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Strain Selection in Carbon-Limited Chemostats Affects Reproducibility of *Thermoanaerobacter ethanolicus* Fermentations

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We found that the reproducibility of chemostat trials can be improved by using chemostat-adapted strains. Our experimental findings are consistent with adaptation that involves an improvement in culture fitness and an alteration of the fermentation genotype.

The potential for use of *Thermoanaerobacter ethanolicus* in industrial alcohol production has been assessed by studying its growth and ethanol production from glucose and xylose (9–13). The motivation for this work was the ability of this bacterium to produce a high ethanol yield (from low concentrations of glucose) and its ability to ferment a wide range of substrates (21).

One unanticipated observation in these studies was the low intertrial reproducibility of the relationship between fermentation products and chemostat growth rate (12). Of particular concern was the fact that in some trials, ethanol was produced in high yields only at the lowest dilution rates.

Culture contamination was discounted as a source of low reproducibility because of the high level of similarity in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein patterns obtained with whole-cell extracts of chemostat samples taken at different time points throughout various trials (9, 11, 14). Variation in environmental conditions also did not seem to be the cause because no correlation was found between the type of fermentation pattern and the small environmental differences in medium composition, temperature, nitrogen gas flow rate, or pH. Therefore, instability of the *T. ethanolicus* genotype was proposed as a major source of the low intertrial reproducibility. Furthermore, it was hypothesized that such instability arose from the selection of fitter variants, strains, or populations, which commonly arise in chemostats (3, 4, 8, 15, 19). The experimental results described below distinguished between the effects of environmental variation and culture selection on intertrial reproducibility.

Experimental trials were carried out by using the equipment and methods described previously (12), except that the inoculating cultures were prepared differently. Whereas previously single-colony isolates from American Type Culture Collection lyophilized stocks ATCC 31550 (17) and ATCC 31938 (16) were used as the sources of inocula, isolates from chemostats that were inoculated with American Type Culture Collection stock isolates 200 h (and 40 volume turnovers) previously were used in the experiments described below. We reasoned that such isolates could have improved levels of fitness compared with the original strain, since Hartl and Dykhuizen found that the most marked increase in *Escherichia coli* culture fitness occurred within the first 100 to 200 h of chemostat operation (7).

The patterns for fermentation products versus dilution rate for three such chemostat-adapted strains, strains ATCC 31550′, ATCC 31938′, and ATCC 31938″ (Fig. 1), were markedly more reproducible than the patterns observed for the parent strains, strains ATCC 31550 and ATCC 31938 (12). This was despite the fact that all of the environmental conditions, including the schedule of sampling, were chosen for trial pairs a plus b and c plus d such that they mimicked the conditions that were used in pairs of previous trials which had given markedly different results. This improvement in reproducibility provides evidence that the major portion of the previously observed intertrial variation (12) did not arise from variation in the environmental conditions, but rather arose from the use of strains which were not chemostat adapted.

The possibility that adaptation involved a genotypic change was suggested by the fact that we isolated strains (from a strain ATCC 31938-inoculated chemostat) which reproducibly yielded batch fermentation patterns that were different than the pattern of the parent (Table 1). The possibility that this apparent genotypic change was driven by improved fitness was implied by the fact that it occurred in a chemostat, which selects for faster-growing strains (3); it was also suggested by the two observations described below. First, the batch growth rate of strain ATCC 31938‴ was greater than that of strain ATCC 31938 in the presence of a growth rate-limiting concentration of xylose (Table 2). The second observation involves the amount of time which needed to pass following cessation of batch growth and commencement of chemostat operation before xylose concentrations of less than 1 mM were once again observed (following an initial increase in concentration). Whereas on average 50 h (or 5 volume turnovers) was needed for chemostats that were inoculated with derivative strains (determined in 10 trials with strain ATCC 31938′, ATCC 31938″, or ATCC 31938‴), 70 h (or 10 turnovers) were needed for those chemostats that were inoculated with the parent strain (determined in 7 trials). Both of these observations are consistent with the selection of one or more isolates which had, compared with the parent, a lower Monod saturation constant or a higher specific growth rate on the substrate which was limiting in the chemostat.

An improvement in culture fitness would have been of little consequence to the reproducibility of chemostat fermentations if it had had no effect on the relationship between fermentation product pattern and chemostat growth rate.

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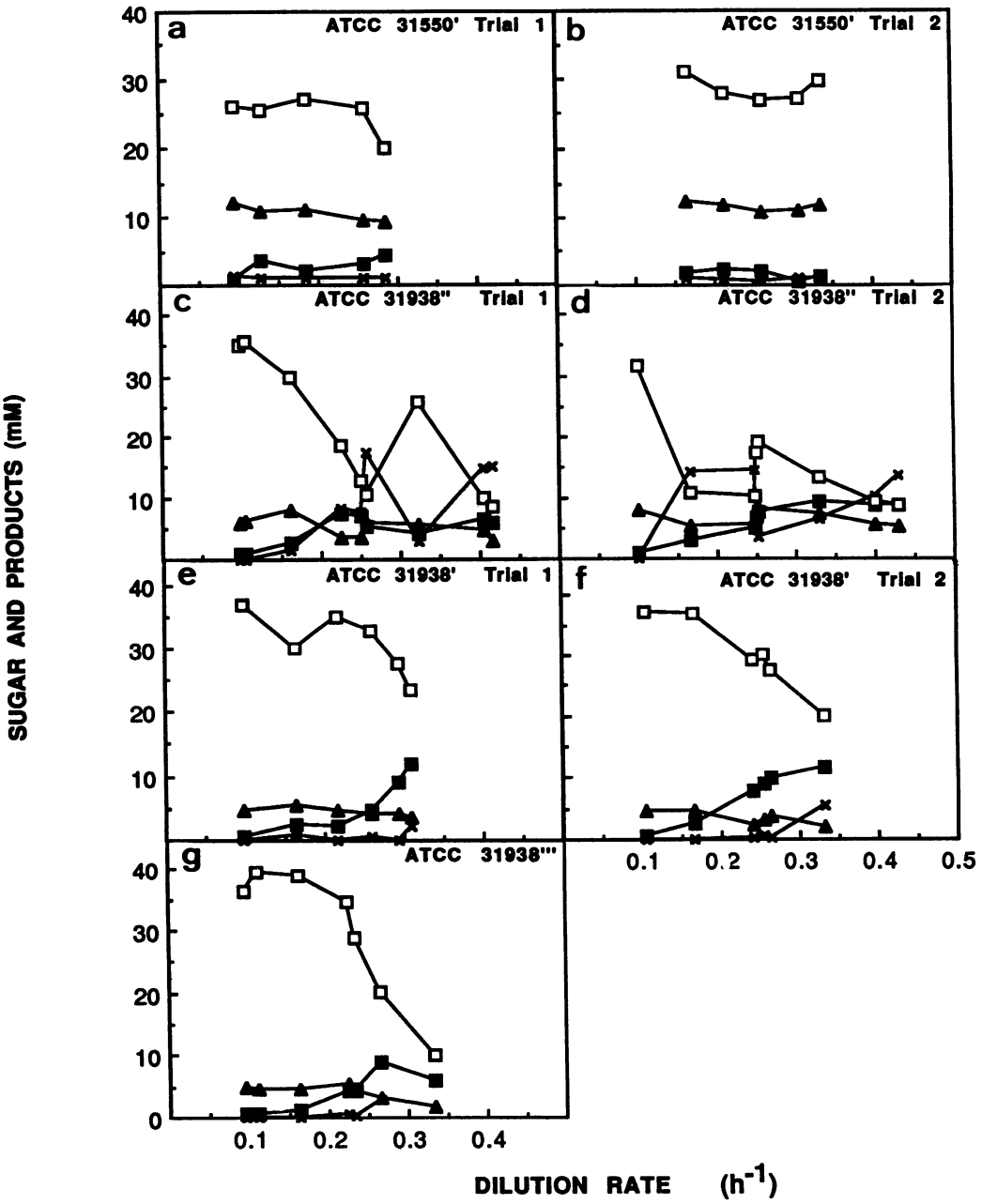

FIG. 1. Results of variable-dilution-rate chemostat trials with different inocula. Cultures were grown batchwise until the sugar was nearly exhausted, and then chemostat operation was initiated. The dilution rate was gradually increased over the course of the experiment, with at least 5 fermentor volume turnovers passing between samples. The media contained either glucose (4 g liter^{-1}) plus yeast extract (2 g liter^{-1}) (a and b) or xylose (4 g liter^{-1}) plus yeast extract (4 g liter^{-1}) (c through g). Symbols: ×, glucose or xylose; ■, lactate; ▲, acetate; □, ethanol.

However, evidence suggests that it did have such an effect. Differences among strain ATCC 31938 derivatives with respect to their fermentation patterns in batch culture correlated with the differences observed in the patterns of chemostat fermentation product versus growth rate. In both batch and continuous cultures, strains ATCC 31938′ and ATCC 31938‴ produced fermentation patterns which were more similar to one another than to the pattern of strain ATCC 31938″ (Table 1 and Fig. 1). While this correlation involved only three isolates, it was probably also valid for the 200-h chemostat population as a whole. A more comprehensive examination of the population's fermentation characteristics, this time by using test tube cultures, revealed changes toward increased lactate and decreased ethanol production (Table 3), changes which were also observed in other experiments (Table 1).

It is readily conceivable that a change in culture fitness could affect chemostat fermentation patterns. The basis for increased fitness could involve improved efficiency at a rate-limiting metabolic step (6, 18, 20). This change could then alter the distribution of internal metabolites and thereby effect a change in fermentation patterns. For example, an

TABLE 1. Comparison of the fermentation products of chemostat isolates and the parent strain in fermentors[a]

Strain	No. of trials	Mean fermentation product concn (mM)		
		Lactate	Acetate	Ethanol
ATCC 31938	3	1.1 (0.4)	6.6 (1.2)	33 (3)
ATCC 31938′	3	6.7 (1.1)[b]	7.5 (2.3)	28 (2)[b]
ATCC 31938″	5	2.6 (1.4)	7.6 (1.8)	33 (2)
ATCC 31938‴	2	7.8 (0.9)[b]	6.0 (1.1)	31 (2)

[a] Fermentors with xylose-containing medium (4 g of xylose per liter, 4 g of yeast extract per liter) were inoculated with overnight cultures of the strains. The concentrations of the fermentation products were measured in samples taken at the end of batch growth. These samples contained less than 2 mM xylose. The values in parentheses are the standard deviations.

[b] The mean is significantly different ($P < 0.01$) than the mean for strain ATCC 31938 and also significantly different ($P < 0.02$) than the mean for strain ATCC 31938″.

increase in the internal concentration of fructose 1,6-diphosphate that arose from a change in the rate-determining step could be expected to increase lactate production. Fructose 1,6-diphosphate levels seem to promote lactate production in bacteria that, like *T. ethanolicus*, have fructose 1,6-diphosphate-dependent lactate dehydrogenases (1, 2, 5, 22).

If we accept the premises for which support has been given above, that (i) within the first 200 h of chemostat operation there was a genotypically based increase in culture fitness and (ii) the chemostat fermentation pattern was affected by the degree of culture fitness, we can conceptualize how selection for improved fitness could result in low intertrial reproducibility when an unadapted strain was used as an inoculum. In a given trial, a determination of the pattern of fermentation product distribution versus dilution rate for such a strain would be influenced not only by dilution rate, but also by (i) the sampling schedule (i.e., how many hours of adaptation or fitness selection had taken place prior to the sampling) and (ii) the randomness of mutation (i.e., the trial-to-trial variation in the sequence and timing of mutations leading to improved culture fitness).

TABLE 2. Batch growth rates in fermentors for strain ATCC 31938 and two derivatives, strains ATCC 31938″ and ATCC 31938‴[a]

Inoculum	Growth rate at an initial xylose concn of:		
	30 mM	80 mM	180 mM
ATCC 31938	0.15 (0.03)[b]	0.16	0.27
ATCC 31938 (chemostat after 9 days)	0.19		
ATCC 31938″	0.17 (0.04)		
ATCC 31938‴	0.29 (0.02)[b]	0.37	

[a] All media also contained 4 g of yeast extract per liter. The nitrogen gas flow rate was set at 5, 15, and 38 ml min^{-1} for xylose concentrations of 30, 80, and 180 mM, respectively. Control batch fermentations, which had an initial xylose concentration of 30 mM and a nitrogen gas flow rate of 38 ml min^{-1}, showed that the concentration of xylose, and not the nitrogen gas flow rate, was growth rate limiting at a xylose concentration of 30 mM (11). In order to conserve fermentor working volume, growth rates were estimated from the rates of product increase instead of from biomass increase. Samples were taken over the period of growth during which the total product concentrations were less than 40 mM. The error of variance was ≥0.96 for all trials. The numbers in parentheses are the standard deviations from two or three trials.

[b] The mean growth rates were significantly different ($P < 0.02$).

TABLE 3. Mean concentrations of fermentation products in test tube cultures of chemostat isolates[a]

Time of sampling (h)	No. of isolates	Mean concn of fermentation products in test tubes (mM)	
		Lactate	Ethanol
Expt 1[b]			
0[c]	4	3.65 (0.26)	25.3 (1.0)
24	57	4.0 (1.1)	25.2 (1.8)
75	45	6.24 (0.75)[d]	21.5 (1.9)[d]
101	38	6.5 (1.0)[d]	20.8 (2.0)[d]
Expt 2[b]			
0[c]	12	0.90 (0.15)	11.2 (1.9)
300	44	4.0 (1.5)[d]	6.0 (2.4)[d]

[a] Broth samples from a chemostat (or other source) were plated. Single-colony isolates were inoculated into test tubes with glucose-containing medium. After 1 day of incubation, these preparations were subcultured into test tubes with xylose-containing medium (4 g of xylose per liter and 4 g of yeast extract per liter in experiment 1; 2 g of xylose per liter and 2 g of yeast extract per liter in experiment 2). These cultures were incubated for 3 days before product concentrations were measured. The values in parentheses are standard deviations.

[b] In experiment 1, the chemostat was operated for the first 75 h at $D = 0.10$ h^{-1}, and thereafter it was operated at $D = 0.16$ h^{-1}. In experiment 2, the chemostat was operated the entire time at $D = 0.16$ h^{-1}.

[c] In experiment 1, the zero-time isolates were obtained from a glycerol stock of strain ATCC 31938; in experiment 2, they were obtained from a strain ATCC 31938-inoculated batch fermentor at the end of exponential growth, just prior to the start of continuous operation.

[d] Significantly different ($P < 0.01$) than the zero-time value, as determined by the Student t test.

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