Folia Microbiol. 29, 1—7 (1984)

Selection of *Escherichia coli* K12 1EA Mutants with Increased Synthesis of Ribitol Dehydrogenase

P. KYSLÍK and B. SIKYTA

Department of Technical Microbiology, Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4

Received March 4, 1983

ABSTRACT. Selection of an interspecific hybrid *Escherichia coli* K 12 1EA in a chemostat on xylitol yielded a stable mutant synthesizing a four-fold amount of ribitol dehydrogenase (EC 1.1.1.56). Subsequent cultivation of the mutant under increased selection pressure resulted in an accumulation of a mutant with 12-fold higher level of ribitol dehydrogenase relative to the parent strain 1EA. A selection during which a UV-mutagenized population of the 1EA mutant was cultivated in a chemostat on xylitol was accompanied by monitoring the activities of ribitol dehydrogenase and D-arabinitol dehydrogenase (EC 1.1.1.11) of two adjacent catabolite operons. A several-fold increase in the activity of the two enzymes was followed by further increase in the activity of ribitol dehydrogenase and a concomitant drop in the activity of D-arabinitol dehydrogenase. The two hyperproducing strains are compared with the parent mutant as to the rate of synthesis of the two dehydrogenases and growth parameters under the conditions of batch cultivation.

Abbreviations: D-Abt D-arabinitol, Rbt ribitol, Xlt xylitol, ADH D-arabinitol dehydrogenase, RDH ribitol dehydrogenase, *dal* catabolite operon for D-arabinitol utilization, *rbt* catabolite operon for ribitol utilization.

In contrast to *Escherichia coli* K 12 (Scangos and Reiner 1978), the wild strain of *Klebsiella aerogenes* can utilize the pentitols Rbt and D-Abt owing to inducible enzymes encoded by two adjacent catabolite operons *rbt* and *dal* (Charnetzky and Mortlock 1974a, b). Some strains that have been isolated can utilize, in addition, the pentitol Xlt which is rarely encountered in nature. These strains synthesize constitutively RDH which catalyzes the conversion of an unnatural substrate, Xlt, to D-xylulose (D-*threo*-2-pentulose) with a high K_M. The K_M value for Xlt is about 100 times higher than that for the naturally occurring substrate Rbt (Mortlock *et al.* 1965) and the oxidation of Xlt catalyzed by RDH is obviously a metabolic reaction limiting the growth of the microorganism.

Rigby *et al.* (1974) limited the growth of *K. aerogenes* in the chemostat by the slowly utilized Xlt and succeeded in isolating hyperproducing mutants which synthesized severalfold higher amounts of RDH. The molecular basis of selection of such strains during growth on an unnatural substrate is obviously a derepression of an operon leading to a constitutive gene expression, and subsequent amplification of the structural gene for the enzyme which catalyzes the reaction limiting the growth of the microorganism (*for reviews cf.* Wu 1978, Lin *et al.* 1976). Under somewhat different conditions, *i.e.* at a variable dilution rate during the selection, Inderlied and Mortlock

(1977) isolated hyperproducing mutants of *K. aerogenes* in which the hyperproduction of a sole enzyme, RDH, was detected. No increase was found in the synthesis of the remaining enzymes of the *rbt* operon or the enzymes encoded in the *dal* operon.

In the present communication the expression of operons participating in the utilization of Xlt is used to describe the course of selection of mutants with enhanced synthesis of RDH. The selection occurred in a heterogeneous population of a parent strain. *E. coli* K 12 (Rigby *et al.* 1976) on Xlt in a chemostat. The growth characteristics were determined in two isolated hyperproducing strains and compared with the parent strain 1EA.

MATERIALS AND METHODS

Microorganisms. Interspecific hybrid strain *Escherichia coli* K 12 1EA was obtained from M.S. Neuberger (Rigby *et al.* 1976). Hyperproducing strains 11EA and 111EA were isolated in this laboratory by selection in a chemostat.

Medium. Batch and continuous cultivations were done in medium M9 containing (g/L): $Na_2HPO_4.12H_2O$ 14.62, KH_2PO_4 3.00, NaCl 1.00, NH_4Cl 1.00, $MgSO_4.7H_2O$ 0.246. Unless otherwise stated, the source of carbon was a pentitol (2 g/L) or casein hydrolyzate (10 g/L).

Cultivation. Batch cultivations were done on a rotary shaker in 500-mL flasks containing 50 mL medium at 37 °C. Continuous cultivations were performed in a 3-L laboratory fermentor LF 2 (*Development Workshops, Czechoslovak Academy of Sciences*) with the following parameters: working volume of fermentor 1.5 L, temperature 37 °C, aeration rate 1.5 L air per min, dilution rate 0.1 h^{-1}, stirrer frequency 10 Hz.

Isolation of mutants. Single-colony isolates obtained by spreading a chemostat population sample on a solid M9 medium supplemented with 4.1 mM Xlt (selection on 13.1 mM Xlt) or 10 g/L casein hydrolyzate (selection on 4.1 mM Xlt) were tested under conditions of a batch culture. High growth rate was used as a criterion for selecting mutants with increased specific RDH activity. Strains were maintained on agar slants in a medium with 11.1 mM glucose at 4 °C. Chemostat selection in a medium with 13.1 mM Xlt followed after mutagenesis of the parent population by UV light: energy fluence of 120 J/m^2 was applied to cells of 1EA strain from the middle of the exponential growth phase in a batch cultivation in a medium with 13.1 mM Rbt. A volume of 45 mL of irradiated culture was used to inoculate a chemostat in which the supply of fresh medium was started at the beginning of stationary growth phase.

Enzyme preparations and assays. The cell extract was prepared at 4 °C. Separated cells were disintegrated mechanically, using glass beads No. 14 in 0.1 M phosphate buffer (pH 7.0) with 1 mM NAD^+. The homogenate was centrifuged at 23 000 ***g*** for 30 min and the activity of RDH and ADH was determined in the supernatant according to Taylor *et al.* (1974) on a Cary 118 spectrophotometer (Varian, USA). The RDH activity was determined with 50 mM Rbt or 500 mM Xlt, ADH activity with 50 mM D-Abt. The activities of both enzymes were expressed as specific activities in nkat/mg sample protein.

Analyses. Proteins were assayed according to Miller (1959). Biomass concentration was measured in a SP 30 UV spectrophotometer (Pye Unicam,

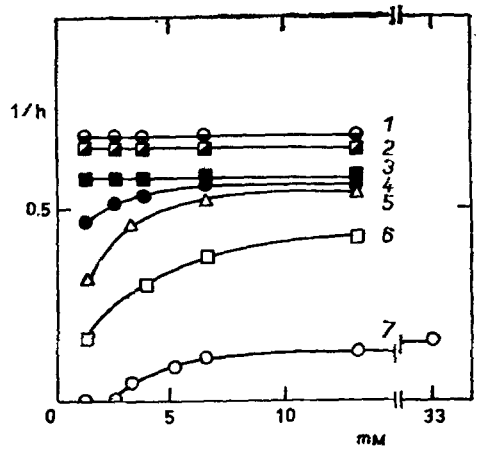

FIG. 1. Dependence of specific growth rate (1/h) of strain 1EA (*circles*), 11EA (*squares*) and 111EA (*triangles*) on initial concentration (mM) of D-arabinitol (*1, 3*), ribitol (*2, 4*) and xylitol (*5, 6, 7*) in batch culture.

England) at 615 nm (A_{615}) and gravimetrically (X, mg/mL) by filtration on Synpor 6 membrane filters (*Synthesia*, Uhříněves, Czechoslovakia). The two quantities are linearly related: $X = 0.37 \cdot A_{615}$. Specific growth rate μ was calculated according to $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_1 and X_2 are biomass concentrations at time t_1 or t_2, respectively, during growth in a batch culture.

RESULTS AND DISCUSSION

The dependence of specific growth rate of strain 1EA on initial concentration of carbon source was studied under conditions of a batch cultivation. The results of growth experiments with Rbt, D-Abt or Xlt as carbon sources are given in Fig. 1. The growth rate of strain 1EA is the same at different

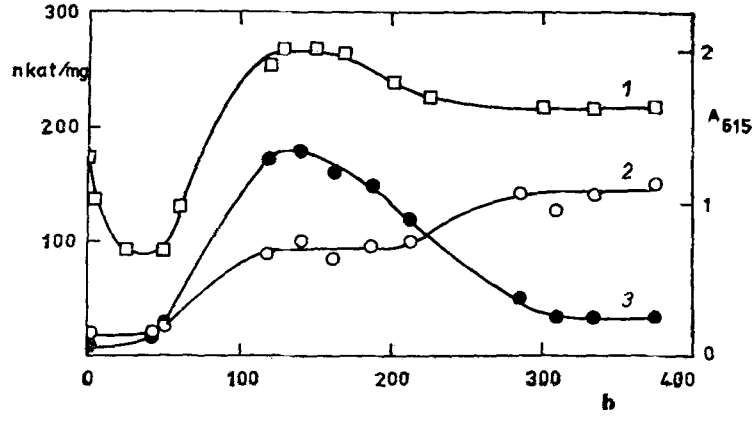

FIG. 2. Specific activity (nkat/mg) of ribitol dehydrogenase (*2*), D-arabinitol dehydrogenase ($3; \times 10^{-1}$) and culture absorbance A_{615} (*1*) during cultivation of UV-mutagenized population of strain 1EA in a chemostat on 13.1 mM xylitol. At time $t = 0$ medium feed was switched on; the culture was in the early stationary phase.

concentrations of natural substrate Rbt and D-Abt whereas with the unnatural substrate Xlt the μ depends on substrate concentration.

Fig. 2 shows the course of cultivation in a chemostat in which a population of strain 1EA mutagenized by UV light was exposed to selection pressure. The selection pressure of the cultivation medium supplemented with Xlt

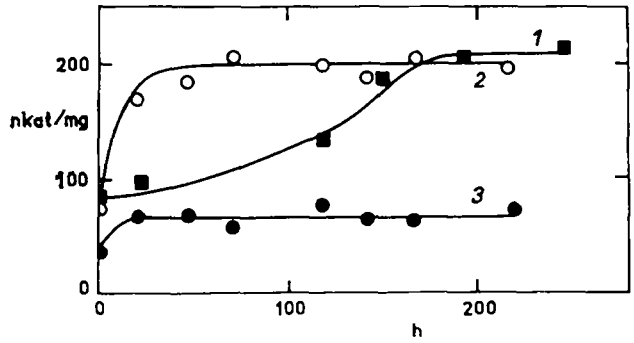

FIG. 3. Specific activity (nkat/mg) ot ribitol dehydrogenase (*1*, *2*) and D-arabinitol dehydrogenase (*3*; $\times 10^{-1}$) of strains 11EA (*squares*), and 111EA (*circles*). At $t = 0$ medium feed was switched on; the culture was in the early stationary phase.

as the growth-limiting factor was increased by a dilution rate of 0.1 h^{-1} which is near the maximum specific growth rate of strain 1EA in a medium with Xlt.

In the course of the selection, specific activities of RDH and ADH were determined. During the first 5 days the specific activities of RDH and ADH increased 5-fold and 20-fold, respectively. This was followed by a gradual decrease in ADH activity and a further rise in RDH activity up to 150 nkat/mg. The increase in cell concentration in the chemostat between the 40th and 60th hour was used to calculate the specific growth rate — 0.46 h^{-1}.

After 187 h a sample was taken from the chemostat and spread on a solid medium with 4.1 mM Xlt to obtain single-colony isolates. At this low Xlt concentration the μ of the parent 1EA strain is still more reduced (Fig. 1). The isolates were tested in batch cultivations in a medium with 13.1 mM Xlt and their μ was determined. The isolate with the highest growth rate was denoted 11EA.

The dependence of μ of strain 11EA on initial concentration of Rbt, D-Abt or Xlt under conditions of a batch cultivation, determined analogously as in strain 1EA, is given in Fig. 1. Since at lower Xlt concentrations strain 11EA grows at a higher μ, selection with this strain could be done in the chemostat at a lower concentration of the growth-limiting Xlt (Fig. 3). Growth was limited by 4.1 mM Xlt at a dilution rate of 0.1 h^{-1}. 200 h after starting of medium feed the population was taken to be homogeneous and the specific activity of RDH was 12-fold higher than with strain 1EA. The accumulated strain was denoted 111EA.

RDH synthesis in strains 11EA and 111EA was studied in a batch culture on Xlt under conditions permitting a maximal expression of the *rbt* operon,

TABLE I. Specific activities of ribitol dehydrogenase and D-arabinitol dehydrogenase in cell-free extract from strains 1EA, 11EA and 111EA

Strain	1EA			11EA			111EA		
Carbon source[a]	Xlt	D-Abt	CH	Xlt	D-Abt	CH	Xlt	D-Abt	CH
Specific RDH activity nkat/mg	20	—	17	83	—	89	83	—	209
Specific ADH activity nkat/mg	2	3	0	3	4	0	4	—	2[b]
X/R[c]	0.040	—	—	0.042	—	—	0.042	—	—

[a] CH — casein hydrolyzate.
[b] High ADH activity in medium supplemented with CH was determined in a sample of culture collected from a chemostat at the time of isolation of strain 111EA.
[c] Ratio of specific RDH activity determined in a reaction mixture with 500 mM Xlt to specific activity with 50 mM Rbt.

on casein hydrolyzate. Table I shows the values of specific activities of RDH in strains 11EA, 111EA and the parent strain 1EA. The ratio of specific RDH activities determined with 500 mM Xlt and with 50 mM Rbt as substrates (X/R) is characteristic for the enzyme (Wu *et al.* 1968). The identical value of the X/R ratio for the three strains under study indicates the hyperproducing character of strains 11EA and 111EA.

In the absence of environmental selection pressure the hyperproduction of RDH in 111EA is unstable. In a batch cultivation in a medium with casein hydrolyzate a high level of the enzyme was found only during strain isolation whereas a low level was determined after transfers of the isolate on a solid medium with Xlt or glucose.

In a batch culture using a medium with Xlt strain 111EA synthesizes a reduced stable amount of RDH comparable with the level of RDH in strain 11EA (Table I). However, the relationship between μ and the initial Xlt concentration in batch cultures of strains 11EA and 111EA is different (Fig. 1).

If the growth of strain 111EA in a chemostat is limited by 4.1 mM Xlt (Fig. 3), the RDH synthesis reaches a high level, in contrast to strain 11EA,

TABLE II. Growth parameters μ_{max} and K_S in 1EA, 11EA and 111EA mutants in batch cultures on Xlt[a]

Mutant	1EA	11EA	111EA
μ_{max}, 1/h	0.20	0.53	0.59
K_S, mM	7.30	6.00	1.20

[a] Parameter values were obtained by a double reciprocal plot of experimental data from Fig. 1 valid for medium with Xlt.

already after 30 h of growth. A sudden rise to about a double value is also found in the specific activity of ADH.

Table I gives ADH activities in strains 1EA, 11EA and 111EA in a medium with Xlt, D-Abt or casein hydrolyzate. In pentitol-containing media the synthesis of enzymes encoded by the *dal* operon is induced (Wilson and Mortlock 1973) whereas on casein hydrolyzate the cells grow in the absence of an inducer. The specific ADH activity in strains 1EA and 11EA grown in a medium with Xlt or D-Abt differs considerably from that on casein hydrolyzate. A relatively high ADH activity in a medium with casein hydrolyzate was found in strain 111EA only immediately after its isolation; it represented 25 % of the value in a sample from the chemostat (Fig. 3). If the parent stock culture was 111EA from agar slant, the ADH activity was identical with that found in strain 11EA.

Table II shows values of growth parameters K_S and μ_{max} in populations of the two hyperproducing strains and the parent strains 1EA in a batch culture on Xlt.

The increase in μ of strain 11EA relative to the parent strain corresponds to the increase of constitutive RDH synthesis. A direct relationship between the amount of the enzyme catalyzing the initial catabolite reaction with a high K_M and μ was also found by Hall and Clarke (1977). On the other hand, no similar relationship was found in a variety of hyperproducing strains of *K. aerogenes* (Rigby *et al.* 1974; Inderlied and Mortlock 1977). The behaviour of the two hyperproducing strains under conditions of a batch culture and in a chemostat permits us to draw only indirect conclusions. The 5-fold lower K_S in strain 111EA determined in a batch culture on Xlt as compared with strain 11EA leads obviously to the accumulation of the former strain in the chemostat. Both hyperproducing strains in a batch culture on Xlt exhibit about the same maximum specific growth rate.

The selection of a mutagenized population of strain 1EA in a chemostat included also the study of the magnitude of DNA segment subject to amplification. The study made use of expression of tightly genetically linked operons *rbt* and *dal*. It is obvious that at initial stages of selection in the chemostat accumulation occurs of strains with activities of the two dehydrogenases increased several-fold. The further course of selection apparently reflects the selection of strains 11EA and 111EA. The highest specific ADH activity exceeds the level of constitutive ADH synthesis in *E. coli* N.C. 260 which carries two copies of the *dal* operon (Neuberger *et al.* 1979).

REFERENCES

Charnetzky W.T., Mortlock R.P.: Ribitol catabolic pathway in *Klebsiella aerogenes*. *J. Bacteriol.* **119**, 162 (1974*a*).

Charnetzky W.T., Mortlock R.P.: Close genetic linkage of the determinants of the ribitol and D-arabitol catabolic pathways in *Klebsiella aerogenes*. *J. Bacteriol.* **119**, 176 (1974*b*).

Hall B.G., Clarke N.D.: Regulation of newly evolved enzymes. III. Evolution of the *ebg* repressor during selection for enhanced lactase activity. *Genetics* **85**, 193 (1977).

Inderlied C.B., Mortlock R.P.: Growth of *Klebsiella aerogenes* on xylitol: implications for bacterial enzyme evolution. *J.Mol.Evol.* **9**, 181 (1977).

Lin E.C.C., Hacking A.J., Aguilar J.: Experimental models of acquisitive evolution. *BioScience* **26**, 548 (1976).

Miller G.L.: Protein determination for large numbers of samples. *Annal.Chem.* **31**, 964 (1959).

Mortlock R.P., Fossitt D.D., Wood W.A.: A basis for utilization of unnatural pentoses and pentitols by *Aerobacter aerogenes*. *Proc.Nat.Acad.Sci. USA* **54**, 572 (1965).

NEUBERGER M.S., PATTERSON A., HARTLEY B.S.: Purification and properties of *Klebsiella aerogenes* D-arabitol dehydrogenase. *Biochem.J.* **183**, 31 (1979).

RIGBY P.W.J., BURLEIGH B.D., HARTLEY B.S.: Gene duplication in experimental enzyme evolution. *Nature* **251**, 200 (1974).

RIGBY P.W.J., GETHING M.J., HARTLEY B.S.: Construction of intergeneric hybrids using bacteriophage PlCM: Transfer of the *Klebsiella aerogenes* ribitol dehydrogenase gene to *Escherichia coli*. *J.Bacteriol.* **125**, 728 (1976).

SCANGOS G.A., REINER A.M.: Ribitol and D-arabitol catabolism in *Escherichia coli*. *J.Bacteriol.* **134**, 492 (1978).

TAYLOR S.S., RIGBY P.W.J., HARTLEY B.S.: Ribitol dehydrogenase from *Klebsiella aerogenes*. *Biochem.J.* **141**, 693 (1974).

WILSON B.L., MORTLOCK R.P.: Regulation of D-xylose and D-arabitol catabolism by *Aerobacter aerogenes*. *J.Bacteriol.* **113**, 1404 (1973).

WU T.T.: Experimental evolution in bacteria. *C.R.C.Crit.Rev.Microbiol.* **6**, 33 (1978).

WU T.T., LIN E.C.C., TANAKA S.: Mutants of *Aerobacter aerogenes* capable of utilizing xylitol as a novel carbon source. *J.Bacteriol.* **96**, 447 (1968).