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Characterisation of Mutants of *Escherichia coli* K12, Selected by Resistance to Streptozotocin

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Summary. From cultures of sensitive bacteria, treated with the antibiotic streptozotocin, two classes of resistant mutants can be isolated: 1) mutants, resistant under all the conditions tested to even the highest doses of the antibiotic. These are either pleiotropic-defective, *pts*-mutants, or more frequently, mutants lacking a transport system (enzyme II^{Nag}-complex of the PEP-dependent phosphotransferase system) encoded by the gene *nag*E. This gene is inducible by N-acetyl-glucosamine and seems to be part of the *nag* operon. The transport system in question is responsible for the uptake of N-acetyl-glucosamine, of D-glucosamine and of streptozotocin; 2) conditional resistant mutants which are unable to energize or to synthesize the streptozotocin transport system under certain growth conditions but do have the transport activity under other conditions. These include a) mutants auxotrophic for amino acids, vitamins, or nucleotides, b) mutants negative or sensitive to carbohydrates in the medium, and c) mutants with defects in energy metabolism such as PEP synthesis.

Introduction

Selection of bacterial mutants, resistant to a number of antibiotics used in modern chemotherapy has become an ever increasing problem. In most cases, the molecular basis of the mutant selection procedure is unknown, since the mechanism of the uptake of the drug into the cells, their biological target, and the actual killing event remain unclear, even for apparently wellknown drugs such as penicillin (Tomasz, 1979) or streptomycin. Resistant cells frequently carry mutations which seem to be unrelated to the mode of action of the drug, like auxotrophic mutants or mutants with regulatory defects isolated after streptomycin (Ishida et al., 1966), nalidixic acid (Helling and Adams 1970; Weiner et al., 1974), kanamycin (Thorbjarnardottir et al., 1978), or fosfomycin (Alper and Ames, 1978) treatment.

The antibiotic streptozotocin, [2d-2-(3-methyl-3-nitrosoureido)-D-glucopyranosid], too can be used to select different bacterial mutants (Lengeler, 1979). After its uptake and phosphorylation in the cell, the drug initially causes a rapid bacteriostasis, followed by irreversible, bactericidal DNA damage (Lengeler, 1980). A series of mutations causing permanent or conditional resistance to the antibiotic will be described and the mechanisms underlying the drug resistance will be discussed.

Material and Methods

Chemicals. Streptozotocin was obtained from Boehringer, Mannheim, and from Sigma GmbH, München. The drug was dissolved in water to 5 mg/ml, the pH adjusted to 5, and the solution kept frozen at −20° C, not longer then 2 months. Since this antibiotic is a strong mutagen and potential carcinogen, it must be handled with adequate security measures. MOPS (morpholinopropane sulfonic acid) was obtained from Sigma GmbH, München, and all labelled compounds from NEN Chemicals GmbH, Dreieich, or from Amersham Buchler GmbH, Braunschweig.

Bacteria. Origin, phenotype, or genotype of the strains used or isolated throughout this paper are given in Table 2. All derivatives of strain *L314*, unless indicated otherwise, have the following genotype: F^-*phoA thi*1 *arg*G6 *met*B *his*1 *gal*T *rps*L *glc*$^+$ *nag*$^+$ *man*$^+$. Strain *CA8445* was kindly provided by J. Beckwith, while strain *L173* has been described before (Lengeler, 1975). Its derivatives *L191* and *L327* lack any detectable enzyme I or HPr activity, due to the integration and subsequent excision of phage Mu cts62, or to the integration of the Tn5 transposon into the *pts* operon.

Culture Media and Growth Conditions. The minimal medium, the minimal and MacConkey indicator plates, and the growth conditions were as described before (Lengeler, 1975). The salt composition of minimal MOPS plates is analogous to the composition of LPM PIPES plates described before (Ruch et al., 1974), PIPES being replaced by MOPS (pH 6.5). In growth determinations, one

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absorbance unit measured at 420 nm corresponds to 4.75×10^8 bacteria/ml. Amino acids (L-form) were added to 20 μg/ml and carbohydrates to 10 mM, except for diauxic growth curves, were the two carbon sources were added to 1.65 mM each. To test streptozotocin sensitivity, the cells were pregrown overnight on N-acetyl-glucosamine (liquid medium or plates) and either streaked on MOPS plates (pH 6.5) containing D-glucosamine as sole carbon source and streptozotocin (50 μg/ml), or inoculated in minimal D-glycerol medium with the antibiotic. By contrast to resistant cells, sensitive cells do not grow out within 8-h incubation at 37° C. Carbohydrate sensitivity was tested by adding to cells, growing exponentially on D-glycerol the carbohydrate to be tested (10 mM) and following the absorbance of the culture.

Transport and Enzyme Tests. Standard uptake tests for D-glucose (5 μM), 2d-D-glucose (100 μM), D-mannose (0.66 μM) and N-acetyl-glucosamine (10 μM), the preparation of cell extracts by lysozyme or ultrasonic treatment, as well as the [enzyme I + HPr] and the enzyme II-complex tests have been described in detail (Lengeler, 1975). The coupled assays for the enzymes phosphofructokinase A and B (E.C. 2.7.1.11.), D-fructose-1, 6-bisphosphate aldolase (E.C. 4.1.2.13) (Lengeler, 1977), sn-glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8) (Ruch et al., 1974) and UTP: α-D-Galactose-1-phosphate uridyl transferase (E.C. 2.7.7.10) (Lengeler, 1966), were as described before. The enzyme N-acetyl-glucosamine deacetylase was measured in whole cells, pregrown on D-glycerol + D-glucosamine, centrifuged, washed and resuspended in minimal medium. To such cells, [^{14}C]-N-acetyl-glucosamine (10 μM) labelled either in the acetyl portion or in the glucosamine portion was added, and samples were taken after different times, filtered, and washed on the filter. In deacetylase-negative mutants, both values are nearly identical, while in wildtype cells the labelled acetyl-portion leaks into the medium, the D-glucosamine labelled portion does not. All transport and enzyme activities are expressed in nmoles · min^{-1} · mg of protein^{-1}.

Genetical Techniques. Mutagenesis, penicillin or streptozotocin selection, conjugations and P1-transductions were performed as described before (Lengeler, 1975, 1979). Mutants, resistant towards carbohydrates or streptozotocin, were selected on MacConkey indicator plates or after growth on a mixture of D-glycerol and the selective agent, using cells pregrown on N-acetyl-glucosamine in the case of streptozotocin.

Table 1. PTS-transport systems for hexoses and hexosamines in *E. coli* K12

Name	Gene symbols	Inducer(s)	Substrates
D-glucose	*glc*A (*pts*G)/ *crr*	Glc	Glc, GlcN, αMeGlc (2d-Glc, Man, Fru)
D-mannose	*man*A (*pts*M)	Man (GlcN)	Man, Nag, GlcN, Fru, Glc, 2d-Glc, αMeGlc
N-acetyl-D-glucosamine	*nag*E	Nag, (GlcN)	Nag, GlcN, Sbs, Stz
D-fructose	*fru*A (*pts*F)	Fru	Fru, (Man, Glc, GlcN, Sbs)
β-glucosides	*bgl*C	β-glucosides	β-glucosides

Carbohydrate symbols used are: Glc, D-glucose; GlcN, D-glucosamine; αMeGlc, α-methyl-D-glucopyranoside; 2d-Glc, 2deoxy-D-glucopyranoside; Man, D-mannose; Fru, D-fructose; Sbs, L-sorbose; Nag, N-acetyl-D-glucosamine; Stz, streptozotocin; PTS, phosphoenolpyruvate-dependent phosphotransferase system; ( ) carbohydrates in parenthesis have low inducing or substrate specificity. The genetic nomenclature is according to Bachmann et al., (1976) and Lengeler and Steinberger (1978). Instead of the recommended, but not unequivocal genetical nomenclature for enzyme II complexes, the elder and clearer notation of Lin (1970) is used throughout this paper, that is *glc*A for *pts*G, *man*A for *pts*M, *fru*A for *pts*F, and the new symbol *nag*E. The symbol *man*I designates the structural gene for the enzyme phosphomannose-isomerase, and is identical to the classical *man* locus

Results

Antibiotics usually enter bacterial cells by means of preexisting carbohydrate-, amino acid-, or ion-specific transport systems, whose natural substrates they mimic (Braun, 1977). Consequently, mutants deficient in one of these transport systems are resistant to an antibiotic entering through this system.

A possible role of one or several transport systems specific for 2d-glucose and amino-glucosides in the uptake of streptozotocin was suggested by the following observations: 1) cells pregrown on N-acetyl-glucosamine (Nag) are sensitized towards streptozotocin; 2) Nag protects sensitive cells against the bactericidal effect of the drug; 3) streptozotocin like D-glucosamine and Nag is a derivative of 2d-glucose. Among the systems, a high-affinity enzyme II^{Man}-complex (Km for D-mannose, ~10 μM) coded for by the gene *man*A (=*pts*M), and an enzyme II^{Nag}-complex, the structural gene of which is still unknown, appeared as promising candidates (Table 1).

1. Effect of Streptozotocin on manA and on glcA Mutants

Owing to a mutation in the gene *man*I, strain *LR2-161* lacks the enzyme phosphomannoseisomerase (E.C. 5.3.1.8) but possesses at least two intact D-mannose transport systems (unpublished results). It is therefore sensitive to this aldose (≦10 μM) in the medium (Table 2). Derivatives resistant to such concentrations have normally lost the *man*A coded transport activity. They are unable to take up D-mannose (0.66 μM) or 2d-glucose (100 μM) at low concentrations, and the mutant locus cotransduces with the markers *eda* and *fad*D, two markers which flank the *man*A locus (Bachmann et al., 1976). As shown for strain *LR2-162*, a representative of fifty such *man*A derivatives of strain *LR2-161*, they transport Nag with a near normal rate (Fig. 1) and grow

Table 2. Genotype, phenotype, and transport activity of different strains

Strain	Origin	Genotype			Phenotype				Transport	
		manA	nagE	others	Man	Nag	Stz	Di.	Nag	Man
L314		+	+	+	+	+	s	−	16.9	1.52
LR2-161	*L314*	+	+	*man*I	s	+	s	−	22.2	1.90
LR2-162	*LR2-161*	−	+	*man*I	−	+	s	−	13.2	0.02
LR2-163	*LR2-161*	+	−	*man*I	s	+	r	+	7.5	0.33
LR-12	*LR2-161*	+	−	*man*I	s	+	r	+	11.4	1.50
LR-29	*LR2-161*	+	−	*man*I	s	+	r	+	11.0	1.90
LR-29Man^r	*LR-29*	−	−	*man*I	r	−	r	n.t.	0.1	0.01
LR2-166	*LR2-161*	+	+	*man*I$^+$	+	+	s	−	8.3	0.57
LR2-167	*LR2-162*	−	−	*man*I	−	−	r	n.t.	0.1	0.01
TdtI	*LR2-167*	−	+	*man*I	s	+	s	−	14.0	0.40
TdtII	*LR2-167*	+	−	*man*I$^+$	+	+	r	+	7.4	1.38
LR-150	*LR2-161*	+	+	*man*I *nag*A	s	s	s	n.t.	10.3	1.48
LR-153	*LR-150*	+	−	*man*I *nag*A	−	−	r	n.t.	2.2	0.32
LR-154	*LR-153*	+	−	*nag*A	+	s	r	n.t.	2.6	1.46
LR-155	*LR-150*	+	−	*man*I *nag*A	s	s	r	n.t.	0.6	n.t.
LR-156	*LR-153*	−	+	*man*I	−	+	s	n.t.	7.2	n.t.
IR-101	*L314*	−	+	*glc*A	+	+	s	−	20.9	n.t.
LR-157	*LR-150*	+	+	*pts*	−	−	r	n.t.	0.0	0.11
L327	*L173*	+	+	*pts*	−	−	r	n.t.	0.0	0.00
L191	*L173*	+	+	*pts*	−	−	r	n.t.	0.0	0.06
L191Nag$^+$	*L191*	+	+	*pts*	−	+	r	−	0.2	0.00
L176	*L173*	+	+	*cya*	+	+	s	n.t.	0.4	1.10
CA8445	*HfrH*	+	+	*cya*^{del} *crp*^{del}	+	+	s	n.t.	1.21	0.34

Carbohydrate and streptozotocin (Stz) sensitivity, diauxic growth (Di.) as well as N-acetyl-glucosamine (Nag, 10 μM) and D-mannose (Man, 0.66 μM) transport activities (nmoles·min^{-1}·mg prot^{-1}) were determined as described in Materials and Methods. The symbols signify: + and − positive or negative phenotype and genotype and presence or absence of a diauxic lag; s, sensitive; r, resistant; n.t., not tested; Tdt, transductant

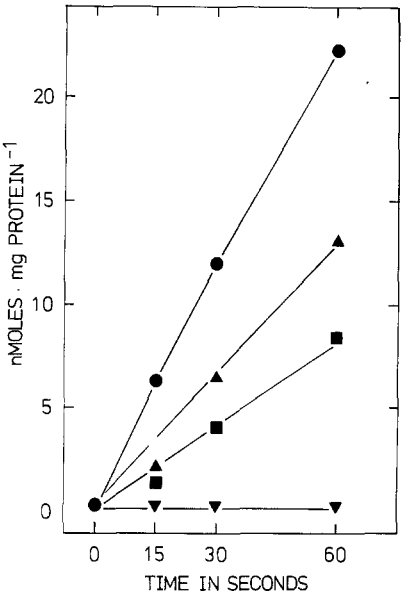

Fig. 1. Uptake of N-acetyl-glucosamine. The uptake (nmoles·min^{-1}·mg prot^{-1}) of NAG (10 μM) was tested in strain *LR2-161* *manA*$^+$ *nagE*$^+$ (●—●), *LR2-162* *manA* *nagE*$^+$ (▲—▲), *LR2-163* *manA*$^+$ *nagE* (■—■) and *LR2-167* *manA* *nagE* (▼—▼)

at normal rates on Nag and D-glucosamine. They all remain sensitive to streptozotocin (Table 2). The drug is, therefore, not taken up by the *man*A coded transport system. Nor does it enter the cells by the main D-glucose transport system, since *glc*A (=*pts*G) mutants like *IR-101* (Table 2) are also sensitive.

2. *All nagE Mutants are Streptozotocin-Resistant*

Streptozotocin-resistant derivatives of strain *LR2-161*, e.g., *LR-12*, *LR-29*, and *LR2-163* (Table 2), are sensitive to D-mannose (≦10 μM) and take up this hexose at a normal rate. In addition, they are able to grow on D-glucosamine and on Nag. Growth on the latter, however, is altered. On MacConkey-Nag plates, the mutant cells form pale colonies with a red center, typical for mutants with a reduced uptake (Lengeler, 1975). Furthermore, during growth on a mixture of D-glucose and Nag (1.6 mM each) they show a pronounced diauxie, not found in wild-type strains or in Nag-positive mutants, lacking the *man*A coded transport system (Fig. 2). This and the

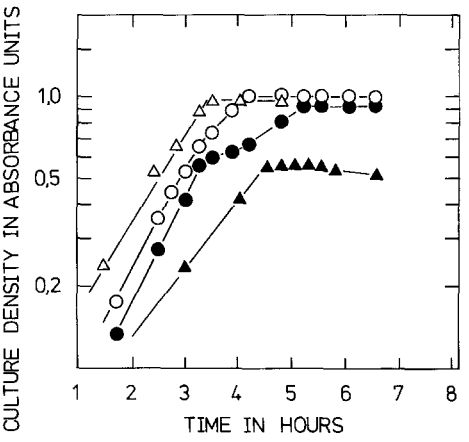

Fig. 2. D-glucose/N-acetyl-glucosamine diauxie. The diauxic growth of strains *LR2-161 man*A$^+$ *nag*E$^+$ (○—○), *LR2-162 man*A *nag*E$^+$ (△—△), *LR2-163* (or several other *man*A$^+$ *nag*E mutants, e.g. *LR-12, LR-29*) (●—●) and *LR2-167 man*A *nag*E (▲—▲) on an equimolar mixture of D-glucose and N-acetyl-glucosamine (1.65 mM each) was measured

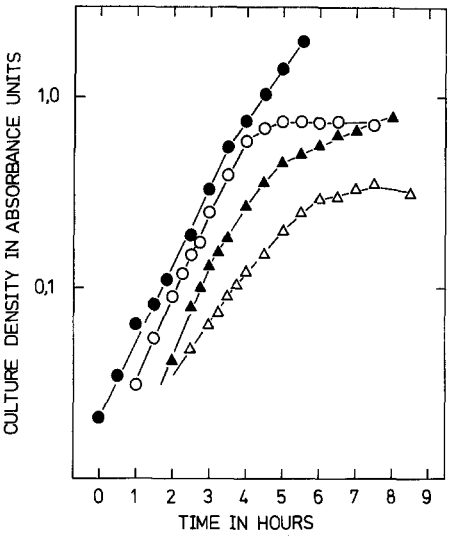

Fig. 3. Streptozotocin sensitivity of *pts* and *cya/crp* mutants. To cells, pregrown in the presence of N-acetyl-glucosamine, and growing exponentially on casamino acids (1%), streptozotocin was added at on optical density of 0.1 and further growth recorded. The strains tested were *LR2-161 man*A$^+$ *nag*E$^+$ (○—○), *LR-29 man*A$^+$ *nag*E (●—●), the mutants *L327, L191* and *L191*Nag$^+$ (●—●), *L167 cya* (△—△) and *CA8445 cya*^{del} *crp*^{del} (▲—▲)

reduced uptake of Nag in such mutants (Fig. 1) indicate the presence of a second Nag transport system in *E. coli* K12, suggested before (White, 1968). D-mannose-resistant derivatives of strains *LR-12, LR-29,* and *LR2-163* always loose in one step the ability to take up D-mannose and Nag together with the ability to utilize Nag as carbon source (*LR-29Man*^{r}, Table 2).

From these data, completed by data from the literature (Postma and Roseman, 1976), it can be concluded that streptozotocin enters *E. coli* by the transport system, coded for by *nag*E, that Nag enters the cells by the systems coded for by *nag*E and by *man*A, while D-glucosamine enters through at least three systems, coded for by *nag*E, *man*A, and *glc*A (Table 1). As predicted by this model, among 500 streptozotocin-resistant mutants of strain LR2-162 (e.g., *LR2-167*, Table 2) the great majority has lost the ability to grow on NAG (10 mM) or to take up this substance (10 μM), but is still able to grow on D-glucosamine. When after a P1-transduction, 96 Nag-positive transductants were isolated from strain *LR2-167* and analyzed, 26 had the Nag$^+$ Man^s Stz^r phenotype of strain *LR2-163*, while 70 had the Nag$^+$ Man^r Stz^s phenotype of strain *LR2-162* (Table 2). Similar results are observed among Nag$^+$-revertants of strain *LR2-167* (data not shown). In a subsequent communication, the exact mapping of *nag*E mutations, leading to streptozotocin resistance and to loss of the major Nag transport activity in *E. coli* K12, as well as data on the properties of this transport system will be reported.

3. *Streptozotocin-Resistant nagE$^+$- Mutants*

Streptozotocin-resistant mutants may be selected on any carbon source (Lengeler, 1979); however, the type of mutant thus obtained depends on the carbon source used. Selection in a wildtype strain can be done, first of all, during growth on a PTS-sugar (a sugar, taken up by the PEP-dependent process of group translocation). If the treated cells are plated immediately on indicator plates, containing the selective carbon source, the majority of the surviving cells phenotypically resemble the *nag*E mutant *LR2-163* (except for D-mannose sensitivity), or they have a pleiotropic defect in carbohydrate metabolism and uptake. The latter can be shown by a series of in vivo and in vitro tests (Lengeler, 1975), to lack functional [enzyme I + HPr]-activity of the PEP-dependent phosphotransferase system (PTS) and to carry mutations in the *pts* operon. Such *pts* mutants are completely resistant to streptozotocin (Table 2; Fig. 3). They do not even show filament formation, characteristic of cells, which take up the drug at a reduced rate (Lengeler, 1980). These properties distinguish them from mutants with defects in the *cya* or in the *crp* genes, mutations frequently associated with an increased general antibiotic resistance (Alper

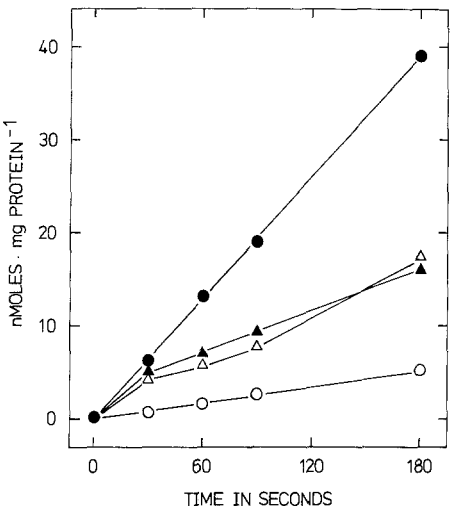

Fig. 4. Test of the enzyme N-acetyl-glucosamine-6-P deacetylase. To cells of strain *LR2-161 nag*A$^+$ (circles) or *LR-150 nag*A (triangles) pregrown on D-glycerol + D-glucosamine and treated as described in Material and Methods, [^{14}C-acetyl]-glucosamine (10 μM) (open symbols) or N-acetyl-D-[^{14}C-glucosamine] (10 μM) (filled symbols) were added, and the amount of radioactively labelled material retained in the cells determined

and Ames, 1978). Thus, the *cya* mutant *L176* and even strain *CA8445*, carrying deletions of *cya* and *crp*, are sensitive to streptozotocin (Fig. 3). Sensitivity, though, is reduced as shown by the extensive filament formation, especially in strain *CA8445*. Mutants with a defect in the *pts* operon are unable to grow on D-mannose, D-glucosamine or Nag and cannot accumulate these carbohydrates (Table 2) or streptozotocin (Lengeler, in prep.). In *cya/crp* mutants, however, growth, transport, and streptozotocin resistance are merely reduced. Finally, a *pts* mutant capable of growth on Nag by means of an ATP kinase (strain *L191Nag*$^+$) remains resistant to the drug (Table 2).

After streptozotocin selection on PTS and on non-PTS carbohydrates, *nag*E or *pts* mutants can easily be counterselected by one to two growth cycles on Nag thus allowing the detection of additional type of resistant mutants, e.g., mutants negative for or sensitive to the carbon source used during the selection. Negative mutants have been found after selection on lactose, melibiose, raffinose, D-xylose, sucrose, and the following carbohydrates for which also sensitive mutants have been isolated and enzymatically characterized (identified mutations are given in paranthesis): D-glucose, D-mannitol, and D-glucitol (*pfk*A); D-glycerol (glpD); D-fructose (*fda*); D-mannose (*man*I); D-galactose (*gal*T). Strains used during selection were wildtype strains or mutants, containing metabolic-plasmids (*raf, scr*) of *E. coli* K12, B and C, *S. typhimurium, Kl. pneumoniae, St. aureus,* and *B. subtilis* (data not shown).

From the *man*I-mutant *LR2-161,* treated with streptozotocin during growth on a mixture of D-glycerol and Nag (1 mM), a mutant *LR-150,* sensitive to Nag was isolated. As shown in Table 2, strain *LR-150* has a high uptake activity for both D-mannose and Nag. It is, however, unable to deacetylate Nag (Figure 4) and resembles in all respects a deacetylase mutant described before (White, 1968). The missing enzyme Nag-6-P deacetylase is essential in the metabolism of Nag, but not in the catabolism of D-glucosamine. It is encoded in the gene *nag*A (Holmes and Russel, 1972). Strain *LR-150* shows normal growth on D-glucosamine and is sensitive to streptozotocin when grown on this amino glucoside. One hundred Nag-resistant derivatives (e.g., *LR-153,* Table 3) become also streptozotocin resistant and again show the reduced uptake of Nag characteristic of *man*A$^+$ *nag*E mutants. The phenotype of these mutants and of several *man*$^+$ and *nag*$^+$ transductants, also listed in Table 2, support the conclusion, that streptozotocin sensitivity in *E. coli* is strictly related to the activity of the *nag*E-coded transport system, even in carbohydrate-negative or -sensitive mutants (see Discussion).

A third class of mutants, found among the survivors of a streptozotocin selection from a culture, growing in minimal medium are mutants with a defect in energy metabolism, e.g., PEP synthesis, or mutants auxotrophic for amino acids, nucleotides, or vitamins (data not shown). In both types the drug is accumulated at a decreased rate, either because the *nag*E transport system is not induced (under the conditions of auxotrophy) or because the system is not energized (no PEP available) (Lengeler, 1980). Therefore, they are inactivated at lower rates then wildtype cells and consequently, are enriched among the survivors of streptozotocin treatment.

Discussion

As shown here and in previous communications (Lengeler, 1979; 1980), streptozotocin does not kill sensitive cells from outside. To inactivate or to mutagenize cells, it must be taken up by a specific transport system. This system, encoded by the gene *nag*E, is inducible by Nag. Consequently, all mutations and all physiological conditions, which reduce the activity of this system render the cells resistant to this antibiotic. The resistance is permanent, if the mutations affect the gene *nag*E, the structural gene for the transport system. Since this system is a membrane-bound enzymeII-complex of the PEP-dependent phospho-

transferase system (White, 1968; Lengeler, in prep.), mutations in the *pts* operon also confer a permanent resistance on the cells. On the other hand, most mutations leading to auxotrophy or to a general block in transport energization cause a conditional resistance. Thus, if the transport system for the drug in auxotrophic mutants is preinduced in minimal medium, lacking the essential nutrient, the number of survivors in a subsequent treatment with the antibiotic increases by a factor greater than 10^4 compared to the same strain preinduced in the presence of the essential nutrient (Lengeler, 1980). Similarly, if carbohydrate-sensitive cells are treated with streptozotocin in the presence of such a carbohydrate, or when cells are treated in the presence of carbon sources they are unable to metabolize, the number of survivors again increases 10^2-to 10^5-times, compared to treatment during growth on carbohydrates, which they can metabolize. A strong catabolite and transient repression, associated with catabolite inhibition and inducer exclusion, which can be regularly observed under such conditions of limited growth, prevent induction and functioning of enzyme II-complexes (Lengeler and Steinberger, 1978; Lengeler, 1980), and consequently uptake of streptozotocin too.

Among mutants resistant to antibiotics such as streptomycin, gentamycin, kanamycin, fosfomycin, or nalidixic acid, similar mechanisms are apparently involved in the selection of auxotrophs or mutants with regulatory defects (for references see introduction and Alper and Ames, 1978). Thus, starvation of essential nutrients in auxotrophic cells increases the tolerance to many antibiotics. Furthermore, mutants carrying mutations in the genes *cya* or *crp* and in the *pts* operon and therefore unable to synthesize inducible transport systems and operons or to grow at a normal rate, also are more resistant to many antibiotics. Nalidixic acid, finally, affects DNA and, in addition, shows a preferential inhibition of inducible operons as compared to non-inducible systems (Smith, et al., 1978; Sanzey, 1979). They most likely all directly or indirectly prevent the drugs reaching the actual 'killing target' on or in the cells. By a sophisticated choice of selection conditions and media, it will be possible to select most wanted mutants by employing one of these drugs. Among them, streptozotocin has the advantage of killing the cells rapidly and irreversibly without causing lysis (Lengeler, 1979).

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