Mutations Conferring Quantitative and Qualitative Increases in β-Galactosidase Activity in Escherichia coli

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Summary. Sodium lactobionate is not utilized as a carbon source by Escherichia coli because it is only poorly bound and hydrolyzed by β-galactosidase and it does not induce the formation of the enzyme. However, treatment with N-methyl-N'-nitro-N-nitrosoguanidine produced 32 independent mutants able to grow on lactobionate. Most of the mutants formed β-galactosidase constitutively, 29 of them having mutations in the regulatory gene and one possibly in the operator. In addition, the mutants possessed quantitatively — or qualitatively — altered β-galactosidase. In 28 mutants the β-galactosidase activity was 1.5 to 4.5 times that of the wild-type. The enzymes of these mutants were unaltered in thermostability and substrate binding. One enzyme that was titrated immunologically possessed a molecular activity identical with the wild-type enzyme. These mutants appear to contain extra copies of the gene for β-galactosidase. The spontaneous mutation rate to constitutivity was $6.3 \times 10^{-3}$ and to the formation of apparently extra genes, $9.2 \times 10^{-3}$.

The β-galactosidases of three mutants were qualitatively changed as judged from their increased thermostability, altered substrate-binding constants and greatly increased ability to hydrolyze lactose and lactobionate. Affinity for O-nitrophenyl-β-galactoside and lactose was increased by the mutations while that for lactose was decreased; maximum velocities for the hydrolysis of O-nitrophenyl-β-galactoside were also decreased. Relative to their rates of hydrolysis of O-nitrophenyl-β-galactoside, these altered enzymes hydrolyzed lactose at 6 to 8 times, and lactobionate up to 23 times, the rate given by the normal enzyme. The mutations appear to increase the hydrophobic nature of the enzyme near the aglycon binding site and facilitate the hydrolysis of more hydrophilic galactosides. The lactobionic acid positive character could be transferred to other bacteria by sexual conjugation when the enzyme changes were qualitative, but not when they were quantitative.

Introduction

The β-galactoside, lactobionic acid, is utilized for growth by Corynebacterium simplex, which possesses a β-galactosidase capable of hydrolyzing the carbohydrate (Bernaerts and de Ley, 1957). In contrast, the affinity and catalytic efficiency of the β-galactosidase of Escherichia coli is too poor with respect to lactobionic acid (Kuby and Lardy, 1953) to permit growth on the compound. Therefore, the mutational acquisition of lactobionic acid utilization appeared to be a suitable character to use in the study of the genetic and biochemical origin of new functions.

Materials and Methods

Strains. Mutations were induced in Escherichia coli 3000, an Hfr strain containing the wild-type genes of the lactose operon; i.e. $i'o'z'y'$, where $i$ is the gene for regulator, $o$ the gene for operator, $z$ the gene for β-galactosidase and $y$ the gene for galactoside permease.
Spontaneous mutation frequencies were also measured in strains 3300 (Hfr $i^{-}o^{+}z^{+}y^{+}$), 2000 ($F^{-}i^{-}o^{+}z^{+}y^{+}$), 2300 ($F^{-}i^{-}o^{+}z^{+}y^{+}$), RV/$Fi^{-}o^{+}z^{+}y^{+}$ (RV is a deletion of the whole lactose operon of the chromosome and the lactose genes are carried in an episome), RV/$Fi^{-}o^{+}z^{+}y^{+}$ and $i^{-}lac^{+}/Fi^{-}lac^{+}$. M-XIII, an $F^{-}$ strain with a deletion of the lactose operon and the adjacent proline $a$ and $b$ genes, was used in crossing experiments. The genetic nature of constitutive mutants was tested by crosses with $i^{s}/F^{s}$, a homogenote containing the super-repressed allele ($i^{s}$) of the regulator gene (Willson et al., 1964) and requiring arginine and histidine.

**Methods.** Mutations to lactobionic acid utilization were induced in strain 3000 by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (M:NN-guanidine) (Langridge and Campbell, 1968). The buffers, growth media, enzyme and protein assays and tests of enzyme sensitivity to heat have also been described earlier (Langridge, 1968 a, b, c).

For routine enzyme assays, bacteria were grown overnight in broth medium without inducer, centrifuged, suspended in phosphate buffer, sonicated and reentrifuged. Unless otherwise stated, $\beta$-galactosidase activity is expressed as $\mu$moles 0-nitrophenyl-$\beta$-galactoside (ONPG) hydrolyzed per minute per $\mu$g of unfractionated bacterial protein in 0.1 M phosphate buffer at pH 7.0.

Lactobionic acid (galactose-$\beta$-D-gluconic acid) was obtained as calcium lactobionate and converted to the sodium salt by treatment with the sodium form of the cation exchange resin, Zeocarb 225. A solution of calcium lactobionate was shaken with the resin until the solution failed to give a precipitate with an ammoniacal solution of 4 percent ammonium oxalate.

Immunological titrations of $\beta$-galactosidase were performed by the method of Horinouchi et al. (1962).

The hydrolysis of the nonchromogenic galactosides, lactose and lactobionate, was measured with the aid of galactose dehydrogenase (EC 1.1.1.48; D-galactose : NAD oxidoreductase) purchased from Boehringer and Sons, Mannheim. The reaction mixture contained $\beta$-galactosidase, lactose ($2 \times 10^{-2}$ M) or Na lactobionate ($10^{-3}$ M), galactose dehydrogenase (10 $\mu$g/ml) and NAD ($2 \times 10^{-3}$ M) in phosphate buffer at pH 7.0. Rates of $\beta$-galactoside hydrolysis were calculated from the increase in optical density at 340 m$\mu$.

Measurements of catabolite repression in constitutive mutants were made as follows. Bacteria were inoculated into minimal liquid medium containing either 0.2 percent glucose or 0.2 percent glycerol as a carbon source and grown overnight with shaking at 37°C. The strains were then reinoculated into fresh medium of the same composition and grown under the same conditions. Samples were taken 60 and 150 minutes after inoculation, the bacterial density read at 600 m$\mu$, made permeable to ONPG by treatment with toluene, and assayed for $\beta$-galactosidase activity.

**Results**

Thirty-two mutants, which had acquired the ability to grow on sodium lactobionate, were isolated after treatment with MNN-guanidine. An enzyme extract of each mutant was prepared by the sonication of bacteria suspended in phosphate buffer. Extracts were assayed for protein content and $\beta$-galactosidase activity to give the specific activity of each mutant, as described in the methods section. The temperature sensitivities of the $\beta$-galactosidases were determined and the mutants were tested for inducibility or constitutivity of the lactose operon. The resultant data are shown in Table 1.

In all but three of the mutants the uninduced specific $\beta$-galactosidase activity was higher than that of strain 3300 which is wild-type with respect to the quantity and quality of $\beta$-galactosidase, and which does not utilize lactobionic acid. Twenty-eight of the mutant enzymes were the same as the normal enzyme in thermostability, the remaining four enzymes (from mutants 3, 5, 13 and 25) being distinctly more sensitive to heat inactivation. As will be shown below, the mutants...