Substrate selection as a possible strategy for amelioration of acid pH by *Rhizobium leguminosarum* biovar *viciae*

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**Abstract**

A possible mechanism for bacteria to ameliorate an unfavorably acidic external pH would be to select for catabolism of those substrates in a mixture that would alkalinize the medium. This hypothesis was tested with cultures of *Rhizobium leguminosarum* biovar *viciae* WSM710 grown at pH 7.0 or 5.5 on binary mixtures of glucose plus fumarate or glucose plus histidine. The results showed no significant increase in the absolute or relative utilization of the alkalinizing substrates (fumarate or histidine) at pH 5.5.

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Bacteria alter the pH of their environment (*pH_e*) during substrate metabolism. Sugar or sugar alcohol utilization often results in acidification with organic acids produced as partial oxidation products, while organic or amino acid use leads to alkalinization, either because of organic acid/OH⁻ exchange or from ammonia release.

Under acidic conditions, amino acids such as lysine or arginine can be decarboxylated as a controlled response to *pH_e* (Gale and Epps, 1942), involving the conversion of lysine to cadaverine in *Escherichia coli* (the cad system; Meng and Bennett, 1992; Watson et al., 1992) or of arginine to agmatine and putrescine (the adi system; Stim and Bennett, 1993), and increasing *pH_e*. In *Clostridium acetobutylicum*, a decrease in *pH_e* caused by the fermentation of sugar to acetic and butyric acids results in a metabolic shift to reduction of butyric acid to butanol (Huang et al., 1986), while low *pH_e* causes *Lactobacillus plantarum* to switch from acidifying lactic acid production from sugars to neutral acetoin formation (Tsau et al., 1992).

While inducible systems are clearly harnessed to modify *pH_e*, can organisms when given a mixture of substrates, whose individual catabolism would inevitably lead to acid or alkali production, elect at acidic *pH* to selectively use those substrates that would result in alkalinization?

*R. leguminosarum* biovar *viciae* co-oxidises both components of a binary mixture of substrates at pH 7.0 (Dilworth et al., 1983) rather than showing the strict catabolite repression and diauxic growth of enterobacteria, and *R. meliloti* (Ucker and Signer, 1978) and *R. leguminosarum* biovar *trifolii* (de Hollaender and Stouthamer, 1979) behave in a similar manner. A *pH_e* change can be prevented by using a suitable mixture of an acidifying substrate (glucose) and a substrate producing alkali (fumarate) (Glenn and Dilworth, 1994). If low *pH* were to modify selection of substrates by cells to include a greater proportion of alkalinizing substrates, *pH_e* could be increased. Such a mechanism might operate in acid soils to create less...
acidic microhabitats and allow organisms to exist (Richardson and Simpson, 1988) in conditions where bulk soil pH would predict that they would not. Whether soil carbon reserves are sufficient for metabolism-induced pH amelioration is, however, questionable. This work sought to determine if acidic pH resulted in greater catabolism of an alkali-producing substrate by *R. leguminosarum* biovar *viciae* from a mixture of alkali and acid producing substrates.

*R. leguminosarum* biovar *viciae* WSM710, a strain isolated from *Pisum sativum* in Japan, was obtained from Agriculture WA, Perth, Western Australia. Cells were grown with rotary mixing at 28°C in the liquid minimal medium of Brown and Dilworth (1975) with 10 mM NH4Cl as the nitrogen source and 5 mM glucose as carbon source to an *A*<sub>600 nm</sub> of approximately 1. They were then centrifuged and resuspended in buffered minimal salts at pH 7.0. An inoculum (1 ml) of this suspension was added to 50 ml buffered minimal salts containing 10 mM NH4Cl at different pH values and the following carbon sources: (a) 5 mM glucose; (b) 5 mM fumarate; (c) 5 mM L-histidine; (d) 5 mM glucose plus 5 mM fumarate; (e) 5 mM glucose plus 5 mM L-histidine. The buffers used were 20 mM HEPES ([N-2-hydroxyethyl]piperazine-N′-[2-ethane sulfonic acid]) at pH 7.0 or 20 mM MES ([2-N-morpholino]-ethane sulfonic acid) at pH 5.5. These two pH values were selected to give a significant pH difference without moving into the “transition” zone for WSM710 (Glenn and Dilworth, 1994), where growth rate falls rapidly over a narrow pH interval. Growth of WSM710 on glucose as a sole carbon source acidifies unbuffered media, whereas growth on fumarate or histidine raises the pH<sub>c</sub>.

Growing cultures were sampled at 1-h intervals for 8 h of logarithmic growth, their absorbance measured at 600 nm (with dilution if necessary) and a culture supernatant retained for substrate assay. Adequacy of buffering was confirmed by lack of pH change during the 8 h growth period.

Glucose concentrations were measured by the glucose oxidase method with 4-aminophenazone (Trinder, 1969), fumarate concentration from its absorbance at 253 nm in 40 mM HEPES buffer (pH 7.2) and histidine concentrations by the method of Ray (1967). To compensate for bacterial growth, the areas under the growth curves were calculated, and substrate concentrations plotted against these values of ∫ *A*<sub>600</sub> dt, resulting in linear plots from which consumption rates could be calculated (de Hollaender and Stouthamer, 1979). These were converted to µmol h<sup>-1</sup> (mg dry weight)<sup>-1</sup> using an experimentally determined value of 0.329 mg dry weight ml<sup>-1</sup> for a culture with *A*<sub>600</sub> = 1. Each consumption rate, therefore, represents a statistically significant regression line (*p* < 0.001 in all cases). The data were then analysed statistically in two different ways: the first using an analysis of variance of two replicate consumption rates for each treatment and calculation of the probability that any two means were significantly different; and in the second, the 16 data points for the two replicates of each treatment were combined into one linear regression line and the resulting slopes compared in pairs. The two approaches yielded the same pattern of significant differences.

*R. leguminosarum* biovar *viciae* WSM710 grows rapidly on either glucose or fumarate as sole carbon source, but more slowly with histidine (Table 1). Both pH values are above the “transition” zone (Glenn and Dilworth, 1994) for this organism, and the adverse effect of lowered pH is, with the possible exception of histidine as a substrate, generally small.

The consumption rates for glucose or fumarate at pH 7.0 are similar (Table 2). For these single substrates at pH 5.5, there is a ca. 33% increase in the rate of glucose catabolism, but no statistically significant change in the rate of fumarate consumption.

When both the substrates are present at pH 7.0, both are catabolized simultaneously, with the decrease in the rate of glucose oxidation being greater than the decrease in the rate of fumarate oxidation. Shifting the pH to 5.5, however, results in a 44% decrease in the rate of fumarate consumption, but no change in the rate of glucose consumption relative to the respective values at pH 7.0. Contrary to what might have been expected if the cells were trying to moderate the acidic pH towards a more neutral one, the balance of substrate consumption is seen to shift towards a greater relative consumption of the acidifying substrate (glucose). The molar ratio between glucose and fumarate consumption rates changes from 0.74 at pH 7.0 to 1.33 at pH 5.5.

Growth was significantly slower when histidine was the sole carbon source (Table 1) at pH 7.0; at pH 5.5 the rate of histidine utilization fell about 25% (but not significantly) (Table 2). The combination of glucose with histidine resulted in a much decreased rate of histidine consumption at pH 7.0, although co-utilization

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Mean generation time (h)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>pH 5.5</td>
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<tr>
<td>Fumarate (5 mM)</td>
<td>2.6 ± 0.15</td>
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<tr>
<td>Fumarate (5 mM) + glucose (5 mM)</td>
<td>2.4 ± 0.07</td>
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<tr>
<td>Glucose (5 mM)</td>
<td>2.5 ± 0.05</td>
</tr>
<tr>
<td>Glucose (5 mM) + histidine (5 mM)</td>
<td>2.4 ± 0.07</td>
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<tr>
<td>Histidine (5 mM)</td>
<td>3.8 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Values represent the means ± SEM of 2–4 determinations.

<sup>b</sup> Significantly different (*p* < 0.05).
still occurred, as previously reported (Dilworth et al., 1983). In the binary glucose–histidine mixture at pH 5.5, there was a significant increase in the rate of glucose consumption compared to that at pH 7.0 (+45%), but a lesser and non-significant change in the rate of histidine utilization (Table 2). The ratio between the rates of glucose and histidine consumption changed from 3.7 at pH 7.0 to 4.1 at pH 5.5. As with the glucose–fumarate substrate mixture, therefore, there was a relative increase in the consumption of the acidifying substrate.

We conclude that *R. leguminosarum* biovar *viciae* WSM710 does not select among the substrates tested for compounds whose catabolism would lead to modification of pH to a more favorable value. It remains to be determined if this finding in WSM710 may hold true for a wider variety of substrate pairs, or indeed for other bacteria able to co-utilize acid- and alkali-producing substrates.

### References


<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH of medium</th>
<th>Fumarate</th>
<th>Fumarate</th>
<th>Glucose</th>
<th>Glucose</th>
<th>Glucose</th>
<th>t-Histidine</th>
<th>t-Histidine</th>
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<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
<td>6.89</td>
<td>ns</td>
<td>5.90</td>
<td>4.35</td>
<td>**</td>
<td>6.75</td>
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<tr>
<td></td>
<td>pH 5.5</td>
<td>6.25</td>
<td>***</td>
<td>3.33</td>
<td>4.40</td>
<td>***</td>
<td>9.40</td>
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</tbody>
</table>

* Each value in the table is derived from the linear regression for combined data (0–7 h) from two replicate measurements of the consumption rate. Symbols between the values indicate the statistical significance of the difference between them: ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.