Evaluation of the stability of biochemical phenotypes of *Escherichia coli* upon subculturing and storage

M. KATOULI,¹* I. KÜHN^{1,2} and R. MÖLLBY¹

¹ Department of Bacteriology, Karolinska Institute, S-104 01 Stockholm, Sweden

² Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden

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Stability of the biotypic characters of 72 enteropathogenic *Escherichia coli* (EPEC) and 21 faecal *E. coli* strains was evaluated after storage and after subculturing using a computerized biochemical fingerprinting method. Sixteen (22 %) EPEC strains and nine (43 %) faecal strains exhibited changes in their biochemical reactions after subculturing. In contrast, strains stored at -70°C and 4°C did not show any measurable changes. Of 23 biochemical markers tested, eight were subject to changes in at least one of these strains. Change in lactulose fermentation was most frequent, occurring in 17 (18 %) strains. A decrease or loss of activity in the fermentation of 5-ketogluconate, arbutin and methyl β -D-glucoside in six strains (6 %), and an increase in the ability to ferment sucrose, raffinose, melibiose and D-arabinose in 20 strains (22 %) were observed. Mean similarity of the strains, when compared pairwise before and after subculturing, was slightly affected by these changes, but the overall biochemical phenotypes of the strains remained constant.

Introduction

Biochemical reactions have been conventionally used for identification of bacteria to the species level. The usefulness of these markers to subdivide strains below the species level, however, is greatly dependent on the variability of these characters within the species and the distribution of these variations (Lockhart & Liston, 1970). Extensive studies of the biochemical reactions of bacteria have been done to introduce biochemical typing systems in epidemiological studies of bacteria (Barr & Hogg, 1979; Krishnan *et al.*, 1987; Crichton & Old, 1982). To achieve maximum discrimination, biotyping together with serotyping (Simoons-Smit *et al.*, 1985; van der Waaij *et al.*, 1976), serotyping and phage typing (Rennie *et al.*, 1978), serotyping and fimbriation analysis (Brauner *et al.*, 1987), serotyping and outer-membrane protein (OMP) analysis (Stenderup & Ørskov, 1983; Achtman *et al.*, 1983) or sero/OMP/electrophoretic analysis of alloenzymes (Ochman & Selander, 1984) have been suggested for the study of epidemiologically important bacterial clones. The value of biotyping in combination with the other bacterial properties used in

these studies, however, relies to a great extent on the reproducibility and discriminative power of the biochemical reactions used. Kühn (1985) developed a biochemical typing method based on the measurement of the kinetics of several biochemical reactions of bacterial strains grown in liquid media in microtitre plates. This method yields a biochemical fingerprint of each strain which consists of several quantitative data which can be used to establish similarity levels among the tested strains using a personal computer. With this method Kühn *et al.* (1985) studied clones of enterotoxigenic *E. coli* (ETEC) strains which had been isolated from Swedish piglets over a period of 20 years and also ETEC strains isolated from humans in different geographical areas (Kühn & Möllby, 1986). They found stable biochemical phenotypes among certain ETEC serogroups, most of them also carrying the relevant virulence factors.

Many studies on the prevalence of virulence factors among *E. coli* strains isolated from different outbreaks or sporadic cases throughout the world have shown that subculturing and/or storage may have a drastic effect on the loss or expression of these factors (Evans *et al.*, 1977; Levine *et al.*, 1983). However, few investigations have assessed the stability of biochemical reactions following such environmental pressures.

The aim of the present study was to measure the

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; F, deep-frozen, N, non-subcultured and S, subcultured strains; OMP, outer-membrane protein.

stability of the biochemical markers of *E. coli* strains after storage and subculturing and to compare the biochemical phenotypes of these strains before and after such treatments.

Methods

***E. coli* strains.** A total of 72 EPEC strains were tested in this study. They belonged to 11 different serogroups, namely O26 (8 strains), O44 (8 strains), O55 (7 strains), O86 (1 strain), O111 (8 strains), O114 (10 strains), O119 (6 strains), O125 (9 strains), O126 (5 strains), O127 (6 strains) and O128 (4 strains). Of these, 68 strains were obtained from the collection of the National Bacteriological Laboratory (NBL), Stockholm, Sweden, and four strains were obtained from K. Wachsmuth, Center for Disease Control, Atlanta, USA. All EPEC strains had been isolated from diarrhoeal cases and had been stored for a period of up to 10 years. Twenty-one faecal *E. coli* strains isolated from healthy adults in Sweden, and stored for 4 years, were provided by A. Brauner, SMCL, Stockholm, Sweden. O antigens of EPEC strains were determined using poly- and monovalent antisera (NBL) and confirmed by tube agglutination. K and H antigens were not determined.

Storage and subculturing. Strains were kept at -70°C in screw-capped tubes, containing nutrient broth and 50% (v/v) glycerol. This set of the strains was called 'deep-frozen' (F). Replicates of these strains on Tryptic Soy Agar (TSA; Difco), kept at 4°C , were designated the 'non-subcultured' (N) set. From these, successive subcultures were made daily on TSA for 21 d, followed by incubation at 37°C overnight. This set was named 'sub-cultured' (S). Each set was plated on MacConkey agar (Difco) and checked for purity before biochemical testing, and a single colony was spread on nutrient agar (Difco) and incubated at 37°C for 24 h.

Biochemical fingerprinting. Preparation of prefabricated microplates (PhP system; BioSys Inova, Stockholm, Sweden) and selection of the appropriate reagents for fingerprinting *E. coli* strains have been described previously (Kühn & Heden, 1976; Kühn, 1985). Table 1 shows a set of 24 reagents that have been specially selected for characterization of *E. coli* strains. One of these reagents, cellobiose, gives negative results for all *E. coli* strains, and was included to check for contamination by other bacteria.

A loopful of the cultures of the organisms on nutrient agar was suspended in 8 ml sterile 0.2% (w/v) proteose peptone (Difco) solution, containing 0.01% (w/v) bromothymol blue as indicator. Aliquots (150 μl) of the suspension were transferred to the 24 wells in a microtitre plate with the aid of a multi-channel pipette, giving a final reagent

concentration of 3.75 mg ml^{-1} in each well. Using 24 reagents, four strains can be tested in each plate. To allow proper rehydration of the reagents, the inoculated plates were kept in a refrigerator overnight before incubation.

Reading and calculation. The plates were incubated at 37°C and the A_{620} of each reaction read after 4, 7, 24 and 48 h using a microplate reader (Titertek Multiskan, Flow Laboratories). The A_{620} values were automatically transferred to a personal computer (Victor-V286C) and multiplied by 10, yielding scores ranging from 0 to 30 for each reaction. Low values represent acidic reactions (yellow), and high values, alkaline reactions (deep blue). The mean value for all four readings of each reaction was calculated, yielding 24 different values scored from 0 to 30 for each strain (the biochemical fingerprint).

Similarities between strains, when compared pairwise, were measured by calculating the correlation coefficient (r) according to the formula:

$$r = \frac{\sum_{i=1}^{24} (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{24} (X_i - \bar{X})^2 \sum_{i=1}^{24} (Y_i - \bar{Y})^2}}$$

where X_i is the value of the i th reaction for the strain X, Y_i is the value of the i th reaction for strain Y, and $\bar{X}$ and $\bar{Y}$ are the mean values of all 24 scores for strains X and Y, respectively. A correlation coefficient close to 1 for a pair of *E. coli* strains means that the two strains have similar biochemical fingerprints, whereas low correlation coefficient values mean different test results and indicate that the strains are less related. Interassay variation for each test was measured by testing 50 strains in duplicate. Values outside the mean ± 2 SD for a test were taken to indicate changes in that particular test. Strains showing changes based on this criterion were retested to rule out the effect of random errors. Clustering of the isolates was done according to the single linkage clustering method (Sneath & Sokal, 1973).

Results

Of 93 *E. coli* strains tested in this study, 25 strains (27%) exhibited changes in one or more of their biochemical reactions after subculturing when compared to the original, N strains. Of these, 16 strains (22%) belonged to EPEC serogroups and nine strains (43%) were control strains ($P = 0.06$). Among the EPEC strains those belonging to serogroups O26, O44 and O127 did not show any changes in their biochemical reactions after subculturing. The mean correlation coefficient of the strains, when compared pairwise before and after subculturing, was affected by these changes (Table 2). To determine whether these changes were due to subculturing rather than to storage, each strain was assayed pairwise without subculturing (N vs N strains), without and with subculturing (N vs S strains), and finally, without subculturing and after deep freezing (N vs F strains). The results showed a clear drift towards lower similarity coefficients when S strains were compared to the other groups (Fig. 1). Further analysis showed that eight out of 23 biochemical test results (cellobiose was not included), had changed in at least one of these strains. Change in

Table 1. Reagents used for the biochemical fingerprinting of *E. coli* strains

D-Xylose	Deoxyribose	Ornithine
Maltose	Sucrose	5-Ketogluconate
Lactose	Raffinose	Melibionate
Sorbitol	Sorbose	Arbutin
Rhamnose	Tagatose	D-Arabinose
L-Fucose	D-Arabitol	Methyl β -D-glucoside
Glycerol	Adonitol	Lactulose
Melibiose	Dulcitol	Cellobiose*

* Used to check for the presence of contaminating species.

Fig. 1. Cumulative relative frequencies of the correlation coefficients (r) obtained from pairwise comparison of 93 *E. coli* strains. Correlation coefficients were grouped into different class intervals to show differences between the distribution of r , between paired strains: O, before subculturing, N vs N strains, i.e. interassay reproducibility; ●, before and after subculturing, N vs S strains.

lactulose fermentation was most frequent, occurring in 17 strains, followed by raffinose in five strains, melibionate and arbutin in four strains each, and finally 5-ketogluconate, methyl β -D-glucoside and D-arabinose in only one strain each. Of 25 strains, 16 showed changes in only one of the biochemical reactions, eight showed changes in two reactions and one showed changes in three reactions (Table 3).

Two distinct patterns of changes in these bacteria were observed. A decrease or loss of activity with fermentation of 5-ketogluconate, arbutin and methyl β -D-glucoside

Fig. 2. Pattern of the increased fermentative ability of 20 *E. coli* strains showing measurable changes in their biochemical reactions upon subculturing. Results are means expressed as a percentage of maximal fermentation, before subculturing (---) and after subculturing (—). □, Lactulose (17 strains); △, raffinose (5 strains); ●, sucrose (2 strains); ▲, D-arabinose (1 strain); ○, melibionate (4 strains).

side (negative values), and an increase or gain of activity with raffinose, sucrose, melibionate, D-arabinose and lactulose (positive values) (see Table 3). Twenty S strains demonstrated an increase in both speed and/or level of fermentation of five different substrates. Utilization of lactulose and sucrose was almost completed within the first 24 h among these strains, whereas with N strains this was achieved after 48 h (Fig. 2). Fermentation of

Table 2. Comparison of the correlation coefficients of *E. coli* strains showing differences in their biochemical reactions before and after subculturing

<i>E. coli</i> strains (sero group)	No. of strains tested (no. showing changes after subculturing)	Mean correlation coefficients $\pm$ SD of the strains tested in duplicate	
		Before subculturing	Before and after subculturing
EPEC strains			
O26	8	0.995 $\pm$ 0.001	0.985 $\pm$ 0.005
O44	8	0.994 $\pm$ 0.001	0.988 $\pm$ 0.004
O55	7 (1)	0.992 $\pm$ 0.002	0.984 $\pm$ 0.008
O86	1	0.992	0.995
O111	8 (2)	0.988 $\pm$ 0.007	0.988 $\pm$ 0.005
O114	10 (5)	0.986 $\pm$ 0.013	0.983 $\pm$ 0.009
O119	6 (3)	0.989 $\pm$ 0.003	0.970 $\pm$ 0.015
O125	9 (3)	0.991 $\pm$ 0.004	0.978 $\pm$ 0.014
O126	5 (1)	0.993 $\pm$ 0.003	0.987 $\pm$ 0.008
O127	6	0.993 $\pm$ 0.001	0.991 $\pm$ 0.003
O128	4 (1)	0.990 $\pm$ 0.002	0.975 $\pm$ 0.008
Subtotal	72 (16)	0.991 $\pm$ 0.005	0.983 $\pm$ 0.01
Faecal strains	21 (9)	0.991 $\pm$ 0.002	0.986 $\pm$ 0.006
Total	93 (25)	0.991 $\pm$ 0.002	0.984 $\pm$ 0.01

Table 3. Pattern of changes in biochemical reactions of *E. coli* strains after subculturing

<i>E. coli</i> strains (sero group)	Biochemical markers*									
	Raffinose	Sucrose	5-Ketogluconate	Melibionate	Arbutin	Methyl β -D-glucoside	D-Arabinose	Lactulose		
EPEC strains										
E14 (O55)	+4.5	-	-	-	-	-	-	+7		
E15 (O111)	-	+4	-	-	-	-	-	+4		
E12 (O111)	-	-	-	-	-	-	+4	-		
E8 (O114)	-	+4	-	-	-	-	-	-		
E10 (O114)	-	-	-	-	-	-	-	+4		
E5 (O114)	-	-	-	-	-	-	-	+4		
E11 (O114)	-	-	-	-	-6.5	-	-	-		
E9 (O114)	-	-	-	-	-	-	-	+4		
E2 (O119)	+4.5	-	-	+4.5	-	-	-	+6.5		
E4 (O119)	-	-	-	-	-	-4.5	-	-		
E3 (O119)	-	-	-	+4.5	-	-	-	-		
E13 (O125)	-	-	-4	-	-	-	-	+4.5		
E16 (O125)	+4	-	-	-	-	-	-	+6		
E6 (O125)	-	-	-	+4.5	-	-	-	+4		
E7 (O126)	-	-	-	-	-	-	-	+5		
E1 (O128)	+4	-	-	-	-	-	-	+7		
Faecal strains										
K9	-	-	-	-	-	-	-	+5.5		
K3	-	-	-	-	-4	-	-	-		
K8	-	-	-	-	-	-	-	+4.5		
K6	-	-	-	-	-	-	-	+4		
K7	-	-	-	+4	-	-	-	+4		
K1	+4	-	-	-	-	-	-	+5		
K2	-	-	-	-	-5	-	-	-		
K5	-	-	-	-	-4	-	-	-		
K4	-	-	-	-	-	-	-	+4		

* -, No change; numerical scores show mean values of changes in each biochemical reaction.

D-arabinose and melibionate on the other hand, was not only faster by S strains during the first 24 h, but was also more complete than by N strains after 48 h. Six strains showed an overall decrease in their ability to utilize arbutin (4 strains), methyl β -D-glucoside (1 strain) and 5-ketogluconate (1 strain) during 48 h of the experiment (Fig. 3). Loss of ability to utilize these compounds was generally not accompanied by an increase in the utilization of other substrates among these strains.

Fig. 3. Pattern of the decreased fermentative ability of six *E. coli* strains, showing measurable changes in their biochemical reactions, upon subculturing. Results are means expressed as a percentage of maximal fermentation before subculturing (---) and after subculturing (—). ○, Arbutin (4 strains); ●, methyl β -D-glucoside (1 strain); △, 5-ketogluconate (1 strain).

To evaluate the effect of these changes in biochemical reactions on the reproducibility of phenotyping of strains before and after subculturing and/or storage, a dendrogram was constructed for 22 strains with recognizable changes (Fig. 4). It was shown that although there was a drift towards a lower similarity when S strains were compared to N and F strains, the S strains were still very closely related to their origins and only one strain fell into another phenotype (strain E2).

Discussion

Extensive studies of the biochemical reactions of *E. coli* strains isolated from different sources have been made in the past (Crichton & Old, 1979; Gargan *et al.*, 1982; Godbout-DeLasalle & Higgins, 1986). To subdivide these strains into more distinct types for epidemiological purposes most authors have employed either conventional test methods (Bettelheim & Taylor, 1969; Crichton & Old, 1979) or commercially available biochemical test systems (Davies, 1977; Cicmanec & Evans, 1980). In practice, most of the strains to be tested for such purposes would inevitably go through different periods of storage and probably a series of subcultures. Our results on the effect of such environmental pressures on the stability of the biochemical reactions of *E. coli* strains showed that subculturing might have some effect on the ability of these strains to express some, if not all, of their biochemical markers. With storage, however, this effect depends to a certain extent, on the conditions in which the strains are stored.

Fig. 4. Dendrogram of 22 strains with measurable changes in their biochemical profile. For each strain, deep-frozen (F), non-subcultured (N) and subcultured (S) variants were analysed.

Using a computerized biochemical fingerprinting method, we compared *E. coli* strains stored at -70°C , to those continuously subcultured or stored at 4°C for 21 d. As expected, the F strains were more similar to the N strains than to the S strains. Crichton & Old (1979) tested many *E. coli* strains from different culture collections, and also from urinary tract infections, for their biotype stability after many months, and even years. They found that many colonies of *E. coli* strains stored on Dorset's egg slants exhibited the same biotype as that originally identified when the strains were isolated from a fresh specimen. Like many other investigators, these workers also used an absolute 'positive' or 'negative' value system to measure the biochemical reaction after a definite incubation time (between 24 and 72 h). Therefore, a prompt early reaction within a shorter period than 24 h was not detected. This could explain the slight differences between our results and those obtained by these workers. Furthermore, the biochemical reactions used in the PhP system have been specifically selected from a large number of reactions so as to have a suitable variability with time (Kühn, 1985).

Differences in biochemical reactions were observed when S strains were compared to N strains. About a quarter of the strains were subject to measurable changes in up to three of the biochemical tests. Eight of the 23 biochemical reactions (cellobiose not included) used in this study showed changed after subculturing, among which two distinct patterns were dominant. With sucrose, raffinose and lactulose, there was a rapid increase in the rate of fermentation within the first 24 h of incubation, reaching approximately the same level as those of N strains after 48 h. With melibionate and arabinose, however, this change occurred in both the speed of reaction and also the level of fermentation. The reason for this more rapid metabolism of these substances among S strains could not be explained simply. The regulatory genes responsible for the synthesis of the necessary enzymes may have been induced during the subculturing process, or S strains may have evolved a more efficient uptake system to assimilate the compounds, or a combination of both processes may have occurred.

Another difference in the metabolic reaction of the S strains was associated with a decrease in the final level and, to a certain extent, with the speed of the reactions. This was observed with arbutin, methyl β -D-glucoside, and 5-ketogluconate. In total, seven *E. coli* strains showed changes in the fermentation of these compounds. The final level of utilization of these compounds among S strains was always much less than that of N strains. This loss of fermentation ability was not accompanied by any increase in fermentation of other substrates among these bacteria.

One of the factors which might have influenced the results is the growth medium. Composition of the media and also their physical states have been shown to have a great effect on the morphology (Iwaya *et al.*, 1978), ultrastructure (Lorian *et al.*, 1982) and growth rate (Brown *et al.*, 1988) of bacteria, and to produce differences in the biochemical constitution of bacteria and their metabolites (Lorian, 1989). Local accumulation of metabolites in the surrounding environment of the bacteria, when they are grown on solid media, has been postulated to be a factor in repression or activation of the genes responsible for the enzymic action of these bacteria (Lorian, 1989). Therefore, it is possible that the *E. coli* strains which showed changes in their biochemical reactions will give a different pattern when they are subcultured on different and/or liquid media.

In comparison with control strains, EPEC strains seemed to be more stable in their metabolic characters after subculturing. Only 22% of the EPEC strains tested in this study showed changes in their biochemical reactions. The equivalent value for faecal *E. coli* strains was almost twice as high (43%), which is not surprising, as the stability of some other phenotypic characters within clonal populations of EPEC strains has also been reported by other investigators (Ørskov & Ørskov, 1983).

Another interesting feature of this study was that some of the S bacteria, without any obvious changes in a single test, had a lower overall similarity when they were compared with N strains. The changes in individual biochemical markers within these bacteria were too small to be spotted, but they were spread over 23 reactions, resulting in slightly different biochemical fingerprint for these strains.

We retested the S strains with measurable changes after another 3 months of storage at 4°C and compared them to the N strains (data not shown). The results indicated that most of the changes, including those of lactulose fermentation, were stable.

In view of the fact that more than 18% of the strains tested in this study showed changes in their lactulose fermentation, we suggest that this test should not be included in biotyping systems for *E. coli* strains, if the system is used for epidemiological studies of isolates from diverse sources. However, if the system is used to detect very small changes in *E. coli* strains, lactulose fermentation is a very good marker.

Kühn (1985) used a minimum correlation coefficient of 0.975 to define identity between *E. coli* isolates. In the present study, the duplicate assays yielded a mean correlation coefficient of 0.991 (Table 2), giving a reproducibility level of the biochemical fingerprinting system of 0.985 (mean correlation coefficient -2 SD). However, when the S strains were compared to the corresponding N strains, 13 (14%) comparisons yielded

lower similarity than 0.975, and if the lactose test was excluded, 7 (8%) comparisons fell below this limit.

The biochemical similarity between different isolates with a common ancestor depends on two factors: firstly, the reproducibility of the assay, and secondly, the extent of the changes the isolates have gone through since their divergence from their common ancestor. The first factor may be measured easily, whereas the second factor cannot be predicted. It is, therefore, important in epidemiological studies, when the bacteria have been isolated from diverse sources, not only to investigate whether they are identical or not, but also to give a measure on how similar the isolates are to each other. This information may easily be shown in a dendrogram such as the one in Fig. 4. On the other hand, if the aim of the study is to detect identical strains from a recent outbreak, as in most nosocomial studies, the same identity level which has been used by Kühn (1985) and Kühn & Möllby (1986) might be applicable.

The need for, and the role and importance of biotyping of bacterial isolates has been presented previously (Balows & Isenberg, 1978; Farmer, 1978). Both conventional biotyping methods and commercial biotyping kits have been employed by many workers. It has been shown that several variables may influence the reproducibility of the biochemical tests. These include inoculum size, time of reading, the interpretation of the results by different workers, temperature of incubation and many others. While certain tests like aesculin hydrolysis are inoculum dependent (Miskin & Edberg, 1978), less effect has been observed with carbohydrates (Crichton & Old, 1979). Most of the test reagents employed in our study were carbohydrates. We also obtained the same results when the strains were retested in plates of different batches. Thus, the computerized biochemical fingerprinting method is not only an easy technique for detecting identity between strains, but also useful for detecting possible changes of biochemical reactions of bacteria due to subculturing, storage and other environmental factors.

In conclusion, our results demonstrate that upon repeated subculturing, *E. coli* strains change their biochemical activity profiles slightly. These biochemical reactions seem to be less stable among normal faecal *E. coli* than among a collection of EPEC strains. It is recommended that the most readily altered biochemical activity, lactulose fermentation, is not included in routine biotyping procedures.

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