

Growth of Fast- and Slow-Growing Rhizobia on Ethanol

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Free-living soybean rhizobia and *Bradyrhizobium* spp. (lupine) have the ability to catabolize ethanol. Of the 30 strains of rhizobia examined, only the fast- and slow-growing soybean rhizobia and the slow-growing *Bradyrhizobium* sp. (lupine) were capable of using ethanol as a sole source of carbon and energy for growth. Two strains from each of the other *Rhizobium* species examined (*R. meliloti*, *R. loti*, and *R. leguminosarum* biovars *phaseoli*, *trifolii*, and *viceae*) failed to grow on ethanol. One *Rhizobium fredii* (fast-growing) strain, USDA 191, and one (slow-growing) *Bradyrhizobium japonicum* strain, USDA 110, grew in ethanol up to concentrations of 3.0 and 1.0%, respectively. While three of the *R. fredii* strains examined (USDA 192, USDA 194, and USDA 205) utilized 0.2% acetate, only USDA 192 utilized 0.1% n-propanol. None of the three strains utilized 0.1% methanol, formate, or n-butanol as the sole carbon source.

The legume-nodulating bacteria have been divided into two genera based on their speed of growth on yeast extract-mannitol medium and various other characteristics (6, 7). The genus *Rhizobium* contains the three fast-growing species, *R. leguminosarum* (consisting of three biovars: *trifolii*, *phaseoli*, and *viceae*), *R. meliloti*, and *R. loti*, while the genus *Bradyrhizobium* contains a single slow-growing species, *B. japonicum*. A new species designation, *Rhizobium fredii*, has been approved (13) for the recently described fast-growing soybean rhizobia (8). While the preferred carbon sources have been reported to be mannitol and sucrose for the fast-growing rhizobia (2) and arabinose for the slow-growing species (1), both groups appear metabolically diverse. Additionally, it appears that although the fast-growing species can use a greater variety of carbohydrate sources (2, 3, 5, 12), the slow-growing ones can catabolize a greater variety of aromatic and hydroaromatic compounds (4, 9). Interestingly, while aldehydes and alcohols have been shown to support acetylene reduction and oxygen consumption in *B. japonicum* bacteroids (10) and acetaldehyde and ethanol have been found in soybean nodules (14, 15), there have been no reports of ethanol utilization by free-living rhizobia in cultures.

Our initial experiments were designed to determine whether fast- and slow-growing rhizobia could use ethanol as a sole source of carbon and energy for growth. Inocula for these studies were prepared by gently washing the cells from YEM (16) agar slants into BIS minimal medium (12) without any carbon source. Cultures were depleted of endogenous carbon substrates by shaking the cultures overnight at 28°C before inoculating them into ethanol-containing medium. Cells were inoculated into 50 ml of BIS medium containing 0.1, 0.25, 1.0, 2.0, 3.0, or 4.0% (vol/vol) of 95% ethanol to give an initial concentration of about 5×10^6 cells per ml. The following cultures were used: *R. fredii* USDA 191, USDA 192, USDA 193, USDA 194, USDA 201, USDA 205, USDA 206, USDA 208, USDA 214, and USDA 217; *B. japonicum* USDA 110, USDA 123, USDA 136, and PRC

113-2, PRC 121-6, PRC 2013, and PRC B15; *Bradyrhizobium* sp. (lupine) CC814s and NZP2021; *R. leguminosarum* Nitragin 92A3 and PRE; *R. leguminosarum* biovar *phaseoli* NZP5253 and NZP5260; *R. leguminosarum* biovar *trifolii* WU95 and NZP560; *R. meliloti* L530 and NZP4013; and *R. loti* NZP2238 and NZP343. Cultures were incubated at 25°C with shaking and were scored for visible growth after 7 days. BIS medium without a carbon source served as the negative control.

Results of these experiments indicated that of the 30 strains examined, only the fast- and slow-growing soybean rhizobia and the slow-growing *Bradyrhizobium* sp. (lupine) CC814s and NZP2021 were capable of growth on 0.1% ethanol. Two strains from each of the other species of *Rhizobium* examined failed to grow on 0.1% ethanol as the sole carbon source. Interestingly, while Rigaud and Trinchant (11) have found a soluble alcohol dehydrogenase in *R. meliloti*, the two strains which we examined failed to grow on ethanol as the sole source of carbon. However, it is not known whether these two strains have an alcohol dehydrogenase. With the exception of *B. japonicum* PRC B15, all of the fast- and slow-growing soybean rhizobia also grew on 0.25% ethanol. Growth of the *Bradyrhizobium* (lupine) strains, CC814s and NZP2021, appeared to be dependent on substrate concentration, since they did not grow on 0.25% ethanol. One *R. fredii* strain, USDA 191, and one *B. japonicum* strain, USDA 110, grew on ethanol up to concentrations of 3.0 and 1.0%, respectively. Although the slow-growing soybean rhizobia examined were able to grow on 0.1 to 0.25% ethanol, the extent of growth was rather limited. There was no difference in the amount of growth produced by the slow-growing Chinese *B. japonicum* strains and the typical slow-growing USDA strains. Typically, viable cell numbers never exceeded about 10^7 cells per ml ($A_{600} < 0.06$) after 18 days of growth. In contrast, *R. fredii* grew quite well on ethanol, with final cell numbers reaching about 10^8 to 10^9 cells per ml ($A_{600} > 2$) after 9 days of growth.

R. fredii USDA 192, USDA 194, and USDA 205 (all members of different serological groups) were also examined for their ability to use other low-carbon-number carbohydrates as the sole source of carbon. Whereas all three of the strains utilized 0.2% acetate for growth, only USDA 192 used 0.1% n-propanol. None of the organisms examined utilized 0.1% methanol, formate, or n-butanol as the sole

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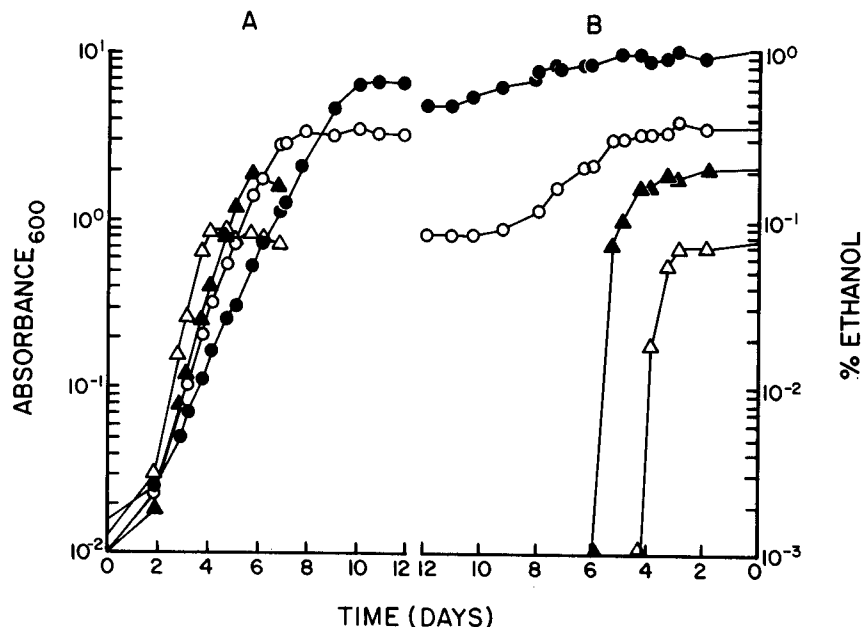


FIG. 1. Growth (A) and ethanol utilization (B) of *R. fredii* USDA 191 in Bishop basal minimal medium containing different concentrations of ethanol. Cultures were grown on 0.1% (Δ), 0.2% ($\blacktriangle$), 0.4% ($\circ$), or 1.0% ($\bullet$) ethanol.

carbon source. Although acetate utilization by fast-growing rhizobia has previously been reported (4), to our knowledge this is the first report of ethanol or propanol catabolism by free-living rhizobia.

To determine whether ethanol concentration affected the growth characteristics of a fast-growing soybean isolate, we inoculated starved USDA 191 cells into BIS medium containing 0.1, 0.2, 0.4, or 1.0% (vol/vol) ethanol. Cultures were incubated at 28°C with shaking, and growth was monitored spectrophotometrically at 600 nm. Total cell numbers were determined with a Petroff-Hauser counting chamber after 7 days of growth for the 0.1 and 0.2% ethanol cultures and after 13 days for the 0.4 and 1.0% ethanol cultures. The disappearance of ethanol from the growth medium was determined by quantitative gas chromatography with a PorPak Q column at 185°C with N_2 as the carrier gas. Our results indicated that ethanol can be used by this isolate as a sole carbon and energy source at all of the concentrations tested (Fig. 1A). These data also indicate that while ethanol can be catabolized by strain USDA 191, it may be a toxic substrate for this organism (Fig. 1A). This toxicity phenomenon is evidenced by a decreased growth rate with increasing ethanol concentrations. At ethanol concentrations of 0.1, 0.2, 0.4, and 1.0%, the mean generation times were 10.1, 13.4, 14.4, and 21.6 h, respectively. The ethanol utilization results indicate that growth is substrate limited with concentrations less than 0.4% (Fig. 1B). At ethanol concentrations of 0.1 to 0.2%, final cell numbers increased from 1.4×10^9 to 2.3×10^9 cells per ml and dry weights increased from 32.6 to 64.6 μ g of cells per ml. At the higher ethanol concentrations (0.4 and 1.0%), final cell numbers and biomass were not proportional to ethanol in the growth medium: cell numbers increased to 2.4×10^9 and 5.7×10^9 cells per ml and dry weights increased to 722 and 1,294 μ g/ml, respectively. Ethanol utilization rates were maximal when cells were grown on 0.1 or 0.2% ethanol (Fig. 1B). The decreased utilization rates which occurred when cells were grown on 0.4 and 1.0% ethanol may have reflected the toxic nature of this substrate.

To determine whether preconditioning cells for growth on a low concentration of ethanol alleviated the toxic effects of higher ethanol concentrations, we inoculated a starved USDA 191 culture to about 10^6 cells per ml in three 500-ml sidearm flasks, each containing 100 ml of BIS medium. Ethanol was added to two of the flasks to a final concentration of 0.1% (substrate limiting), while the remaining flask received 1.0% ethanol. Cultures were incubated at 25°C with shaking, and growth was monitored spectrophotometrically. One of the flasks initially containing 0.1% ethanol received an additional 0.1% ethanol at the beginning of each stationary phase of growth. The other flask with 0.1% ethanol received a single 1.0% ethanol addition. The flask which originally contained 1.0% ethanol received no further additions. The results of this experiment (Fig. 2) indicated that the relatively high concentration of ethanol similarly affected the growth characteristics of strain USDA 191 whether cells were originally grown on 1.0% ethanol or on ethanol concentrations raised from 0.1 to 1.0%. In addition, there was no effect on the growth rates of cells in the flask receiving multiple additions of 0.1% ethanol. Thus, the preconditioning of cells for growth on a low concentration of ethanol does not appear to alleviate the toxic effects of the higher substrate concentration.

Interestingly, while ethanol is a toxic growth substrate for rhizobia, it does not appear to act as a competitive inhibitor for the catabolism of other carbon compounds. Whether cells were grown on limiting concentrations of both ethanol (0.2%) and mannitol (0.02%) or on mannitol alone (0.02%), there was no apparent difference in the growth characteristics of this organism (Fig. 3). However, once mannitol became exhausted (after about 2 days), the growth rate declined to that seen for cells grown on ethanol alone. These results suggest that ethanol does not act as a competitive inhibitor of carbon utilization but most likely inhibits some essential metabolic functions.

In summary, free-living soybean rhizobia and *Bradyrhizobium* sp. (lupine) have the ability to catabolize ethanol. However, at the relatively high concentrations examined,

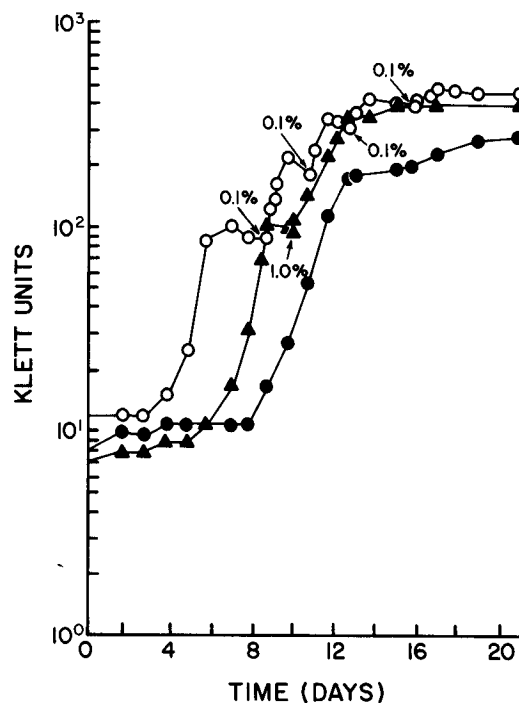


FIG. 2. Ethanol concentration step-up for *R. fredii* USDA 191 growing in two concentrations of ethanol. Cultures started in 0.1% ethanol (○ and ▲) received an additional 0.1 or 1.0% ethanol, respectively, at arrows. The culture that was started in 1.0% ethanol (●) received no extra ethanol.

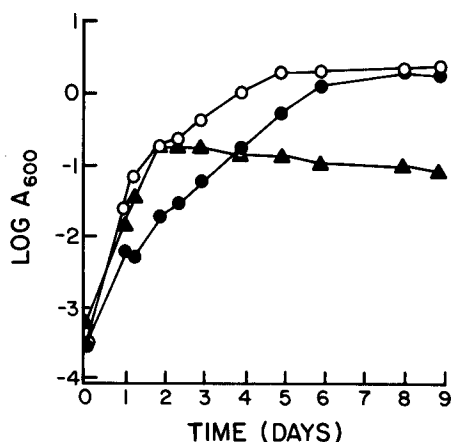


FIG. 3. Growth of *R. fredii* USDA 191 on mannitol and ethanol. Growth was on 0.2% ethanol (●), 0.02% mannitol (▲), or 0.2% ethanol plus 0.02% mannitol (○).

ethanol can act as a toxic substrate and appears to affect cell growth in a noncompetitive manner.

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