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Physiology of L-Asparaginase Synthesis in Recombinants of *Escherichia coli* A-1

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A mating between *Escherichia coli* 4318 (*thi leu* Las$^-$ Hfr) and *E. coli* A-1 (Met$^-$ Las$^+$ F$^-$) resulted in the formation of prototrophic recombinants having L-asparaginase activities at three distinct levels. The physiology of L-asparaginase synthesis in these recombinants is described. One class of recombinants produced significantly more L-asparaginase than *E. coli* A-1. L-Asparaginase synthesis in the recombinants was inhibited by the presence of dissolved oxygen in the medium and was transiently repressed by the presence of glucose in the same manner as that observed in the parental strains. L-Asparaginase activity was increased by the addition of oxalacetate as well as other members of the tricarboxylic acid cycle.

A number of reports have appeared in recent literature describing the conditions affecting synthesis of L-asparaginase in several genera of bacteria. This literature reveals that a complex and variable set of culture conditions is required for optimum synthesis of this enzyme (2–6, 8, 9, 12–15). In a previous study (1), it was shown that the synthesis of L-asparaginase by *Escherichia coli* A-1 requires very exacting nutritional and environmental conditions. Briefly, optimum enzyme synthesis required the following: (i) an exogenous supply of amino acids such as that found in yeast extract or nutrient broth, (ii) high levels of glutamic derivatives such as L-glutamine or monosodium glutamate (MSG), (iii) moderate aeration but no dissolved oxygen in the medium, and (iv) absence of any readily oxidizable carbon source such as glucose. Under these conditions, *E. coli* A-1 produces approximately 20 IU of L-asparaginase per ml of culture fluid when grown in a small fermentor. In contrast, the majority of cultures described in the literature produce 1 to 4 IU/ml (6–8).

L-Asparaginase is a nonessential enzyme and provides the cell with no known selective advantage over organisms that do not synthesize it. For this reason, selective or indicator media capable of revealing mutants that produce large quantities of L-asparaginase have not been produced. To detect overproducers, colonies must be picked, purified, cultured, and then tested. The majority of colonies that grow after mutagenic treatment produce the same level of enzyme as untreated cells; i.e., the majority of survivors are not mutants. Furthermore, maximum enzyme levels are achieved only under the rigorous conditions described above, and little or no enzyme is produced by colonies grown on solid medium. This means that a "fermentor run" must be performed to adequately examine each clone after treatment with mutagenic agents. To avoid this step, we started with cultures known to produce L-asparaginase and attempted to increase the level of production by selective matings. The efficiency of gene transfer was determined by using auxotrophic strains and selecting for prototrophic recombinants. In this paper, we report on recombinants obtained by mating an L-asparaginase producer (F$^-$) with a nonproducer (Hfr). The purpose of this mating was to convert the F$^-$ to Hfr in preparation for matings of producer strains, but the results were more interesting from the general point of view of genetic phenomena.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* A-1 (Met$^-$ F$^-$) and *E. coli* HAP were obtained from the stock culture collection at Wadley Institutes of Molecular Medicine. The designation Las$^+$ is suggested to indicate L-asparaginase production, and the phenotype of *E. coli* A-1 is given as Met$^-$ Las$^+$ F$^-$. *E. coli* CGSC 4318 (*thi leu* Hfr) was kindly supplied by Royston Clowes, Department of Molecular Biology, University of Texas at Dallas. We amended the designation to *E. coli* CGSC 4318 (*thi leu* Las$^-$ Hfr). Stock cultures were maintained on slants of 3% nutrient agar (3 g of commercial

nutrient agar powder per 100 ml of water) in a refrigerator. Working cultures were obtained by streaking stock cultures and picking isolated colonies.

Media. Test tube cultures of parental and recombinant strains were routinely grown in 3% nutrient broth containing 1% (wt/vol) reagent-grade monosodium glutamate (MSG). Minimal agar was prepared by adding NH_4Cl (5 g), Na_2SO_4 (2 g), K_2HPO_4 (3 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), and glucose (10 g) per liter of distilled water. Fermentor cultures were grown in 3% nutrient broth plus 1% MSG or 0.5% glucose. Tricarboxylic acid intermediates were sterilized by filtration, adjusted to pH 7.0 with 0.1 N NaOH, and added to 3% nutrient broth.

Mating procedure. *E. coli* 4318 and A-1 were grown overnight at 37°C in test tubes containing 4 ml of 3% nutrient broth in the shaker-incubator. Each culture was diluted 1:20 with sterile broth and reincubated until mid-log-phase growth (2×10^8 cells per ml) was attained. Two milliliters of each culture was mixed and allowed to incubate without agitation at 37°C for 90 min. The donor and recipient cultures and the mating mixture were centrifuged and washed twice with 0.9% saline, and then 0.1 ml of each was spread on minimal agar plates. These were incubated for 4 to 5 days at 37°C. Donor and recipient cells plated in this manner failed to yield colonies in all experiments performed. Organisms that formed colonies on this medium were known to be genetic recombinants. They were subcultured five times on minimal agar and then tested for enzyme activity.

Test tube cultures. Stock cultures of parental and recombinant strains were streaked on plates of 3% nutrient agar and checked for purity. Tube cultures (4 ml each) were prepared with inoculum obtained from isolated colonies and were incubated for 20 h at 37°C in the shaker-incubator.

Fermentor cultures. A 1,000-ml fermentor with attached pH and dissolved-oxygen controls (Bioflow model C-30, New Brunswick Scientific Co., New Brunswick, N.J.) was used to confirm test tube results and for additional studies. Fermentor temperature was maintained at 37°C, air flow was maintained at 560 cm^3/min, and agitator (propeller) speed was maintained at 400 rpm. Foaming was controlled with Corning Antifoam B reagent.

L-Asparaginase assay. Whole cells were assayed for L-asparaginase activity by nesslerization or on a Technicon Autoanalyzer (Technicon Corp., Inc., Tarrytown, N.Y.) by methods previously described (11, 14).

RESULTS

E. coli A-1 was recognized as an F^- by mating experiments with known F^- and Hfr *E. coli* strains. This organism was able to receive donor markers for *met* with a recombination frequency of about 0.05%. Using the nutritional markers as proof of recombination, we obtained 350 isolates after primary plating on minimal agar. Between 60 and 70 were subcultured five times on the minimal agar, and stock cultures were then prepared. The synthesis of L-asparaginase was measured in each of these isolates, and they were divided into three categories according to the amount of enzyme produced. Table 1 shows enzyme activities of the parental strains and three recombinants representing the three categories. *E. coli* HAP is the parental strain from which *E. coli* A-1 was derived (1) and is included only for comparison. Recombinants, like parental strains, produced L-asparaginase but not L-glutaminase in the presence of MSG (Table 1). A comparison of L-asparaginase activity in international units per milliliter shows that the isolate designated R-17 produced very low levels of enzyme compared with *E. coli* A-1. If the comparison takes cell growth into consideration (international units per milligram of cell dry weight), it becomes evident that R-17 cells produced almost as much enzyme as the parental strain. On the other hand, although *E. coli* R-26 grew relatively well in the test medium, L-asparaginase activity was very low. Again, R-69 produced levels of L-asparaginase approximately 100% higher than *E. coli* A-1, whereas the increase in growth was only 15%.

To confirm the results from these studies, a series of experiments with the Bioflow fermentor was carried out. Previous studies with *E. coli* A-1 have shown that optimum enzyme levels are

TABLE 1. *L-Asparaginase synthesis in test tube cultures of parental and recombinant strains grown in 3% nutrient broth plus 1% MSG*

E. coli strain	Phenotype or genotype	L-Asparaginase (IU/ml of culture fluid)	Cell dry wt (mg/ml)	L-Asparaginase (IU/mg of cell dry wt)	L-Glutaminase (IU/ml)	pH of supernatant
HAP	Prototrophic	2.5	2.60	1.0	0.0	7.8
A-1	$Met^- Las^+ F^-$	10.8	2.20	4.9	0.2	8.1
4318	*thi leu* Las^- Hfr	0.0	1.60	0.0	0.0	7.6
R-26 (1)[a]	Prototrophic	0.2	1.80	0.1	0.0	7.7
R-17 (2)[a]	Prototrophic	3.2	0.76	4.2	0.0	7.7
R-69 (3)[a]	Prototrophic	20.3	2.53	8.1	0.3	8.0

[a] (1) Includes all recombinants that produce <1 IU/ml; (2) includes those that produce >1 but <10 IU/ml; (3) includes those that produce >10 IU/ml.

achieved in 3% nutrient broth plus 1% MSG medium when the pH is maintained at 7.5 and the dissolved-oxygen level is at 0%. In our study, enzyme levels of parental and recombinant strains were generally found to be greater than those obtained in test tube cultures, but they maintained the same relationship to each other (Table 2). The increase in L-asparaginase activity of *E. coli* R-69 can be explained on the basis of better growth in the fermentor, but the amount of L-asparaginase per unit of cell dry weight remained essentially the same as that of *E. coli* A-1. Nevertheless, the 54% increase in international units per milliliter when compared with *E. coli* A-1 justifies the use of this strain for large-scale production. Although L-asparaginase levels for *E. coli* R-17 did not increase significantly, growth in the fermentor improved by 58% and, whereas growth of R-26 improved only 17%, enzyme synthesis increased by 50%.

Additional characterization of the recombinant strains is described in Fig. 1. Previous studies on the parental recipient *E. coli* A-1 have shown that maximal enzyme synthesis is attained in pregrown cells after 20 h of MSG induction by maintaining the pH at 7.5 and allowing the dissolved-oxygen level to drop to zero. *E. coli* R-69 produced L-asparaginase at a higher rate than *E. coli* A-1, whereas *E. coli* R-17 and *E. coli* R-26 produced much less enzyme under the same conditions (Fig. 1).

Because *E. coli* R-69 produced significantly higher levels of L-asparaginase than A-1, the organism currently used for commercial produc-

TABLE 2. *Characterization of parental and recombinant strains grown in 3% nutrient broth plus 1% MSG in the Bioflo fermentor*

E. coli strain	Phenotype or genotype	L-Asparaginase (IU/ml of culture fluid)	Cell dry wt (mg/ml)	L-Asparaginase (IU/mg of cell dry wt)	L-Glutaminase (IU/ml)	Supernatant pH[a]
A-1	Met^- Las^+ F^-	19.8	2.30	8.7	0.3	7.5
4318	*thi leu* Las^- Hfr	0.0	1.89	0.0	0.0	7.5
R-26 (1)[b]	Prototrophic	0.3	2.10	0.1	0.0	7.5
R-17 (2)[b]	Prototrophic	4.1	1.20	3.3	0.0	7.5
R-69 (3)[b]	Prototrophic	30.5	3.10	9.8	0.4	7.5
HAP	Prototrophic	3.2	3.50	0.9	0.0	7.5

[a] Controlled automatically with 0.1 N HCl or 0.1 N NaOH.

[b] (1) Includes recombinants that produce <1 IU/ml; (2) includes those that produce >1 but <10 IU/ml; (3) includes those that produce >10 IU/ml.

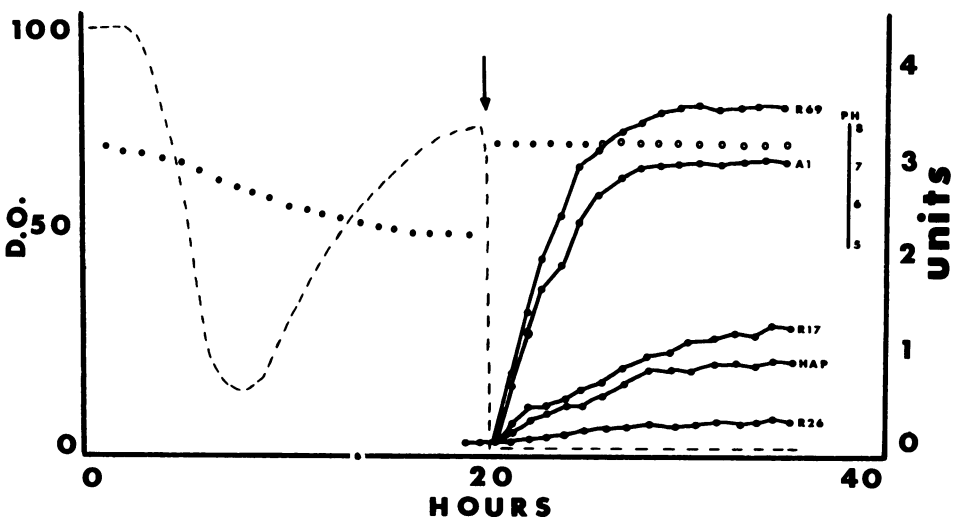

FIG. 1. *Kinetics of L-asparaginase synthesis by E. coli A-1, HAP, R-69, R-26, and R-17 in a Bioflow fermentor. Symbols: (— —) percent dissolved oxygen (D.O.), (——) international units of asparaginase per milligram of cell dry weight, and (· · · · ·) culture pH. Medium pH was adjusted (arrow) and controlled automatically thereafter. Enzyme synthesis was initiated by adjusting pH, decreasing dissolved oxygen to 0%, and adding 0.5% (wt/vol) MSG.*

tion, it was selected for further studies. L-Asparaginase synthesis for this recombinant was inhibited by dissolved-oxygen levels greater than zero in exactly the manner seen in *E. coli* A-1. As with A-1, the inhibition of enzyme synthesis by oxygen dissolved in the medium was found to be reversible (Fig. 2). The addition of glucose, once enzyme synthesis was induced, produced a transient repression (Fig. 3). Enzyme synthesis continued only after the glucose had been consumed in the same manner as occurred in A-1. Recent studies by Netrval (12, 13) have shown that many of the tricarboxylic acid cycle intermediates enhance L-asparaginase activity in *E. coli*. Table 3 shows that, in every case, L-asparaginase levels were higher in R-69 than in A-1.

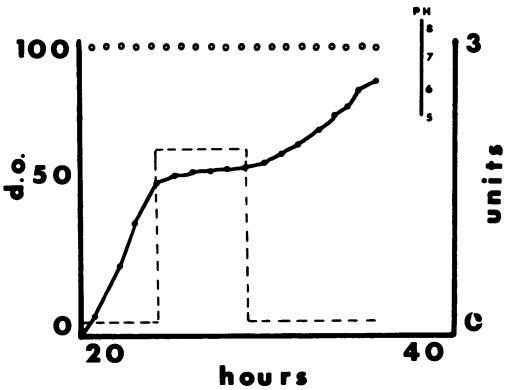

FIG. 2. *Effect of a 50% dissolved-oxygen (d.o.) pulse on the kinetics of asparaginase synthesis in E. coli R-69. Dissolved oxygen was adjusted by increasing or decreasing agitator speed. Symbols:* (- - -) *dissolved-oxygen pulse,* (—) *international units of asparaginase per milligram of cell dry weight, and* (· · · ·) *culture pH.*

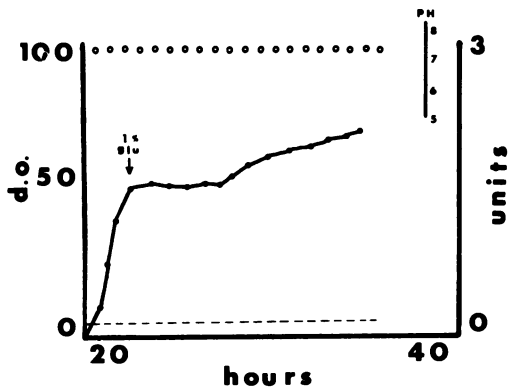

FIG. 3. *Effect of 1% glucose on the kinetics of L-asparaginase synthesis in E. coli R-69. Symbols:* (- - -) *percent dissolved oxygen (d.o.),* (——) *international units of asparaginase per milligram of cell dry weight, and* (· · · ·) *culture pH.*

DISCUSSION

In contrast to the many reports on the physiological conditions that affect the kinetics of L-asparaginase synthesis, little work has been reported on the genetics of *E. coli* or any other bacteria with respect to synthesis of this enzyme. The isolation of strains that produce large amounts of enzyme is made difficult by the lack of suitable media and adequate techniques for selection. Selection of mutants has also been made impossible by these deficiencies. In view of this, it was assumed that a genetic selection procedure could be designed to yield strains of *E. coli* that produce large amounts of L-asparaginase.

The mating experiment described in this report resulted in the isolation of recombinant progeny that had varying degrees of enzyme activity. Approximately 10% produced greater levels of L-asparaginase than the parental strains. The remainder produced lower levels but are considered to be recombinants since they are prototrophic organisms obtained by mating auxotrophic parental strains and also because the amounts of L-asparaginase are different from those produced by either parent strain. The isolation of recombinants capable of producing 30 IU/ml that are derived from parental strains such as A-1, which produces 20 IU/ml, and 4318, which is a nonproducer, raises many questions regarding the genetics of L-asparaginase synthesis in *E. coli*. These recombinants also differed from the parental strains in growth characteristics. Fermentor cultures showed that variations in total cell yield were independent of L-asparaginase synthesis. Examination of many recombinants showed that the two traits were transmitted randomly from 4318 to A-1. It is obvious that *E. coli* possesses a complex physiological

TABLE 3. *Effect of various tricarboxylic acid cycle intermediates on the synthesis of L-asparaginase in E. coli A-1 and E. coli R-69 in test tube cultures*

Addition (0.5%) to 3% nutrient broth	L-Asparaginase synthesis (IU/mg of cell dry wt) in:	
	E. coli A-1	*E. coli* R-69
None	2.7	2.9
MSG	4.8	7.7
Oxalacetate	4.6	6.5
Pyruvate	4.0	6.1
Acetate	4.0	6.0
Malate	3.2	5.0
Succinate	3.1	5.0
α-Ketoglutarate	2.9	4.6
Fumarate	2.8	4.5
Citrate	2.7	3.3
Isocitrate	2.7	3.2

mechanism for the production of L-asparaginase, but the brief study presented here indicates that the genetic mechanism involved may be far more complicated. Many questions, including the following, have become very pressing. (i) Does 4318 have genes that are not expressed? (ii) Does 4318 transmit a derepressor? (iii) Why do most (90%) recombinants produce less L-asparaginase than does the parent A-1? These interesting, unanswered questions will be examined later. On a more practical note, however, the work reported here has yielded a new prototrophic recombinant, R-69, which is now being used in the large-scale (1,000-liter) commercial production of L-asparaginase. This organism regularly produces 30 IU/ml of culture fluid, an increase of 10 IU/ml over production by A-1. The total yield from R-69 was improved by some 10^7 IU of L-asparaginase per run of the fermentor. The average therapeutic dose of L-asparaginase in acute leukemias ranges from 3,000 to 9,000 IU/kg of body weight (10); the use of R-69 represents an increase of some 35 to 100 daily doses per culture for a person weighing 30 kg. The time, cost, and work involved are identical for R-69 and A-1. We think that conjugation experiments may become the preferred method for work of this kind.

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