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RESEARCH PAPER

# Acetate utilization pathways in *Mycobacterium tuberculosis*, a potential pathogen in maize silage

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**Abstract:** Acetic acid is an important component of maize silage, which imparts stability to silage against damage by aerobic conditions. Inoculation of maize fodder with heterofermentative lactic acid bacteria results in formation of acetic acid. The drop in pH that results in ensiled fodder inhibits growth of undesirable organisms. However, certain organisms have developed mechanisms to survive in hostile conditions. Of particular interest is the notorious human pathogen, *Mycobacterium tuberculosis* which had been implicated as a potential pathogen of maize silage. The conditions in silage like availability of magnesium confer advantage to many species of the *Mycobacterium tuberculosis* complex by stabilizing certain proteins, enabling the organism to survive under the otherwise harsh conditions. In this article, the possible routes of acetate conversion in ten strains of *M. tuberculosis*, to acetyl-Coenzyme A, an important intermediate in energy metabolism have been deciphered from a curated metabolic pathway repository. The results implicate that metabolic flexibility in *M. tuberculosis* strains constitutes a strategy for enhanced pathogen survival under adverse conditions. The diversity in utilization of acetate and other carbon sources necessitate an investigation of mycobacterial survival strategies for a more informed Quality Control of maize silage for applications in dairy industry.

**Keywords:** Acetate · Maize · Metabolic flexibility · Mycobacterium tuberculosis · Silage

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## Introduction

Maize is an important feed crop that can supplement dairy industry for increased efficiency and remuneration. Processing of maize in the form of silage provides a rich source of energy to the cattle and results in enhanced milk yields (Khan *et al.,* 2015). Maize silage is considered to be a solution to scientific livestock management. The low productivity of cattle in India, which is due to improper nutrition, can be solved through increased use of silage in dairy industry. Silage making involves a process of ensiling, whereby the green fodder is preserved and becomes suitably available for cattle for a long time. During ensiling, the maize fodder is compressed and is subsequently kept under anaerobic conditions. The ensiled fodder may be left to ferment on its own or actively fermented by addition of lactic acid bacteria. The use of inoculants results in a high-quality silage. The pH of the ensiled fodder drops soon and prevents growth of spoilage-causing microbiota. When the silos are opened for taking silage, air enters and results in oxidation of fermentation via growth of acid-tolerant microorganisms (Danner *et al.,* 2003). The difference in stability of fodder inoculated by homofermentative or heterofermentative lactic acid bacteria, towards aerobic deterioration had been observed previously (Weinberg and Muck, 1996). It has been noticed that inoculation with heterofermentative lactic acid bacteria like *Lactobacillus brevis* or *Lactobacillus buchneri* is stable against deterioration caused by aerobic conditions (Danner *et al.,* 2003). Acetic acid has been reported to be responsible for higher stability in the above silages.

The drop in pH that results in maize silage inhibits growth of non-desirable microbes. Spoilage organisms like *Clostridia* result in butyric acid formation and conversion of proteins to ammonia. While this reduces the quality of silage, it also poses challenges for downstream processing

of dairy products. The presence of *Clostridia* spores results in diminished cheese quality, leading to economic losses (Garde *et al.,* 2013). Another potential pathogen group is the *Mycobacterium tuberculosis* complex species. Its presence in maize silage has already been reported earlier (Garnett *et al.,* 2003). A pathogen of high concern in the complex is *Mycobacterium tuberculosis*, which infects humans and can also infect other animals (Grange, 2001 and Hlokwe *et al.,* 2017). Reports of *M. tuberculosis* and *M. bovis* presence in cattle herd settings (Cezar *et al.,* 2016a) and also in end-products like cheese have been obtained (Cezar *et al.,* 2016b). This necessitates investigation of factors that may lead to mycobacterial survival in ensiled conditions and development of intervention strategies to control the same. *M. tuberculosis* is known to survive in low pH also. The growth disadvantage of *M. tuberculosis* is partially rescued by the presence of high magnesium content (Piddington *et al.,* 2000). *M. tuberculosis* is also known to utilize acetate as a carbon source (Rücker *et al.,* 2015). In view of the above, it becomes imperative to understand the ability of acetate utilization of different *M. tuberculosis* strains. We have used a comprehensive metabolic pathway repository for *in vitro* analysis of metabolic flexibility for acetate utilization in ten *M. tuberculosis* strains.

## Materials and methods

*Database used for selection of M. tuberculosis strains*

BioCyc collection of genome databases was used to retrieve data of *M. tuberculosis* strains (Karp *et al.,* 2019). A total of 10 *M. tuberculosis* strains, *viz.*, *M. tuberculosis* 02_1987, *M. tuberculosis* 7199-99, *M. tuberculosis* Beijing, *M. tuberculosis* CDC1551 (TIGR 2014), *M. tuberculosis* H37Rv, *M. tuberculosis* Haarlem, *M. tuberculosis* KZN 605, *M. tuberculosis* M0002959-6, *M. tuberculosis* SCAID 252.0 and *M. tuberculosis* SUMu008 were taken for the analysis. BioCyc platform allows analysis, storage and sharing of data after logging in the server.

*Determination of metabolic pathways of acetate utilization*

The above ten strains of *M. tuberculosis* were analyzed in the Metabolic Route Search module of BioCyc program. The parameters used involved acetate as 'Start Compound' and Acetyl-CoA as 'Goal Compound'. The metabolic route was searched by setting 'Switching Organisms' in penalty mode. Amongst other parameters, the number of routes (reaction) and maximum route length were kept as 5 and 9, respectively, in the BioCyc program.

## Results and discussion

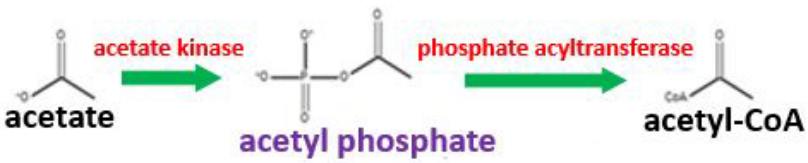
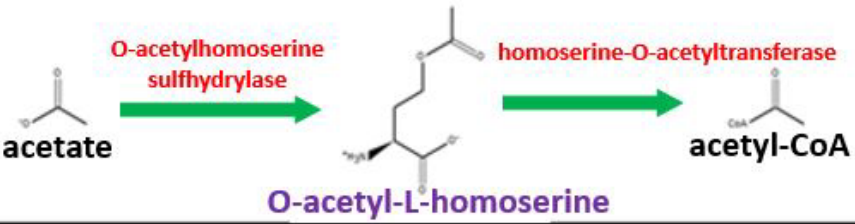
We have used the information in BioCyc program to understand the inherent diversity of acetate utilization in *M. tuberculosis* strains through *in vitro* analysis (Karp *et al.,* 2019). A total of 10 *M. tuberculosis* strains, *viz.*, *M. tuberculosis* 02_1987, *M. tuberculosis* 7199-99, *M. tuberculosis* Beijing, *M. tuberculosis* CDC1551 (TIGR, 2014), *M. tuberculosis* H37Rv, *M. tuberculosis* Haarlem, *M. tuberculosis* KZN 605, *M. tuberculosis* M0002959-6, *M. tuberculosis* SCAID 252.0 and *M. tuberculosis* SUMu008 were taken for the analysis of metabolic routes that convert acetate to acetyl-CoA in the above strains. *M. tuberculosis* Beijing genotype is associated with high virulence and multiple drug resistance, although its global frequency is not precisely known (Lillebaek *et al.,* 2003). *M. tuberculosis* Haarlem genotype is ubiquitously present and is thought to be linked to post-Columbus European colonization (Kremer *et al.,* 1999). *M. tuberculosis* H37Rv is the laboratory strain (Bifnai *et al.,* 2000), while *M. tuberculosis* CDC1551 is the clinical isolate that induces a rapid response in host (Manca *et al.,* 1999). The above ten strains of *M. tuberculosis* were analyzed in the Metabolic Route Search module of BioCyc program for conversion of acetate as 'Start Compound' to Acetyl-CoA as 'Goal Compound'. Acetyl-Coenzyme A (Acetyl-CoA) is an important molecule, which determines the cellular fate. Acetyl-CoA plays role in carbohydrate, protein and lipid metabolism. Due to its involvement in diverse metabolic processes, it is a key indicator of cellular health (Shi and Tu, 2015). Under 'fed' state, Acetyl-CoA participates in lipid metabolism, while under 'starved' state, it mobilizes to mitochondria for energy production (Shi and Tu, 2015). In *M. tuberculosis*, Acetyl-CoA activates Isocitrate lyase 2, which regulates carbon flux and plays an essential role in bacterial growth as well as virulence (Bhusal *et al.,* 2019). Analysis of top five least-cost routes from acetate to acetyl-CoA showed that the *M. tuberculosis* strains utilize acetate via different routes. Table 1 depicted the top four routes preferred by mycobacterium for conversion of acetate to acetyl-CoA. The reactions occur at comparable metabolic cost, indicating that any of the routes may be taken by the cell, depending on circumstances. The theoretical metabolic cost ranges from 105-110, as mentioned by the BioCyc program. The enzymes acetate CoA-transferase, acetyl-CoA synthetase, acetate kinase,

phosphate acetyltransferase, O-acetyl homoserine sulphydrylase and O-homoserine acetyltransferase are present in all the 10 strains analyzed. The above analysis shows metabolic flexibility for acetate utilization exist in different *M. tuberculosis* strains. This indicates that the enzymes revealed in the present analysis may, under the adverse conditions of maize silage, play a role in acetate utilization as well, besides fulfilling other functions. Gene expression of the deciphered enzymes in different mycobacterial strains can be used to assess relative acetate utilization in different species and the contribution of an individual strain to overall mycobacterial load and its persistence under the acidic conditions of silage.

In addition to this, however, a fifth reaction at a comparable metabolic cost of 115, occurs in *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551. Figure 1 represents the route from acetate to acetyl-CoA mediated via two intermediates N-acetyl-L-ornithine and N-acetyl-L-glutamate. This reaction occurs only in the above two, out of the ten, *M. tuberculosis* strains analyzed.

The comparable metabolic cost of the conversion of acetate to acetyl-CoA via N-acetyl intermediates indicates the inherent diversity in mycobacterial species to utilize different substrates as carbon sources. The above results were obtained with a high penalty imposed to 'Switching organisms cost' in the BioCyc program. 'Switching organisms' refers to the condition where metabolites can be shared amongst the different organisms or species, leading to desired goal molecules in any organism. *M. tuberculosis* is known to share metabolites. For example, it extracts nicotinamide from host during infection (Young *et al.*, 2015). In scenarios, where multiple mycobacterial strains may be capable of supporting each other by sharing of metabolites, the survival range of the otherwise susceptible species would also enhance.

**Table 1.** Analysis of the top four routes (reactions) converting acetate to acetyl-CoA in the ten *M. tuberculosis* strains. The reactions occur at comparable metabolic cost

| S. No. | Metabolic Cost | Reaction |
|---|---|---|
| 1. | 105 |   |
| 2. | 105 |   |
| 3. | 110 |   |
| 4. | 110 |   |

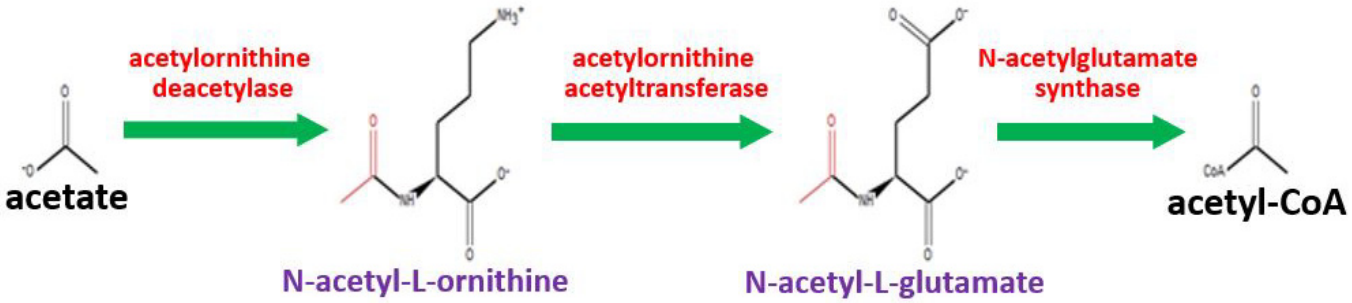



**Figure 1.** Conversion of acetate to acetyl-CoA via N-acetyl intermediates. Acetate is converted to N-acetyl-L-ornithine, N-acetyl-L-glutamate and acetyl-CoA by enzymes acetylornithine deacetylase, acetylornithine acetyltransferase and N-acetylglutamate synthase.

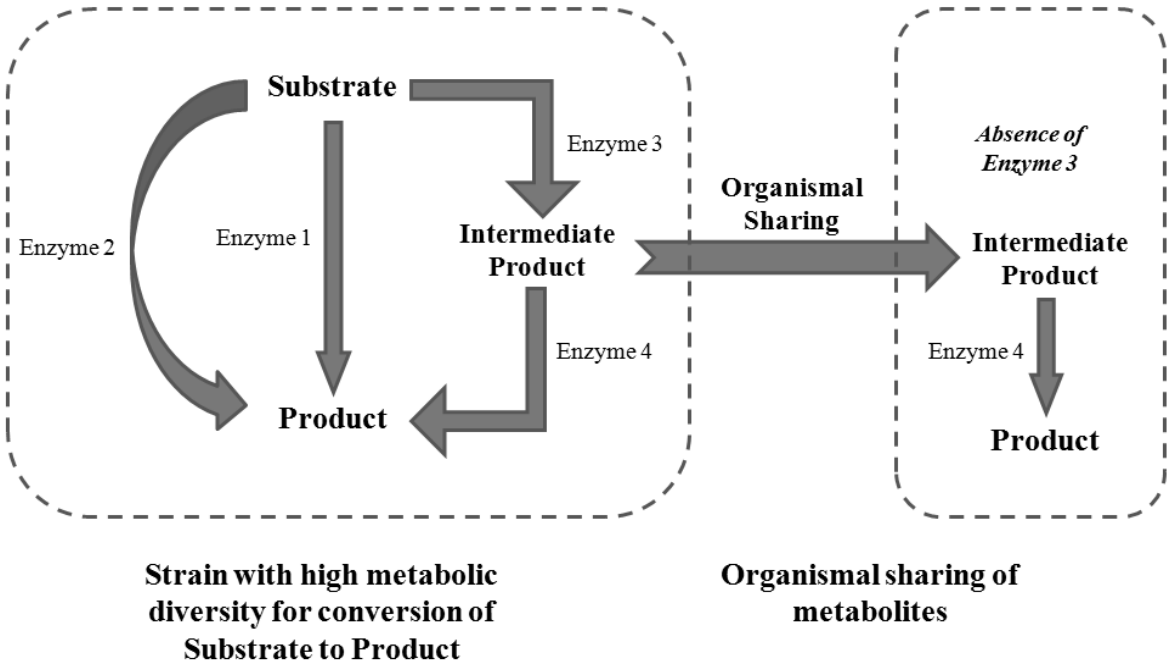



**Figure 2. A model of enhanced pathogen survival under adverse conditions by metabolic flexibility and organismal sharing.** Metabolic flexibility and organismal sharing of metabolites are two mechanisms whereby enhanced pathogen survival in hostile conditions may be mediated. The strain on the left has enzyme diversity for substrate conversion. Organismal sharing of a key metabolite (Intermediate Product) allows propagation of the strain on the right, which otherwise lacks enzyme diversity for substrate conversion.

The diversity of routes leading to molecules of energy metabolism is important, as under environmental conditions, many confounding factors may be present, all of which may together influence the outcome of bacterial growth towards active division, sporulation or inhibition. The acidity of silage is not a favourable environment for mycobacteria to grow. However, with the availability of different enzymes to utilize energy sources and the possibilities of sharing of metabolites amongst the different strains, enhanced mycobacterial survival under the adverse conditions of maize silage is possible. Figure 2 presents a conceptual model, whereby an interplay of metabolic flexibility and organismal sharing increases the energy source options available to mycobacteria. This may explain the presence of *M. tuberculosis* in cattle herd settings and their end-products, being primarily transmitted from maize silage used as feed.

The availability of more options in the form of increased number of enzymes is obviously to the advantage of microorganism. Evolutionary processes whereby different microbes may get involved via metabolite sharing is another factor that must be considered, when Quality Control

parameters for maize silage are reviewed. Mycobacteria also associate with each other in the form of biofilms. In case of *M. smegmatis*, horizontal transfer of DNA occurs between organisms in the biofilm (Nguyen *et al.*, 2010). The above model provides a framework to understand enhanced mycobacterial survival through an increased repertoire of enzymes for substrate utilization and sharing of metabolic products.

## Conclusion

Metabolic flexibility and organismal sharing of metabolites, together with the biofilm habitat, have the potential to allow diverse mycobacterial strains to survive in the adverse acidic conditions of maize silage. It is necessary to estimate the survival potential of strains with diverse molecular functions, under natural conditions as well as artificially low pH, high magnesium conditions of maize silage to determine the likely salvage points, which may be utilized by the microorganism to grow. An understanding of the survival mechanism will help in designing effective strategies to prevent the spread of tubercle bacilli in maize silage.

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