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**A metabolic switch is involved in life-style decisions in
*Photorhabdus luminescens***

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1 **A metabolic switch is involved in life-style decisions in *Photorhabdus***
2 ***luminescens*.**

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1 Summary

2 *Photorhabdus luminescens* is a species of Gram negative bacteria that is pathogenic to
3 insects whilst also maintaining a mutualistic association with nematodes from the
4 family *Heterorhabditis*. *P. luminescens* elaborates an extensive secondary metabolism
5 during the post-exponential phase of growth that includes the production of an
6 antibiotic called 3-5-dihydroxy-4-isopropylstilbene (ST), an anthraquinone pigment
7 (AQ) and bioluminescence. In this study we identified a mutant that was unable to
8 produce ST, AQ and light. This mutation was found to be in the *mdh* gene, encoding
9 malate dehydrogenase, a key enzyme in the TCA cycle. Interestingly the *mdh* mutant
10 was unaffected in virulence but was unable to support nematode growth and
11 development *in vivo* or *in vitro*. This clearly establishes that secondary metabolism in
12 *P. luminescens* is required for the mutualistic interaction with the nematode.
13 Furthermore the construction of mutations in key genes in other central metabolic
14 pathways confirmed the critical role for the TCA cycle in both secondary metabolism
15 and mutualism, but not in virulence. Therefore we conclude that the TCA cycle is
16 required for the transition of *P. luminescens* from pathogen to mutualist implicating
17 the involvement of a metabolic switch in the regulation of life-style decisions in this
18 bacterium.

1 Introduction

2 *Photorhabdus* is a genus of bioluminescent, Gram negative bacteria that is highly
3 virulent to insect larva whilst also maintaining a mutualistic relationship with soil-
4 dwelling nematodes from the family *Heterorhabditis* (Clarke, 2008, Waterfield *et al.*,
5 2009). *Photorhabdus* is normally found in gut of the infective juvenile (IJ) of the
6 heterorhabditid nematode (Ciche & Ensign, 2003). The IJ is a specialized, non-
7 feeding stage of the nematode that is active for dispersal in the soil and infection of
8 new insect hosts (Ciche, 2007). Once inside the insect the IJ migrates to the
9 hemolymph where the bacterial symbionts are regurgitated. The bacteria replicate in
10 the nutrient-rich hemolymph reaching densities of $>10^8$ cfu/insect after 48-72 h at
11 which time the insect succumbs to septicemia. As a consequence of bacterial growth,
12 and due to the secretion of a range of hydrolytic enzymes, the internal organs of the
13 insect are converted into bacterial biomass and it is this biomass that is used by the
14 developing nematodes as food. The first step in nematode development is the
15 recovery of the IJ into a self-fertile adult hermaphrodite. This process is initially
16 stimulated by low molecular weight signals present in the insect hemolymph and,
17 subsequently, by a bacterial food signal that indicates the presence of a high density
18 of *Photorhabdus* (Joyce *et al.*, 2008, Ciche & Ensign, 2003). With the appropriate
19 conditions inside the insect cadaver the adult hermaphrodite nematode lays eggs that
20 hatch and develop through 4 juvenile molts (L1-L4) to give a new generation of adult
21 worms. As the nematode numbers increase (and food levels decrease) conditions
22 within the insect cadaver become sub-optimal and the juvenile nematodes are
23 stimulated to develop into the IJ, an alternative L3 stage. There can be $>100,000$ IJs in
24 a single insect cadaver and each IJ carries a population of approximately 100 colony

forming units (cfu) of *Photorhabdus* arising from 1-2 bacterial cells that originally colonize the IJ in a complex transmission process (Ciche *et al.*, 2008).

Photorhabdus is both a pathogen and a mutualist and these different life-styles can be temporally separated according to the growth phase of the bacteria (ffrench-Constant *et al.*, 2003). *Photorhabdus* have been shown to grow exponentially in the insect and LT₅₀ values are tightly correlated with *in vivo* bacterial growth rates (Clarke & Dowds, 1995, Watson *et al.*, 2005). On the other hand the nematodes develop and reproduce in the insect cadaver only when *Photorhabdus* have reached a high cell density and the bacteria have entered the post-exponential (stationary) phase of growth (Hu & Webster, 2000). *Photorhabdus* produce a range of activities during the post-exponential phase of growth that appear to be important for the mutualistic association with the nematode. These activities have been called symbiosis factors and they include bioluminescence, an anthraquinone pigment (AQ), and a stilbene based antibiotic called 3,5-dihydroxy-4-isopropylstilbene (ST) (Joyce *et al.*, 2008, Brachmann *et al.*, 2007, Joyce & Clarke, 2003). Therefore pathogenicity is associated with exponentially growing bacteria whilst mutualism is associated with bacteria in the post-exponential phase of growth.

In this study, we identify a mutant in *P. luminescens* TT01 that is unable to produce ST, AQ and bioluminescence. This mutation is in *mdh*, encoding the TCA cycle enzyme malate dehydrogenase. We show that the *mdh* mutant is not able to support nematode growth and development *in vivo* or *in vitro* but is as virulent to insect larvae as the wild-type. Therefore, although the *mdh* mutant can grow in the insect, this mutant cannot undergo the change from pathogen to mutualist implying that a

1 metabolic switch, requiring a functional TCA cycle, controls this important bacterial
2 life-style transition.

3

4 **Results**

5 **The *mdh* gene is required for the production of ST, AQ and light.** We recently
6 described the biochemical pathways required for the production of ST and AQ in *P.*
7 *luminescens* TT01 and, during the course of these studies, we undertook several Tn5-
8 based mutant screens to identify genes involved in these biosynthetic pathways (Joyce
9 *et al.*, 2008, Brachmann *et al.*, 2007, Williams *et al.*, 2005). One mutant identified as
10 a non-producer of both ST and AQ during these studies was mapped to the *mdh* gene,
11 encoding malate dehydrogenase (Mdh). *P. luminescens* is bioluminescent and light
12 production is concomitant with ST and AQ biosynthesis. Further examination of the
13 *mdh::Tn5* mutant suggested that this mutant was not capable of producing light (data
14 not shown). To confirm these phenotypes we constructed a non-polar deletion
15 mutation of the *mdh* gene in TT01. As expected, the Δmdh mutant was unable to
16 produce ST, formed non-pigmented colonies on LB agar, and did not produce light
17 (see Figure 1). All of these defects were complemented by the expression of *mdh* in
18 trans confirming that *mdh* is required for the production of ST, AQ and
19 bioluminescence in *P. luminescens* (see Figure 1).

20

21 **The Δmdh mutant is defective for growth in LB.** It is well documented that the
22 production of ST, AQ and light occurs during the post-exponential phase of *P.*
23 *luminescens* growth in LB broth (Brachmann *et al.*, 2007, Joyce & Clarke, 2003,
24 Daborn *et al.*, 2001). Therefore, it was possible that the non-production of these
25 phenotypes in the Δmdh mutant was the result of a defect in growth. To test this we

grew TT01 and the Δmdh mutant in LB broth and, although both strains had a very similar growth rate over the initial 9 h of incubation, it is clear that the Δmdh mutant stopped growing sooner, and at a lower OD₆₀₀, than TT01 (see Figure 2A). This cessation in growth of the Δmdh mutant was very abrupt and occurred at a time that corresponded to the entry into the post-exponential phase of the wild-type culture (9 h post-inoculation). Importantly the growth characteristics of the Δmdh mutant were restored to wild-type by the expression of *mdh in trans* confirming that the defect in growth was due to the deletion of the *mdh* gene. Viability counts showed that the Δmdh mutant stopped dividing at a cell density of 8.9×10^8 cfu ml⁻¹ whilst the wild-type entered the post-exponential phase at a cell density of 1.7×10^9 cfu ml⁻¹, achieving a final cell density of 2.7×10^9 cfu ml⁻¹ after 24 h (see Figure 2B).

The Δmdh mutant is defective in the assimilation of amino acids. During growth on peptone-based media such as LB bacteria need to be able to assimilate amino acids. Some amino acids require the TCA cycle for their effective catabolism. Mdh catalyses the interconversion of malate and oxaloacetate and is an important enzyme in the TCA cycle. Therefore the defect in growth observed with the Δmdh mutant may be due to an inability to catabolize certain amino acids. To test this we measured the disappearance of amino acids from LB broth during the growth of TT01 and the Δmdh mutant. Over the first 9 h (a period that corresponds to exponential growth (see Figure 1)) both the wild-type and the Δmdh mutant have very similar utilization profiles with the complete assimilation of serine and proline and partial assimilation of threonine, alanine, glutamate, glycine, aspartate and arginine. In the wild-type cultures assimilation of these amino acids continues until they are largely exhausted from the LB after 24 h (note: cysteine, leucine, isoleucine, valine, methionine, lysine and

phenylalanine levels were unchanged after 24h indicating that these amino acids are not effectively assimilated by *Photorhabdus*). In contrast, after 9 h, the Δmdh culture exhibits very low rates of assimilation of threonine and alanine and no further assimilation of the other amino acids (see Figure 3). Interestingly serine, alanine and threonine are metabolized via pyruvate and assimilation does not require a functional TCA cycle. On the other hand proline, glutamate, arginine and aspartate are metabolized to TCA cycle intermediates and require a functional TCA cycle for complete assimilation. Therefore the defect in growth observed with the Δmdh mutant in LB broth is likely due to the inability of this mutant to assimilate certain amino acids as the result of a non-functional TCA cycle.

The defect in the production of symbiosis factors is due to a defect in the TCA cycle. Mdh is required in the TCA cycle, the glyoxylate pathway and also, when cells are growing on amino acids, gluconeogenesis. To determine what role of Mdh relates to the phenotypes reported here we constructed deletion mutants in key genes encoding proteins involved in these pathways: *aceA* (encoding isocitrate lyase (required for the glyoxylate pathway)), *fumC* (encoding fumarase C (required for the TCA cycle)) and *fbp* (encoding fructose-1,6-biphosphatase (required for gluconeogenesis)). It is important to note that although *E. coli* has 3 genes predicted to encode fumarase activity (*fumA*, *fumB* and *fumC*) the genome of TT01 is only predicted to have a single fumarase gene, *fumC* (Duchaud *et al.*, 2003). These mutants were then tested for their ability to produce a range of activities associated with the post-exponential phase of growth in *Photorhabdus* (see Table 1). The $\Delta aceA$ and Δfbp mutants were essentially identical to the wild-type for all activities tested except for a significantly reduced level of ST production in the Δfbp mutant, suggesting that

gluconeogenesis may have a specific role in ST production (see Table 1). Interestingly the *ΔfumC* mutant was non-pigmented and exhibited the same abrupt cessation of growth shown with the *Δmdh* mutant (see Table 1 and supplemental Figure S1). Indeed phenotypic analyses suggest that the *ΔfumC* mutation is even more defective than the *Δmdh* mutation in relation to the production of these activities (see Table 1). Therefore we can conclude that the TCA cycle is required for the production of a range of activities during the post-exponential phase of growth in *Photorhabdus*.

The acetate switch is not involved in the production of the symbiosis factors. The assimilation of serine, alanine and threonine results in the excretion of acetate into the growth medium. At some point during growth the excreted acetate can be taken up by the growing cells and this change from acetate dissimilation to acetate assimilation is called the acetate switch (Wolfe, 2005). The acetate switch has been shown to play an important regulatory role in *E. coli* and other bacteria and acetate assimilation (and therefore the acetate switch) requires a functional TCA cycle (Wolfe *et al.*, 2003, Wright *et al.*, 2009). Therefore we wanted to determine if the acetate switch was involved in the phenotype of the *Δmdh* mutant. As expected both wild-type and *Δmdh* mutants excreted acetate into the growth medium during the first 6 h of growth (corresponding to the utilization of serine) (see Figure 4). Between 6-8 h post-inoculation wild-type cells began to consume the acetate indicating that *Photorhabdus* do exhibit an acetate switch during growth in LB broth. In contrast the *Δmdh* mutant does not undergo the acetate switch and these cells continue to excrete acetate resulting in the accumulation of 10.3 mM acetate in the growth medium after 25 h incubation (see Figure 4). As expected a similar profile for acetate production was observed with the *ΔfumC* mutant (see supplemental Figure S2). This data confirmed

the non-functionality of the TCA cycle in the Δmdh (and $\Delta fumC$) mutant and implicated the acetate switch as a potential player in the regulation of the post-exponential phase phenotypes. To test this we constructed mutants in the *ackA-pta* locus ($\Delta ack-pta$) and the *acs* gene in TT01 encoding the major acetate dissimilation and assimilation pathways (Wolfe, 2005). We confirmed that the $\Delta ack-pta$ mutant did not excrete acetate and the Δacs mutant could not assimilate acetate (see supplemental Figure S2). Interestingly although the Δacs mutant is expected not to be able to assimilate acetate the levels of this organic acid remained constant after approx. 9 h suggesting that, unlike the Δmdh and $\Delta fumC$ mutants, the Δacs cells are no longer metabolizing acetogenic amino acids. However phenotypic analysis of both the $\Delta ack-pta$ and Δacs mutants revealed that there were no difference between the mutants and wild-type cells (see Table 1). In addition a $\Delta mdh \Delta ack-pta$ double mutant still exhibited all of the phenotypes of the Δmdh mutant (data not shown). Therefore the assimilation and/or dissimilation of acetate is not involved in the phenotypes observed with the Δmdh mutant.

The TCA cycle is not required for pathogenicity. *P. luminescens* is a highly virulent insect pathogen and bacterial growth within the insect has been shown to be strongly correlated with virulence (as measured by LT_{50}) (Watson *et al.*, 2005, Clarke & Dowds, 1995). Therefore to test for any affect on virulence we injected wild-type and the mutant strains of TT01 into final instar larvae of *Galleria mellonella*, the Greater Waxmoth. All of the mutants tested, with the exception of the $\Delta ack-pta$ mutant, had similar LT_{50} values when compared with the wild-type (see Table 2). The $\Delta ack-pta$ mutant appeared to be attenuated in virulence with an extended LT_{50} of 59.1

h compared to 46 h for TT01 suggesting that acetate dissimilation may be important for growth in the insect (see Table 2). Interestingly, similar to what we had observed *in vitro*, the Δmdh mutant only achieved a final cell density in the insect that was approx. 50% of that achieved by the wild-type bacteria (2.79×10^8 cfu/insect for TT01 compared to 1.49×10^8 cfu/insect for Δmdh) (see Figure 5A). Moreover the insect cadavers infected with the Δmdh mutant were dark brown rather than the characteristic bright orange pigmentation (indicative of AQ production) resulting from infection with wild-type bacteria (see Figure 5B). The virulence, final cell density within, and pigmentation of the insect cadaver infected with the $\Delta fumC$ mutant was similar to the Δmdh mutant (data not shown). On the other hand, the LT_{50} values for the $\Delta aceA$ and Δfbp mutants were indistinguishable from the wild-type bacteria (see Table 1). Therefore this data shows that mutations in the TCA cycle, whilst affecting the final bacterial cell density in the insect, do not affect virulence against the insect host.

The TCA cycle is required for *P. luminescens* to be able to support nematode growth and development. *Photorhabdus* also maintains a mutualistic association with *Heterorhabditis* nematodes. Therefore we decided to examine the mutants constructed in this study for a role in this symbiosis. Each of the strains was plated on lipid agar and, after 3 days, the bacterial biomass was inoculated with 40 surface-sterilized IJs. The recovery and growth of the nematode population within the bacterial biomass can then be followed *in situ*. The first step in nematode growth and development is the recovery of the IJ to an adult hermaphrodite (Joyce *et al.*, 2008, Ciche, 2007). Recovery was completely absent when IJs were inoculated onto either Δmdh or $\Delta fumC$ mutant bacteria (data not shown). All of the other mutants tested ($\Delta aceA$,

1 *Afbp*, *Δack-pta*, *Δacs*) supported normal IJ recovery on lipid agar plates (data not
2 shown) and produced a new generation of IJs. These IJs (now carrying the bacteria on
3 which they were cultured) were used to infect insects and, after insect death, the
4 cadavers were placed in White traps in order to collect the IJs that will emerge after
5 nematode growth and development *in vivo*. Enumeration of these IJs facilitates a
6 quantitative assessment of how effective the mutants are at supporting nematode
7 growth and development in the insect. Therefore the IJs were collected and counted at
8 15, 18 and 21 days post-infection and all of the mutants supported the development of
9 a high number of IJs in each insect ($>10^5$ IJs/insect) confirming that the glyoxylate
10 pathway, acetate metabolism and gluconeogenesis are not essential for nematode
11 growth and development *in vivo* (see supplemental Figure S3). However it is
12 noteworthy that the cumulative IJ yield of each mutant was only approximately 50-
13 60% of the IJ yield from insects infected with TT01 suggesting that, although not
14 essential, these metabolic pathways might be important for the full exploitation of the
15 insect by the bacteria-nematode complex. Finally we also confirmed that the *ΔaceA*,
16 *Afbp*, *Δack-pta* and *Δacs* mutants were competent for transmission to the new
17 generation of IJs (see Table 3). Therefore these genes (and their associated metabolic
18 pathways) are not required during the complex transmission process that involves
19 bacterial replication in both the hermaphrodite and the IJ (Ciche *et al.*, 2008).
20
21 We have previously shown that ST production is required for IJ recovery during *in*
22 *vitro* symbiosis assays (Joyce *et al.*, 2008). Therefore the absence of recovery with the
23 *Δmdh* and *ΔfumC* mutants might be simply explained by the inability of these cells to
24 produce this molecule (see Figure 1). In the insect, IJ recovery is independent of ST
25 and is stimulated by uncharacterized factors present in the insect hemolymph.

Therefore to confirm that the defect observed during *in vitro* symbiosis with the Δmdh mutants was not simply due to low levels of IJ recovery we co-infected insect larvae with axenic IJs and either TT01 or Δmdh bacteria and 7 days later the insect cadavers were dissected to observe the nematode population. As expected there was a significant mixed-stage nematode population present within insects killed by the wild-type bacteria (see Figure 6). Moreover the bacteria were present as a structured, pigmented mass that was difficult to disperse. We also observed nematodes grazing throughout this structured mass. On the other hand we could only detect a few nematodes present in insects infected with the Δmdh mutant (see Figure 6). These nematodes were larger than the IJ, indicating that recovery had been initiated, but were invariably dead suggesting that conditions within the cadaver were not permissive for nematode growth and development. Therefore we can conclude that *Photorhabdus* require a functional TCA cycle in order to be able to support nematode growth and development in the insect. Interestingly the appearance of the Δmdh mutant biomass within the cadaver was very different from that observed with wild-type bacteria i.e. it was neither pigmented nor structured and dispersed very easily in the buffer. This suggests that the TCA cycle is also important for driving significant morphological developments within the bacterial population that may be important for the mutualistic interaction with the nematode.

Discussion

In this study we have shown that the TCA cycle is required in order that *P. luminescens* can change its lifestyle from pathogen to mutualist. We have shown that this lifestyle switch is concomitant with the transition from exponential to post-exponential growth and, moreover, that the TCA cycle is required for the production

1 of symbiosis factors during the post-exponential phase of bacterial growth. These
2 symbiosis factors include, but are not limited to, light, AQ and ST. The coordinated
3 production of these symbiosis factors during post-exponential growth (notably when
4 bacterial cell densities are high) is typical of secondary metabolism but is also
5 reminiscent of a phenomenon called group behavior. The molecular mechanisms
6 controlling group behavior in model species such as *Pseudomonas aeruginosa* and
7 have been well characterized and it is now established that this behavior involves
8 complex regulatory networks that are controlled by signaling molecules such as
9 acylhomoserine lactones (AHL) and c-di-GMP (Schirmer & Jenal, 2009, Williams &
10 Camara, 2009, Hengge, 2009, Romling *et al.*, 2005). Remarkably analysis of the
11 genome sequence of *P. luminescens* TT01 does not identify any typical AHL
12 synthases and *Photorhabdus* has not been reported to have any AHL-based quorum
13 sensing networks (Duchaud *et al.*, 2003, Derzelle *et al.*, 2002). Moreover
14 *Photorhabdus* does not encode any gene with a predicted role in the synthesis,
15 turnover or sensing of the c-di-GMP molecule (Duchaud *et al.*, 2003, Galperin, 2004).
16 Therefore, in the absence of these well characterized signaling molecules it was
17 possible that a metabolic switch (perhaps associated with the transition from
18 exponential to post-exponential growth) might be involved in coordinating the
19 expression of the symbiosis factors. In this study we have identified the TCA cycle as
20 part of the metabolic switch that controls life-style decisions in *Photorhabdus* and we
21 have thus established a link between central metabolism and group behavior in this
22 bacterium.
23
24 *Photorhabdus* have been shown to grow exponentially in the insect and the bacterial
25 growth rate is closely correlated with LT₅₀ values (Watson *et al.*, 2005, Clarke &

1 Dowds, 1995). Therefore mutants that are affected in their *in vivo* growth rate are
2 attenuated in virulence as they have extended LT₅₀ values compared to the wild-type
3 (Watson *et al.*, 2005). All of the mutants analyzed in this study, with the exception of
4 the $\Delta ack-pta$ mutant, were unaffected in their LT₅₀ suggesting that the TCA cycle,
5 gluconeogenesis and the glyoxylate pathway are not required for *in vivo* growth and,
6 therefore, pathogenicity. The reason for the attenuation observed with the $\Delta ack-pta$
7 mutant may be related to the accumulation of acetyl-CoA and pyruvate that would be
8 expected to occur in this mutant. We also have preliminary evidence that a mutation
9 in *pfkA*, encoding 6-phosphofructokinase (an enzyme specific for glycolysis) is
10 unaffected in its LT₅₀ suggesting that glycolysis is not required for growth in the
11 insect (our unpublished data). Therefore it appears that abrogation of any single
12 metabolic pathway has little or no affect on the *in vivo* growth rate of TT01. The
13 insect hemolymph is a rich source of nutrients including amino acids, such as serine,
14 and glycolytic sugars. Therefore it appears that *P. luminescens* has a robust
15 metabolism during infection, a phenomenon that has also been observed in
16 *Salmonella* (Becker *et al.*, 2006). As a result it is likely that *Photorhabdus* expresses
17 redundant metabolic pathways during the early stages of infection (corresponding to
18 exponential growth) in order to exploit all of the different nutrients available in the
19 insect. However, we did observe that, similar to what was observed during growth in
20 LB broth, the $\Delta amdh$ (and $\Delta fumC$) mutant did not reach the same cell densities inside
21 the insect cadaver as the wild-type (see Figure 5). Moreover, during post-exponential
22 growth on LB agar the $\Delta amdh$ (and $\Delta fumC$) mutant failed to produce significant
23 amounts of light, AQ or ST (see Figure 1). Similarly we observed that the insect
24 cadaver infected with $\Delta amdh$ (or $\Delta fumC$) mutant cells failed to pigment suggesting that
25 the symbiosis factors are not being produced in these cadavers (see Figure 5).

1 Therefore it is clear that the TCA cycle plays a key role in controlling post-
2 exponential growth and secondary metabolism in *P. luminescens* both *in vivo* and *in*
3 *vitro*.

4

5 Our data shows that, in LB broth, *Photorhabdus* achieve exponential growth through
6 the assimilation of acetogenic amino acids, primarily serine. Moreover exponential
7 growth in LB broth appears to be independent of the TCA cycle, gluconeogenesis and
8 the glyoxylate pathway. Although the TCA cycle is not required for the exponential
9 growth of *P. luminescens in vivo* or *in vitro* it is clear that this metabolic pathway is
10 important during post-exponential growth. Mutations in either *mdh* or *fumC* resulted
11 in an abrupt and early cessation in growth *in vitro* and *in vivo*. In *E. coli* a similar *in*
12 *vitro* growth phenotype was reported in cells carrying a mutation in the *nuo* locus,
13 encoding NADH dehydrogenase I (Pruss *et al.*, 1994). It was proposed that the growth
14 defect in the *E. coli nuo* mutants was due to the accumulation of NADH in the cell
15 and the subsequent inhibition of key TCA cycle enzymes such as Mdh. Therefore the
16 requirement for an active TCA cycle during post-exponential growth is not restricted
17 to *Photorhabdus*. Indeed the TCA cycle has been shown to be required for the post-
18 exponential production of capsule in *Staphylococcus aureus* and *P. aeruginosa*
19 (Somerville *et al.*, 2002, Sadykov *et al.*, 2010, Hassett *et al.*, 1997). Nonetheless it is
20 clear that, in *Photorhabdus*, the TCA cycle is linked to an elaborate post-exponential
21 (i.e. secondary) metabolism that is required for the interaction between the bacterium
22 and its nematode host.

23

24 Secondary metabolism has been best studied in *Streptomyces*, an important source of
25 bioactive compounds and a metabolic switch has been implicated in the transition

1 from primary to secondary metabolism in this bacterium (Nieselt *et al.*, 2010). This
2 switch is controlled by environmental conditions (e.g. nutritional status) and is linked
3 to complex changes in gene expression mediated by master regulators such as PhoP
4 and AfsR (Martin & Liras, 2010, Sola-Landa *et al.*, 2003, Santos-Beneit *et al.*, 2009).
5 In *P. luminescens* the UvrY response regulator has been identified as a positive
6 regulator of AQ, ST and light production (Krin *et al.*, 2008). Therefore a mutation in
7 the *uvrY* gene in *P. luminescens* resulted in lower expression levels of the *ant* operon
8 (responsible for AQ production), *stlA* (encoding phenylalanine ammonia lyase,
9 catalyzing the first step in ST biosynthesis) and the *luxCDABE* operon (responsible
10 for bioluminescence) (Krin *et al.*, 2008). Interestingly secondary metabolism in
11 *Pseudomonas fluorescens* CHAO, an important antagonist of phytopathogenic fungi
12 found in the rhizosphere of corn, is also controlled by the BarA/UvrY homologues,
13 GacS/GacA (Hassan *et al.*, 2010, Kay *et al.*, 2005, Lapouge *et al.*, 2008). Moreover
14 mutations in genes encoding key TCA cycle enzymes (i.e. *pycAB* encoding pyruvate
15 decarboxylase and *fumA*, encoding fumarase A) have been shown to affect the activity
16 of the GacS/GacA pathway in *P. fluorescens* CHAO suggesting a link between the
17 TCA cycle and secondary metabolism (Takeuchi *et al.*, 2009). In *P. fluorescens* these
18 mutations were shown to affect GacS/GacA activity by altering the levels of TCA
19 cycle intermediates such as α -ketoglutarate, succinate and fumarate (Takeuchi *et al.*,
20 2009) and it is tempting to speculate that, in a similar way, the BarA/UvrY pathway in
21 *Photorhabdus* may be responding to changes in the pool of TCA cycle intermediates.
22 However the TCA cycle is also required for the production of reducing equivalents
23 (i.e. NADH) that can be converted to energy via the electron transport chain. In a
24 recent report it was shown that increasing the levels of proline enhances the
25 production of ST and AQ by *P. luminescens* in both rich media and filtered insect

hemolymph (Crawford *et al.*, 2010). It was proposed that proline, an amino acid that is present at relatively high levels in insect hemolymph, acts as a signal for the bacterium to indicate that it is in the insect host (Crawford *et al.*, 2010). Proline assimilation requires proline dehydrogenase (encoded by *putA*) and results in the generation of both glutamate and NADH. It was shown that the proline-dependent increase in AQ production was linked to an increase in the production of reducing equivalents suggesting that the production of NADH may be an important requirement for secondary metabolism in *Photorhabdus* (Crawford *et al.*, 2010). Interestingly, Mdh activity generates NADH when converting malate to oxaloacetate and the Δmdh and $\Delta fumC$ mutants do not appear to reduce the tetrazolium salt present in the NBT agar plates suggesting that these strains are compromised in their ability to generate reducing equivalents (see Table 1). However, Table 1 also shows that the $\Delta fumC$ mutant exhibits a more extreme phenotype than the Δmdh mutant and, as fumarase activity does not generate NADH, this would not be expected to be the case if the sole role for the TCA cycle in secondary metabolism in *Photorhabdus* was the generation of reducing equivalents. Therefore the exact nature of the interface between primary metabolism, secondary metabolism and mutualism in *Photorhabdus* remains to be determined.

Experimental procedures

Bacterial strains and growth conditions.

A spontaneous rifampicin-resistant mutant of *Photorhabdus luminescens* subsp *laumondii* TT01 was used as the wild-type in all experiments (Bennett & Clarke, 2005). Bacteria were cultured in LB broth or on LB agar (LB broth + 1.5% (w/v) agar) at 30°C. All LB agar plates were supplemented with 0.1% (w/v) pyruvate,

unless otherwise stated. When required antibiotics were added at the following concentrations: ampicillin (Amp): 100 $\mu\text{g ml}^{-1}$; chlormaphenicol (Cm): 20 $\mu\text{g ml}^{-1}$; kanamycin (Km): 30 $\mu\text{g ml}^{-1}$; rifampicin (Rif): 100 $\mu\text{g ml}^{-1}$.

Construction of deletion mutants

The *mdh::Tn5* insertion mutant was originally identified during a previously described screen for mutants that were unable to produce the ST antibiotic (Williams *et al.*, 2005). Specific deletion mutants were constructed using a 3-step PCR protocol developed in our laboratory (Joyce *et al.*, 2008, Brachmann *et al.*, 2007). Deletion mutants are constructed so that the predicted ORF is completely deleted leaving only the start and stop codons to minimize polarity. Briefly primer pairs A+B and C+D (see supplemental Table S1) were used to amplify approx. 500bp of the upstream and downstream flanking DNA the gene to be deleted. These amplicons were designed to have complementary ends (i.e. the 5' end of primers B and C for each gene are complimentary) so that, when mixed, the PCR fragments formed a full-length chimeric molecule that could be amplified using the appropriate primers (i.e. primers A and D). This full-length amplicon was cloned into the mobilisable suicide vector pDS132 and the plasmid was conjugated from *E. coli* S17-1 (λpir) into *P. luminescens* TT01 as previously described (Easom & Clarke, 2008). After overnight incubation of the conjugation mix on LB agar at 30°C exconjugants were selected by plating the resuspended cells on LB agar plates supplemented with Rif and Cm. The pDS132 plasmid contains the *sacB* gene and, therefore, a second recombination event was selected for by picking a colony into LB broth and culturing the cells at 30°C without any antibiotic selection. After 18-24 h serial dilutions of the culture were prepared and 100 μl of each dilution was spread onto LB agar supplemented with

1 0.2% (w/v) sucrose. Suc^R mutants were replica plated on agar plates containing Cm
2 and Suc^R Cm^S colonies were screened for the presence of the deletion mutation by
3 colony PCR and the primers designated F and R for each gene (see supplemental
4 Table S1).

6 *Cloning of mdh into pTRC99a*

7 The *mdh* gene was amplified from *P. luminescens* TT01 chromosomal DNA using
8 PCR and the primers JW132 [5'-ttattccatggaagttgcagttctcggtgcagc-3'] and JW133 [5'-
9 tatttctagattagtattaataaaattttcgcctaattc-3']. The amplicon was digested with *Nco*I and
10 *Xba*I and ligated into pTRC99a digested with the same restriction enzymes resulting
11 in the plasmid, pBMM935. The integrity and accuracy of the insert was confirmed by
12 DNA sequencing. The preparation of electrocompetent cells of *P. luminescens* TT01
13 and mutant derivatives was carried out as previously described (Bennett & Clarke,
14 2005).

16 *Phenotypic tests*

17 For all phenotypic tests cells were cultured overnight in LB broth at 30°C, the OD₆₀₀
18 of the culture was adjusted to 1 and 5 µl of each cell suspension was spotted onto the
19 surface of a LB agar plate and incubated for 3 days at 30°C. AQ production was
20 qualitatively assessed on the basis of the level of colony pigmentation compared to
21 wild-type colonies. These colonies were also assessed for light production using an In
22 Vitro Imaging System (IVIS-100, Xenogen Alameda, Ca.). ST production was
23 assessed using a *Micrococcus* overlay assay. Briefly colonies were grown on LB agar
24 for 3 days at 30°C before being overlaid with soft agar (LB broth supplemented with
25 0.3% (w/v) agar) containing *Micrococcus luteus*. The level of ST production was

determined by measuring the size of the halo surrounding each colony (*Williams et al.*, 2005). Siderophore production was measured using LB agar supplemented with CAS (*Watson et al.*, 2005). NBT agar is nutrient agar supplemented with 25 $\mu\text{g ml}^{-1}$ bromothymol blue and 30 $\mu\text{g ml}^{-1}$ 2,3,5-tetrazolium salt. Lipase production was measured using nutrient agar supplemented with Tween 80 and CaCl_2 . Protease production was measured using skimmed milk agar. Motility was determined using swim agar (LB broth supplemented with 0.3% (w/v) agar) (*Easom & Clarke*, 2008). Catalase production was detected using the fizz test whereby cells from an overnight culture were mixed with H_2O_2 and the formation of bubbles is indicative of catalase production (*Joyce & Clarke*, 2003).

Amino acid analysis and determination of acetate levels

Single colonies were picked into 5 ml LB broth and grown overnight at 30°C. The overnight culture was then inoculated into 50 ml of LB broth to achieve a starting OD_{600} of 0.05 and cultures were grown, shaking at 200 rpm, in 250 ml flasks at 30°C. After 3, 6, 9 and 24 h a 5 ml aliquot was taken from each flask, the cells were removed by centrifugation and the supernatants were filter-sterilised, through a 0.2 μm filter, and stored at -20°C until required. For amino acid analysis, samples were deproteinised by mixing equal volumes of 24% (w/v) trichloroacetic acid (TCA) and sample. The mixtures were allowed to stand for 10 minutes before centrifuging at 14,400 x g (Microcentaur, MSE, UK) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nm/ml. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK)

1 fitted with a Jeol Na⁺ high performance cation exchange column. Acetate levels were
2 determined by high performance liquid chromatography (HPLC) using an Agilent
3 1200 HPLC system with a refractive index detector. A REZEX 8 μ 8%H, Organic
4 Acid Column 300 x 7.8 mM (Phenomenex, USA) was used with 0.01N H₂SO₄ as the
5 elution fluid, at a flow rate of 0.6ml/min. The temperature of the column was
6 maintained at 65°C. Acetate peaks were identified by comparison of their retention
7 times with those of pure compounds and concentrations were determined from
8 standards of known concentrations.

9

10 *Pathogenicity assay*

11 The pathogenicity of *P. luminescens* strains was assessed using *Galleria mellonella*
12 larvae, purchased from Livefood (UK), as the model insect host. Briefly, overnight
13 cultures of *P. luminescens* TT01 and mutant derivatives were washed 3 times in 1 x
14 PBS before the OD₆₀₀ was adjusted to 1.0 (equivalent to 4 x 10⁸ cfu ml⁻¹). The culture
15 was then diluted to a cell density of 2 x 10⁴ cfu ml⁻¹ and 10 μ l (equivalent to 200 cfu)
16 was injected into the hemolymph of a *G. mellonella* larva using a Hamilton syringe
17 and a BD MicrolanceTM 3 30G x 1/2" needle. The injected insect larvae were placed
18 into a Petri dish containing some Whatman paper and incubated at 25C and, at the
19 appropriate times, the insects were checked for signs of life by gentle prodding with a
20 sterile yellow tip.

21

22 *In vitro symbiosis assay*

23 *In vitro* symbiosis assays were carried out as previously described (Easom & Clarke,
24 2008). Briefly an overnight culture of the appropriate bacteria was diluted to an OD₆₀₀
25 =1 and 50 μ l was spread, in a Z pattern, onto the surface of a lipid agar plate (/L: 10 g

1 corn syrup, 5 g yeast extract, 25 g nutrient agar, 5 ml cod liver oil, 2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)
2 using an inoculating loop. The plates were incubated at 30°C for 3 days before 50
3 surface sterilised IJ nematodes were added to the bacterial biomass. Nematodes were
4 surface-sterilised by washing in a solution (0.4% (w/v)) of hyamine (Sigma).
5 Nematode recovery was assessed 7 days later by counting the number of
6 hermaphrodites on the lipid agar plate. After 21 days, the new generation of IJs was
7 collected and the number of IJs present (i.e. the IJ yield) was determined. The number
8 of bacteria present levels in the IJ (i.e. colonization levels) was determined by
9 crushing individual, surface-sterilised IJ nematodes in 100 μl 1 x PBS using a hand-
10 held homogeniser. At least 10 IJs were crushed for each mutant strain and the bacteria
11 were enumerated by plating the homogenate onto LB (Rif) agar.

12

13 *Co-infection symbiosis assays*

14 To determine the ability of mutant bacteria to support nematode growth and
15 development *in vivo* we co-infected insect larvae with the bacteria being tested and
16 axenic IJ nematodes. Axenic nematodes were produced by growing *H. bacteriophora*
17 TT01 nematodes on a bacterial lawn of *P. temperata* K122. Under these conditions
18 the nematodes grow well but the *P. temperata* K122 bacteria are unable to colonize
19 the *H. bacteriophora* IJ (our unpublished data). Axenicity was confirmed by a)
20 crushing 1000 surface-sterilized IJs and plating the homogenate on LB agar and b)
21 allowing the IJs to infect insects to confirm that there was no insect death. To
22 establish co-infections insects were first injected with the appropriate bacteria using
23 the same procedure outlined for pathogenicity assays. The injected insects were then
24 placed in a Petri dish containing Whatman filter paper that had been previously
25 wetted with 1 ml PBS containing 1000 axenic IJs. The insects were left on the filter

1 paper for 3 days at 25°C (until insect death) and then transferred to fresh Petri dishes.
2 After a further 4 days incubation at 25°C the insect cadavers were surface sterilized
3 by dipping the cadaver in ethanol before removing the ethanol by flaming. The
4 cadavers were then dissected by placing in a Petri dish containing 10 ml sterile 1 x
5 PBS and disrupting the cuticle using a sterile scalpel blade.

6

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15

16 **Figure Legends**

17 **Figure 1.** The Δmdh mutant does not produce AQ, ST or light. TT01 and Δmdh cells
18 carrying either pTRC99a (vector) or pBMM953 (*pmdh*) were cultured overnight at
19 30°C. The OD₆₀₀ of the culture was adjusted to 1.0 and 5 µl of each cell suspension
20 was spotted onto the surface of a LB agar plate and incubated for 3 days at 30°C for
21 AQ production (as indicated by level of pigmentation), ST production and
22 bioluminescence.

23

24 **Figure 2.** Growth of the Δmdh mutant in LB broth. A) Overnight cultures of TT01
25 carrying pTRC99a (filled circle) and Δmdh carrying pTRC99a (open circle) or

pBMM953 (open squares) were inoculated into fresh LB broth (+Amp) supplemented with 0.1mM IPTG and incubated, with shaking at 200 rpm, at 30°C. Samples were taken at regular time intervals to measure OD₆₀₀ and the graph shown is a representative curve. B) Overnight cultures of TT01 (filled circle) and *Δmdh* (open circle) were inoculated into fresh LB broth and samples were withdrawn at regular intervals and spread onto LB agar to enumerate colony forming units (cfu). The results shown are the mean of 3 experiments and error bars represent the standard deviation.

Figure 3. Assimilation of amino acids during growth in LB. TT01 and *Δmdh* cells were grown overnight and inoculated into 50 ml fresh LB broth and incubated, with shaking, at 30°C. Aliquots were taken at regular intervals, the cells were removed by centrifugation and the supernatant was analyzed for amino acid levels as described in Experimental procedures. The results shown are the mean values from 3 replicates inoculated with independent overnight cultures and cultured in the same batch of LB broth. Standard deviations (<1% in all cases) are not shown for clarity.

Figure 4. Determination of acetate levels during growth in LB. TT01 cells (dark bars) and *Δmdh* cells (light bars) were cultured as described in the legend for Figure 3. Samples were withdrawn at regular intervals and supernatants were analyzed for acetate levels using HPLC. Experiments were carried out three times using independent cultures and the mean acetate levels are shown with the standard error. A representative growth curve for TT01 (filled circle) and *Δmdh* (open circle) is also shown for reference.

1 **Figure 5.** *In vivo* growth of TT01 and Δmdh . A) Insects were injected with 200 cfu of
2 each strain from an overnight culture. The infected larvae were incubated at 30°C and
3 the density of bacteria within the cadavers after 72 h was determined by
4 homogenizing the cadaver and enumerating the bacteria present by spreading the
5 homogenate on LB agar. The results shown represent the mean cfu/insect calculated
6 from 3 cadavers and the error bars represent the standard deviation. B) The difference
7 in the level of pigmentation observed in insect cadavers 72 h after being infected with
8 either TT01 or Δmdh bacteria.

9

10 **Figure 6.** Nematode growth and development in the insect. *G. mellonella* larvae were
11 co-infected with axenic *H. bacteriophora* IJ nematodes and either TT01 or Δmdh (as
12 described in Experimental procedures). The cadavers were dissected 7 days after
13 infection and examined for the presence of nematodes. Nematodes are abundant in the
14 cadaver infected with TT01 bacteria whilst only 1 nematode (indicated with black
15 arrow) was seen within the cadaver infected with Δmdh bacteria. In addition, within
16 insects infected with TT01, the bacterial biomass was clearly evident as a structured,
17 pigmented mass (indicated with white arrow). On the other hand this mass was not
18 observed in insects infected with Δmdh bacteria.

19

Table 1. Phenotypes of mutants used in this study.

	TT01	Δmdh	$\Delta fumC$	$\Delta aceA$	Δfbp	Δacs	$\Delta ack-pta$
OD ₆₀₀	6.5	2.4	1.7	6.6	7.3	6.6	6.5
AQ	+++	-	-	+++	++	+++	++
ST	+++	-	-	+++	-/+	+++	+++
bioluminescence	+++	-	-	+++	+++	+++	++++
siderophore	+++	-	- ^a	+++	++	+++	+++
Colour on NBT agar	red	yellow	yellow	red	red	red	red
Lipase	+++	++	-	+++	+++	+++	+++
Protease	+++	+++	- ^a	+++	+++	+++	+++
Catalase	+++	+++	-	+++	+++	+++	n.d.
Motility	+++	+++	++++	+++	+++	+++	+++

^a: poor growth

n.d.: not determined

Table 2. LT₅₀ values for TT01 and mutant derivatives.

Strain	LT ₅₀ (h) ^a
TT01	46 ± 2.0
<i>Δmdh</i>	48.7 ± 0.9
<i>ΔfumC</i>	48.8 ± 2.3
<i>ΔaceA</i>	43.5 ± 0.6
<i>Δfbp</i>	48.6 ± 2.4
<i>Δack-pta</i>	59.1 ± 0.64 ^b
<i>Δacs</i>	42.2 ± 1.13

^a: values shown are the mean ± standard deviation resulting from the direct injection of 100 cfu of each mutant into 10 insect larvae (n≥3).

^b: the LT₅₀ values was calculated from only 2 experiments as only 4 insects (out of 10) died within 80 h in the third experiment.

Table 3. Colonization levels of TT01 and mutant derivatives in IJ nematodes.

Strain	cfu/IJ ^a
TT01	88 (26-114)
<i>Δmdh</i>	- ^b
<i>ΔfumC</i>	- ^b
<i>ΔaceA</i>	53 (2-128)
<i>Δfbp</i>	63 (26-256)
<i>Δack-pta</i>	47 (14-90)
<i>Δacs</i>	56 (10-192)

^a: values shown are the median (range is in parenthesis) of cfu enumerated from 10 individual IJs for each strain (with the exception of TT01 (n=5)).

^b: colonization levels could not be calculated as nematodes did not grow on these mutants

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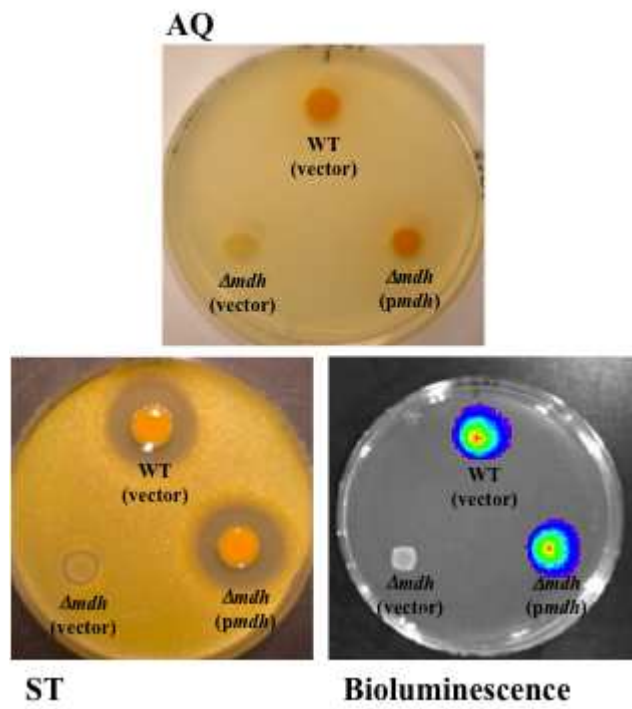
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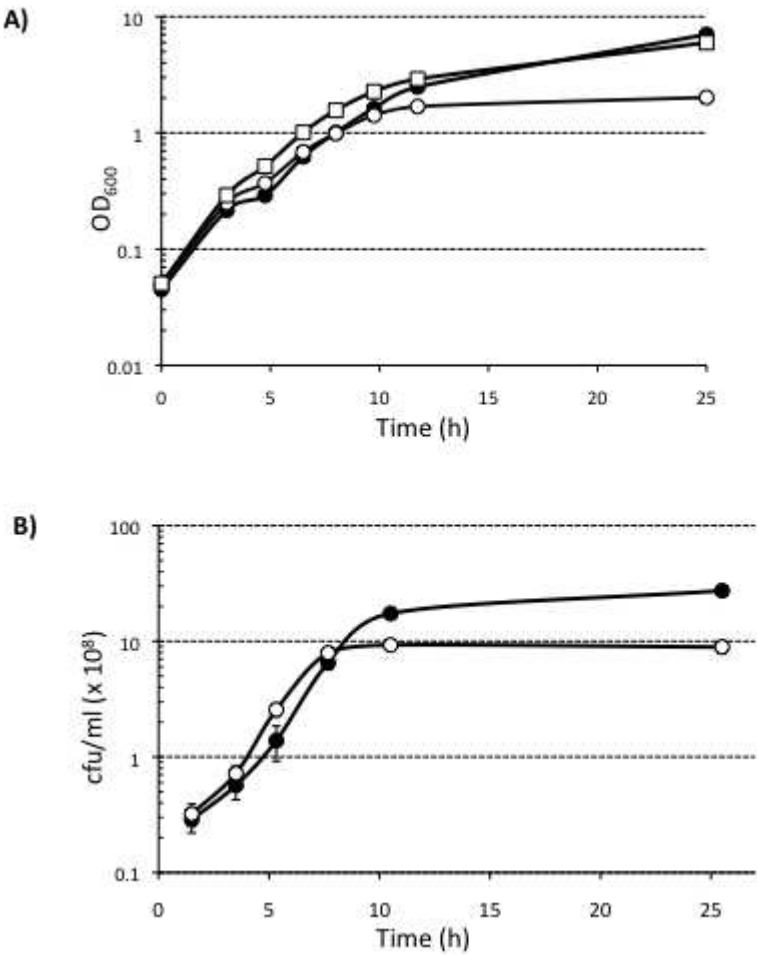
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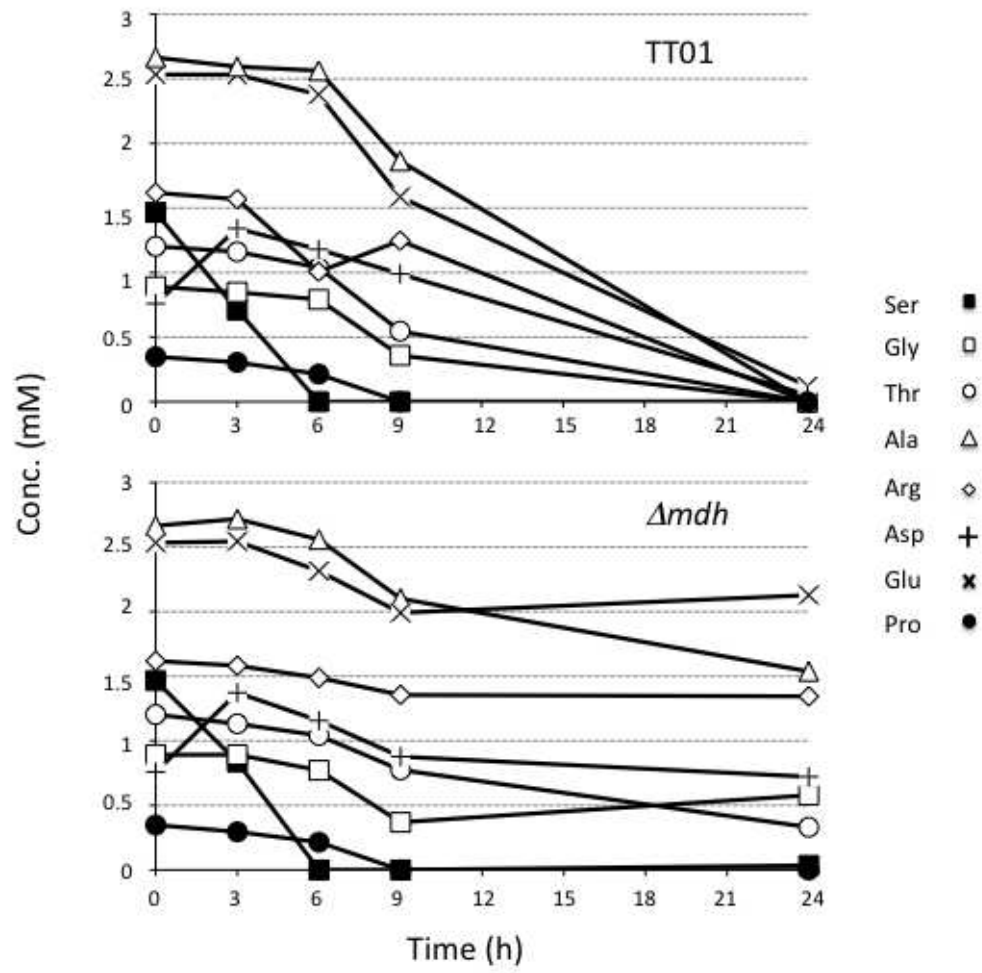
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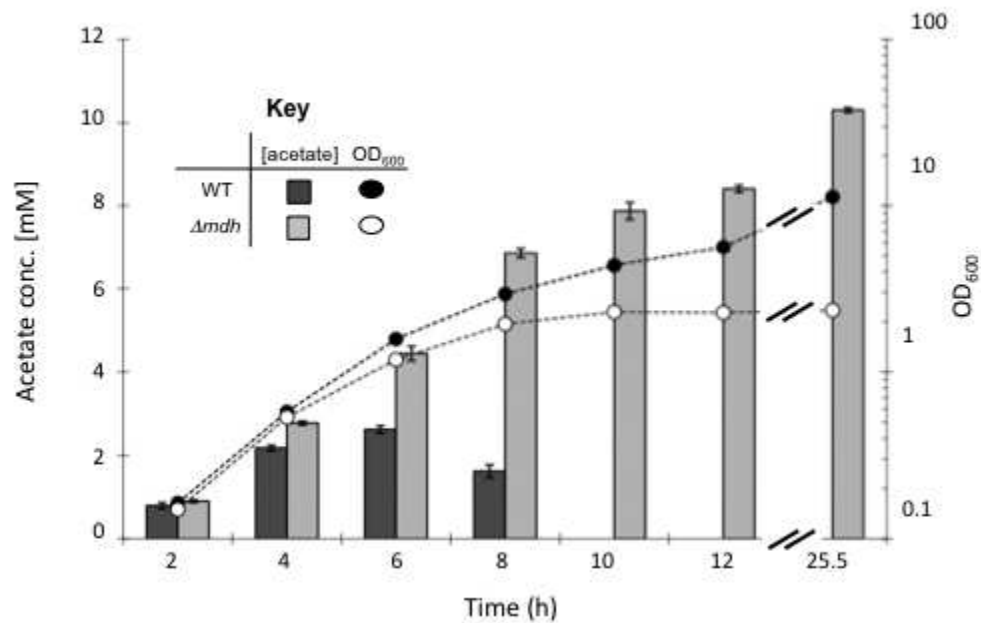
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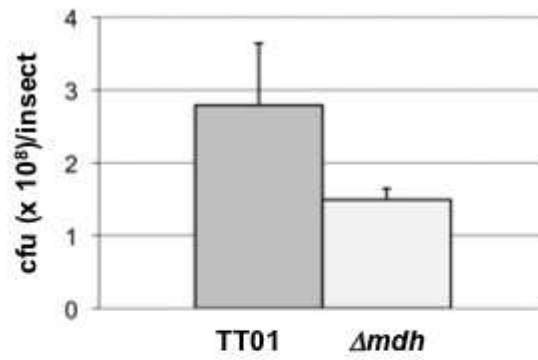
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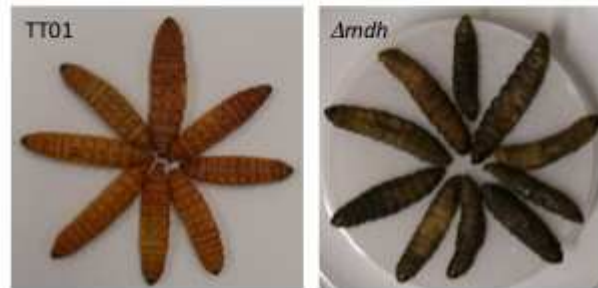
176x124mm (72 x 72 DPI)

review

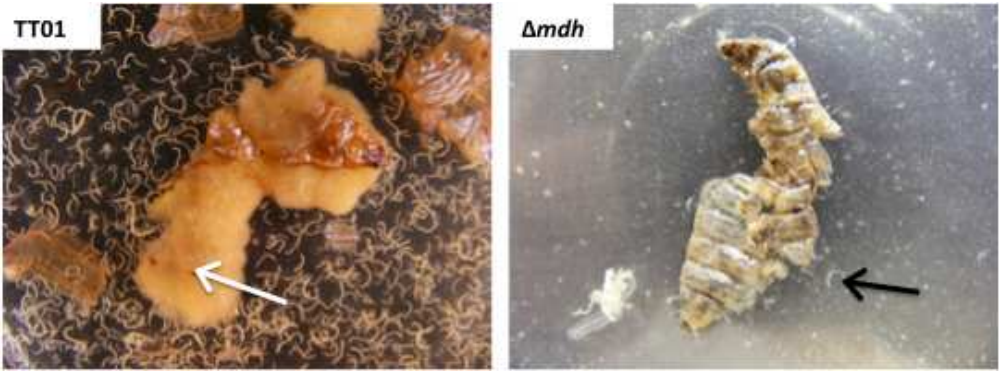
A)



B)



133x159mm (72 x 72 DPI)



190x70mm (72 x 72 DPI)

Peer Review