>-1.4</td>
<td>sbp</td>
<td>ABC transporter periplasmic sulfate-binding protein</td>
<td></td>
</tr>
<tr>
<td>Bmul_2482</td>
<td>1.4</td>
<td>urtA</td>
<td>urea ABC transporter urea binding protein</td>
<td></td>
</tr>
<tr>
<td>Bmul_4146</td>
<td>1.3</td>
<td>narK</td>
<td>major facilitator superfamily MFS_1</td>
<td></td>
</tr>
<tr>
<td>Bmul_4147</td>
<td>1.7</td>
<td>nirB</td>
<td>nitrite reductase (NAD(P)H), large subunit</td>
<td></td>
</tr>
<tr>
<td>Bmul_4148</td>
<td>2.0</td>
<td>nirD</td>
<td>nitrite reductase (NAD(P)H), small subunit</td>
<td></td>
</tr>
<tr>
<td>Bmul_4149</td>
<td>1.3</td>
<td>nos</td>
<td>molybdopterin oxidoreductase</td>
<td></td>
</tr>
</tbody>
</table>

### Carbon metabolism and energy production

| Bmul_2649 | -1.9 | - | oxidoreductase FAD/NAD(P)-binding domain protein |
| Bmul_3307 | -1.4 | cydA | cytochrome bd ubiquinol oxidase subunit I |
| Bmul_3795 | 2.2 | - | TonB-dependent siderophore receptor |
| Bmul_5321 | -1.7 | - | 2-amino-3-ketobutyrate coenzyme A ligase |

### Posttranslational modification, protein turnover, chaperones

| Bmul_0546 | -1.2 | - | peptidase M48 Ste24p |
| Bmul_0776 | -1.3 | clpS | ATP-dependent Clp protease adaptor protein ClpS |
| Bmul_1348 | -1.2 | tig | trigger factor |
| Bmul_1351 | -1.3 | lon | ATP-dependent protease La |
| Bmul_1426 | -1.3 | clpB | ATP-dependent chaperone ClpB |
| Bmul_2055 | -1.5 | - | heat shock protein Hsp20 |
| Bmul_2056 | -1.3 | - | heat shock protein Hsp20 |
| Bmul_2384 | -1.4 | - | heat shock protein Hsp20 |
| Bmul_2528 | -1.5 | groEL | chaperonin GroEL |
| Bmul_2529 | -1.6 | groES | chaperonin Cpn10 |
| Bmul_2633 | -1.7 | dnaK | chaperone protein DnaK |
| Bmul_2635 | -1.4 | grpE | heat shock protein GrpE |
| Bmul_3087 | -1.3 | hslU | ATP-dependent protease ATP-binding subunit HslU |

### Secondary metabolism

| Bmul_5943 | -1.4 | - | deoxyxylulose-5P synthase |
| Bmul_5944 | -1.4 | - | methyltransferase type 12 |
| Bmul_5945 | -1.5 | - | methyltransferase type 12 |
| Bmul_5946 | -1.6 | - | Rieske (2Fe-2S) domain containing protein |
| Bmul_5947 | -1.5 | - | hypothetical protein |
| Bmul_5948 | -1.4 | - | cytochrome P450 |
| Bmul_5949 | -1.4 | - | Rieske (2Fe-2S) domain containing protein |

*LB-FC, lower bound of fold change.*
### Table 3 Strains and plasmids used in this work.

<table>
<thead>
<tr>
<th>Strain and plasmid*</th>
<th>Relevant characteristic</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. multivorans ATCC 17616</td>
<td>Soil isolate, USA; EPS+</td>
<td>(42)</td>
</tr>
<tr>
<td>B. multivorans ΔldhR</td>
<td>ATCC 17616-derivative with the ldhR gene replaced by a trimethoprim resistance cassette</td>
<td>This work</td>
</tr>
<tr>
<td>B. multivorans D2095</td>
<td>Cystic fibrosis isolate, Canada; EPS+</td>
<td>(16)</td>
</tr>
<tr>
<td>B. multivorans D2095-nmv</td>
<td>Nonmucoid variant obtained under nutrient starvation; EPS-</td>
<td>(11)</td>
</tr>
<tr>
<td>B. multivorans VC5602</td>
<td>Cystic fibrosis isolate, Canada; EPS+</td>
<td>(31)</td>
</tr>
<tr>
<td>B. multivorans VC5602-nmv1</td>
<td>VC5602-derivative with a frameshift mutation in bceF gene, EPS-</td>
<td>Moreira LM (unpublished)</td>
</tr>
<tr>
<td>B. multivorans CF-A1-1</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC9159</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC8086</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC10037</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans JTC</td>
<td>Chronic granulomatous disease, USA</td>
<td>(43)</td>
</tr>
<tr>
<td>B. multivorans VC3161</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC7495</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC6882</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC12539</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans VC12675</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. contaminans IST408</td>
<td>Cystic fibrosis isolate, Portugal; EPS+</td>
<td>(44)</td>
</tr>
<tr>
<td>B. contaminans IST408-nmv</td>
<td>Nonmucoid variant obtained under nutrient starvation; EPS-</td>
<td>(11)</td>
</tr>
<tr>
<td>B. contaminans strain E</td>
<td>Cystic fibrosis isolate, Argentina</td>
<td>J. Degrossi</td>
</tr>
<tr>
<td>B. anthina FC0967</td>
<td>Cystic fibrosis isolate, Canada; EPS+</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. anthina FC0967-nmv</td>
<td>Nonmucoid variant obtained under nutrient starvation; EPS-</td>
<td>(11)</td>
</tr>
<tr>
<td>B. anthina J2552</td>
<td>Rhizosphere, UK</td>
<td>(45)</td>
</tr>
<tr>
<td>B. vietnamiensis PC259</td>
<td>Cystic fibrosis isolate, USA; EPS+</td>
<td>(46)</td>
</tr>
<tr>
<td>B. vietnamiensis PC259-nmv</td>
<td>Nonmucoid variant obtained under nutrient starvation; EPS-</td>
<td>(11)</td>
</tr>
<tr>
<td>B. vietnamiensis FC0441</td>
<td>Chronic granulomatous disease, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. vietnamiensis G4</td>
<td>Industrial waste treatment facility, USA</td>
<td>(47)</td>
</tr>
<tr>
<td>B. dolosa CEP0743</td>
<td>Cystic fibrosis isolate, Canada; EPS+</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. dolosa CEP0743-nmv</td>
<td>Nonmucoid variant obtained under nutrient starvation; EPS-</td>
<td>(11)</td>
</tr>
<tr>
<td>B. dolosa CEP0021</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. dolosa CEP1010</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. cepacia LMG 17997</td>
<td>Urinary tract infection, Sweden</td>
<td>(43)</td>
</tr>
<tr>
<td>B. cepacia FC1101</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. cepacia ATCC 25416</td>
<td>Allium cepa, USA</td>
<td>(43)</td>
</tr>
<tr>
<td>B. cepacia PC184</td>
<td>Cystic fibrosis isolate, USA</td>
<td>(48)</td>
</tr>
<tr>
<td>B. cepacia CEP0571</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. cepacia FC0447</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. cepacia ATCC 17765</td>
<td>Urinary tract infection, UK</td>
<td>(43)</td>
</tr>
<tr>
<td>B. cepacia J2315</td>
<td>Cystic fibrosis isolate, UK</td>
<td>(43)</td>
</tr>
<tr>
<td>B. stabilis C7322</td>
<td>Cystic fibrosis isolate, Canada</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. stabilis LMG 14294</td>
<td>Cystic fibrosis isolate, Belgium</td>
<td>(43)</td>
</tr>
</tbody>
</table>
B. ambifaria FC0168  Cystic fibrosis isolate, Canada  D. P. Speert
B. ambifaria CEP0958  Cystic fibrosis isolate, Canada  D. P. Speert
B. ambifaria CEP0996  Cystic fibrosis isolate, Australia  (45)
P. aeruginosa FC1061  Cystic fibrosis isolate, Canada  D. P. Speert
P. aeruginosa 005  Cystic fibrosis isolate, Canada  D. P. Speert
Escherichia coli DH5α  DH5α recA1, lacU169, o80dλacZ ΔM15  Gibco BRL

Plasmids
pRK2013  Tra+ Mob+ (RK2) Km::Tn7 ColE1 origin, helper plasmid, Km’  (49)
pBCKS  3.4-kb phagemid derived from pUC19; lac promoter, Cm’  Stratagene
pUC-TP  pUC-GM derivative with a 1.1-kb Tp’ gene cassette, Ap’ Tp’  (50)
pUK21  3089-bp pUC21 derivative, Km’  (51)
pBBR1MCS  4,717-bp broad-host-range cloning vector, Cm’  (52)
pAT312  pBCKS derivative containing the 1719-bp HindIII/XbaI fragment upstream of ldhR gene  This work
pAT812  pAT312 derivative containing the 1800-bp XbaI/SacI fragment downstream of ldhR gene  This work
pAT812-Tp  pAT812 derivative containing the trimethoprim resistance cassette  This work
pLM135-5  pUK21 derivative containing a 0.4-kb HindIII/NdeI fragment with the bce genes promoter region  (44)
pMM137-1  pLM135-5 derivative containing a 1070-bp NdeI/XbaI fragment with the ldhR gene  This work
pLM016-1  pLM135-5 derivative containing a 1002-bp NdeI/XbaI fragment with the ldhA gene  This work
pMM137-2  pBBR1MCS derivative containing the bce promoter and ldhR gene from pLM137-1  This work
pLM016-2  pBBR1MCS derivative containing the bce promoter and ldhA gene from pLM016-1  This work
pARG015-1  pBBR1MCS derivative containing a HindIII fragment expressing the ldhRA gene from their own promoter  This work

Abbreviations: Tp’, trimethoprim resistance; Cm’, chloramphenicol resistance; Km’, kanamycin resistance; Ap’, ampicillin resistance; EPS’, exopolysaccharide producer; *Due to the high number of strains tested in Figure 9, only the ones forming planktonic cellular aggregates were here included.
Table 4 List of primers used in this work.

<table>
<thead>
<tr>
<th>Gene region</th>
<th>Forward*</th>
<th>Reverse*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmul2557L</td>
<td>GAAATCTAGACATGGTCTGAAATCTGG</td>
<td>CCTAAGCTTGCTTTCAGATATGGC</td>
</tr>
<tr>
<td>Bmul2557R</td>
<td>AGCGAGCTCGTTCTGAGCATCGGGCTT</td>
<td>GACTCTAGACCGGCGGCTCGAATGTA</td>
</tr>
<tr>
<td>P1</td>
<td>GCAACATAAGACCCAGATTCAGACCAATG</td>
<td>TCTTCCTAGATGAACGAAATCGTCGTCGTA</td>
</tr>
<tr>
<td>P2</td>
<td>CATGCAATAAGCCTGATCGTTCTGCAGC</td>
<td>CGTTCTAGAGCGGAATCAGCAGGCTC</td>
</tr>
<tr>
<td>P3</td>
<td>GCAGAAGCTGCGCGCCGATTGGA</td>
<td>AGGAAGCTTGCGGAAAGGCCGGAAG</td>
</tr>
<tr>
<td>2557/2558_RT</td>
<td>TCGAACATGCGATCGAGCATT</td>
<td>TCGAGCCTGTCGTTGACGAA</td>
</tr>
<tr>
<td>2558/2559_RT</td>
<td>CGACGACATCGAGCAGCTGAT</td>
<td>CGACGAGAATCGCCGCAATGT</td>
</tr>
<tr>
<td>qRT_2557</td>
<td>CACTCGTCAGCGTTCGAT</td>
<td>GCTGAGACTAGCCGCGAT</td>
</tr>
<tr>
<td>qRT_2558</td>
<td>CGTGTCCTGCGAGATCGAATG</td>
<td>TACCACGCGCAGCTGAA</td>
</tr>
<tr>
<td>qRT_trpB</td>
<td>GACTGGTTCAGGAACATCGAGAA</td>
<td>ACCGGAATGCGTCGATGA</td>
</tr>
</tbody>
</table>

*, restriction sites are in italics.
Fig. 2

A

B

C

D2055
IST408
FC067
PC259
CEP0743

Fold-change

0.2
0.4
0.6
0.8
1.0

Gene

lux-box

scmR

AGACAAGCC
CTGT
GCT
GATC
CGA
CAG
CCG-----CGGCTCGCCATGATCG

ldhR

CGATACGAGCGGGAATCACGCGCCGCGCAATGTTTTTTCCGCGCTGCACG

Start codon

** * *  * *   * *  ** *   *           ***  **  **  *

smR

AAGGCGAAAAACACAACATAATCATTTAATGGGCCAACGAAAGCT------ATG

ldhR

ATG-AAAAATCACAACCATAATCAGTCAACCGGGCCAAAAATACGCAAGCCATG

* *   ***     * ******** * **  ** * *  **  *  

Gene

Bmul. 2556
Bmul. 2557
Bmul. 2558
Bmul. 2559
Fig. 4

**EPS dry weight (g/l)**

**A**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>WT</th>
<th>/+pBBR</th>
<th>/+ldhRA</th>
<th>WT/+ldhRA</th>
</tr>
</thead>
</table>
| D-glucose | ![Graph](image)
| D-galactose | ![Graph](image)
| D-fructose | ![Graph](image)
| D-mannitol | ![Graph](image)
| D-mannose | ![Graph](image)

**B**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>WT</th>
<th>/+pBBR</th>
<th>/+ldhRA</th>
<th>WT/+ldhRA</th>
</tr>
</thead>
</table>
| D-glucose | ![Graph](image)
| D-mannitol | ![Graph](image)
| D-mannose | ![Graph](image)
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9

<table>
<thead>
<tr>
<th>Burkholderia species</th>
<th>With aggregates</th>
<th>Without aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cepacia</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>B. multivorans</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>B. cenocepacia</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>B. stabilis</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B. vietnamiensis</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>B. dolosa</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>B. ambifaria</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>B. anthina</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>B. contaminans</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>