Molec. gen. Genet. 128, 349—357 (1974)
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Pleiotropic Mutations in *Escherichia coli* Conferring Tolerance to Glycine and Sensitivity to Penicillin

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Received October 17, 1973

Summary. Four different mutants of *Escherichia coli* were isolated after selection for an increased tolerance to glycine (3 to 4%). The mutants showed additional effects, notably an increased sensitivity to penicillin G. The interpretation is that permeability to several agents is changed as a result of some distorted function in the cell envelope. Reversion analysis showed that the complex change in phenotype is the result of a single point mutation. The four mutants represent four previously undescribed genes termed *qme*, of which *qmeC* could be genetically located between *aroB* and *str*, *qmeD* near to *lys* and *thy* in the order *qmeD-lys-thy*, *qmeE* near *aroD*, and *qmeF* in the 17/22 min region.

Introduction

Glycine in concentrations between 1 and 5% can induce spheroplast formation in *Escherichia coli* (Mc Quillen, 1960). Enzymes with a role in cell wall synthesis and sensitive to glycine are L-alanine-adding enzyme, D-alanyl: D-alanine ligase, and alanine racemase (Lugtenberg, 1972). The genetic loci for these three enzymes have been established (Wijsman, 1972b, c; Miyakawa *et al.*, 1972). Furthermore, a gene for an enzyme mediating transport for glycine as well as for D-alanine and the antibiotic D-cycloserine has been described (Wargel, Shadur, and Neuhaus, 1970, 1971; Curtiss *et al.*, 1965; Russell, 1972; Wijsman, 1974). Mutants were selected tolerant to 3 to 4% glycine, to find changes in one of the enzymes mentioned. Preliminary mapping of the mutations involved revealed several new loci, which was explained as pointing to aspecific changes in permeability to glycine in the mutants. Accordingly, other agents were tested, and the mutations were shown to have pleiotropic effects. One of these was a decrease in tolerance to penicillin.

The combination of several physiological alterations (especially changes in permeability) as a result of a single point mutation is a recurring theme in the description of cell surface mutants (Nagel de Zwaig and Luria, 1967; Hill and Holland, 1967; Hirota, Mordoh, and Jacob, 1970; Ennis 1971; Whitney, 1971; Bernstein, Rolfe, and Onodera, 1972; Egan and Russell, 1973). The usual explanation is that a change in one of the strongly interdependent constituents of the cell surface may affect many other functions for reason of their structural relations. Certain enzymes bound to the cell envelope may also have a role in maintaining the cell's structure. For a complete description of the intricate bacterial envelope structure, mutants may reveal the number of proteins involved in maintaining the envelope's integrity, and some of them even regulatory mechanisms concerned with their synthesis. Therefore a full description is given of the glycine-tolerant mutants obtained.

Material and Methods

Strains. The bacterial strains of *Escherichia coli* K-12 used in this study are listed in Table 1.

Media. The synthetic minimal medium as well as the complete media Peptone agar (no NaCl added) or Nutrient broth have been described (Wijsman, 1972b). Glycine was added at concentrations depending upon the strains used in the experiment (cf. Table 2). Rifampicin was a gift of Lepetit, Holland. Gentianviolet was obtained commercially from Edward Gurr, London, and penicillin G from Mycofarm, Delft.

Table 1. List of strains

Strain	Relevant characters	Source
AB1157	F^- *thr leu proA his thi argE lac gal ara xyl mtl str tsx*	A. Rörsch
GIA58	AB1157 *qmeC*	this paper
GIA37	F^- *leu his argF argI thy pyrF lac tonA tsx str azi*	,,
GIA63	GIA37 *qmeD*	,,
GIA64	GIA37 *qmeF*	,,
GIA65	GIA37 *qmeE*	,,
PC0615	Hfr KL19	Phabagen collection
PC0512	Hfr B11 *metB cml*	,,
KA6	F'8 *gal*$^+$/*gal met*	A. Rörsch
JP5048	Hfr C *purA met ilv ampA1 cycA*	R. R. B. Russell
PC0515	Hfr KL16	Phabagen collection
AB2826	F^- *aroB351*	A. J. Pittard
RE113	F^- *aroE nal tsx*	S. Normark
GIA44	F^- *thr leu pyrF lys thi his arg ilvA lac tonA tsx*	this paper
GIA12	Hfr H *aroD6 his* (AB1360 × KMBL171)	,,
AB1360	F^- *proA his arg aroD6 thi tsx lac gal xyl mtl*	A. L. Taylor
KMBL171	Hfr H *met azi*	A. Rörsch

Genetic symbols are defined by Taylor and Trotter (1972).

Table 2. Response to glycine of *qme* mutants

Strain	Minimal inhibitory concentrations of glycine (%)				
	Nutrient broth 37°C	peptone agar 28°C	peptone agar 37°C	peptone agar 42°C	Minimal agar 37°C
AB1157 (wild)	2.0	1.5	1.5	1.5	1.0
GIA58 *qmeC*	5.0	3.5	4.0	2.5	3.5
GIA37 (wild)	2.5	1.5	1.6	1.5	1.5
GIA63 *qmeD*	>2.5	2.5	3.0	3.0	2.5
GIA64 *qmeF*	>2.5	3.0	3.5	3.0	2.5
GIA65 *qmeE*	>2.5	3.5	3.5	3.0	2.5

In broth increase of turbidity was observed; on solid medium colony formation. That growth in broth of the mutants at 2.5% glycine is normal can be seen in Fig. 1, but only in the case of GIA58 it has been attempted to find a mutant's upper limit.

Selection of Mutants. Because growth in Nutrient broth is nearly absent at concentrations glycine of about 2.0%, after mutagenesis the bacteria were grown for several generations at 1.0% glycine; then in serial transfers the concentration was increased in steps of 0.5%, with several generations growth at each step, until 3% was reached; mass increase had by then become very slow. Next, the culture was plated on complete agar medium containing 2.5% glycine. From each culture treated, one mutant colony was isolated, after which each strain's maximum concentration tolerated was determined. In the case of GIA58, the mutagen used was *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; for GIA63, 64, and 65 nitrous acid been applied.

Genetic Techniques. Recombinants from conjugation were selected by plating a mixture containing Hfr cells and F^- cells in a ratio of 1 to 10, without interruption. For transduction phage P1 was used. For propagating P1 a single plaque was isolated and suspended in 1 ml of an exponentially growing culture, concentrated by a factor 10 in LC Broth containing 2.5×10^{-3} M $CaCl_2$. After incubation for 35 min at 37° C without aeration soft LC agar was added to the suspension, and it was poured on to an LC agar plate. After 15 h incubation at 37° C, the top was harvested in 2 ml bidestilled water. Next, dilutions of the supernatant were made and titrated. Plates with confluent lysis were harvested after 6 to 8 h incubation. The supernatant was used for transduction; titers on *qme* mutants are normal (1 to 5×10^9). In transduction a ten times concentrated exponentially growing culture was mixed with phage; after 30 min incubation at 37° C, the cells were centrifuged, resuspended in saline, and plated on selective media.

Recombinant colonies were picked and restreaked before being tested for the presence of unselected markers. Originally, the Qme phenotype was scored on plates containing glycine in a range of concentrations. Probably owing to the long time needed for full expression of glycine resistance, in every experiment some colonies could not be allocated to one of the two parental genotypes. This difficulty was solved, however, by also making use of penicillin sensitivity for scoring the presence of *qme*, since on plates containing penicillin no intermediary situations arose.

Results

Physiological Properties of the Mutants

In the wild type strains tested, glycine at a concentration of 1.75% inhibits the formation of colonies on solid complete media. After the isolation from AB1157 of the first mutant strain resistant to glycine (GIA58, carrying *qmeC*), further mutants were isolated in GIA37. Three of the latter type are used in this study; GIA63, 64, and 65, carrying *qmeD*, *qmeF* and *qmeE*, respectively. The mutants are tolerant to at least 2 to 3% glycine both when colony formation on agar is scored (Table 2), or when growth of a culture in broth is followed (Fig. 1), and at different temperatures.

Table 3. Minimal inhibitory concentration for *qme* mutants of several agents

Strain	Penicillin G (I.U./ml)	Rifampicin (μg/ml)	Deoxycholate (%)	Gentian Violet (μγ/ml)
AB1157 (wild)	40	5.0	0.6	>500
GIA58 *qmeC*	15	2.0	0.6	150
GIA37 (wild)	30	2.0	0.6	>500
GIA63 *qmeD*	20	1.0	0.6	40
GIA64 *qmeF*	15	1.0	0.1	>500
GIA65 *qmeE*	15	1.0	0.1	500

Colony formation at 37°C was observed. For gentian violet, 500 μg/ml was the maximum concentration which could be reached.

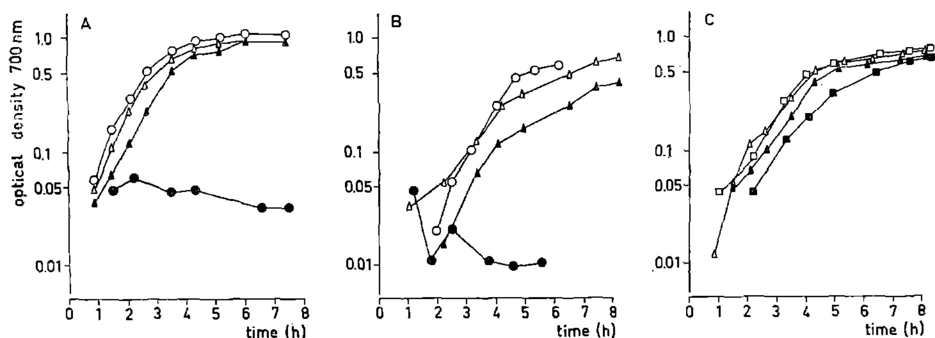

Fig. 1A—C. Growth of wild and mutant strains in Nutrient broth (open symbols) or in Nutrient broth with glycine added at 2.5% (closed symbols) at 37° C. A. Strains AB1157 (○, wild) and GIA58 (△, *qmeC*). B. Strains GIA37 (○, wild) and GIA63 (△, *qmeD*). C. Strains GIA64 (▽, *qmeF*) and GIA65 (□, *qmeE*)

Several mutants in which the membrane system is supposed to be affected show a response different from that of the wild type to several dyes, detergents, and antibiotics (Nagel de Zwaig and Luria, 1967; Hill and Holland, 1967; Hirota, Mordoh, and Jacob, 1970; Ricard, Hirota, and Jacob, 1970; de Graaf and Stouthamer, 1970; Tamaki, Sato and Matsuhashi, 1971; Whitney, 1971; Bernstein, Rolfe and Onodera, 1972). After the increased tolerance of the mutants presently studied had been ascribed to aspecific changes in the permeability barrier, the cell's response was tested to the effects of the following agents; the dyes methylene blue, eosin, acridine orange, gentian violet; the detergents sodium deoxycholate and sodium dodecyl sulphate, and the chelating agent ethylenediamine tetraacetate; the antibiotics chloramphenicol, rifampicin and penicillin G. Effects were found in the case of gentian violet (GIA58, 63), deoxycholate (GIA64, 65), rifampicin and penicillin (tolerance about halved in all the mutants). Addition of $MgSO_4$ (0.08 M) to complete medium did not change the sensitivity spectrum of the strains.

Since an effect of the pH of the medium on growth has been observed in some membrane mutants (Steenbakkers, personal communication) the growth pattern was tested under conditions of low pH. Between AB1157 and GIA58 no difference was found. However, GIA37 is able to form colonies on complete agar medium at pH 5.0 and even at pH 4.7, while pH 5.3 is the lowest pH at which strains GIA63, 64 and 65 could grow on agar. Examples of growth in broth are given in Fig. 2; the difference with the wild type is small, but consistent with the results on solid media.

No changes in comparison with the wild type in the fermentation pattern of different carbon sources could be observed. In cultures of the mutants normally more "chains" of two or more cells can be observed than in the parents, but it has not been attempted to establish that the difference was statistically significant.

It is important to prove that the pleiotropic effects found are the result of not more than one mutation. To test this, revertants were selected on penicillin containing medium. In the case of GIA58 and GIA63 the original wild phenotype

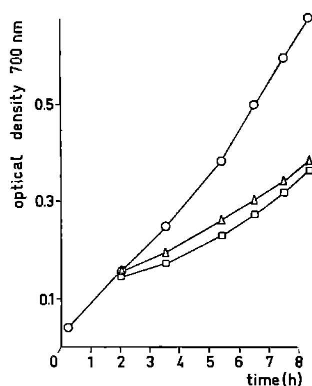

Fig. 2

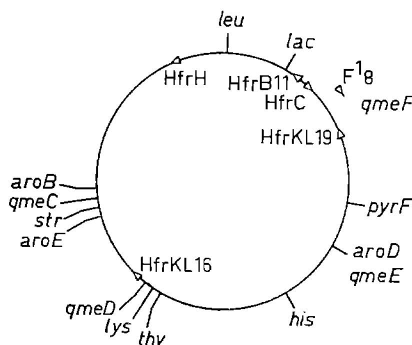

Fig. 3

Fig. 2. Effect of pH on growth. Strains GIA37, GIA63 and GIA65 were grown at pH 5.0 in Nutrient broth at 37° C

Fig. 3. Genetic map of *Escherichia coli* showing relevant loci according to Taylor and Trotter (1972), and origin of transfer of Hfr strains

was restored in all revertants, which provides conclusive evidence that the physiological changes involved are due to a single point mutation. However, in GIA64 and GIA65 some of the revertants on penicillin plates had not lost glycine resistance. They can be considered pseudo-revertants in the sense of Whitney (1971), who ascribed their occurence to interactions between different proteins controlling parts of the cell envelope.

Genetic Analysis

The genetic location of the mutations (Fig. 3) was assessed by conjugal crosses, indicating linkage of *qmeC* to *str*, of *qmeD* to *thy*, location of *qmeE* between *his* and *pyrE*, and location of *qmeF* between the origins of Hfr B11 (13 min) and Hfr KL19 (22 min).

The possibility of P1-mediated cotransduction with suitable markers was investigated (Table 4). The gene *qmeC* can be cotransduced with *aroB* and *str*. In view of the absence of cotransduction of *aroB* and *str* this points to a gene order *aroB-qmeC-str* (Fig. 4), which places *qmeC* at about 65 min. Out of 40 *str* transductants, 6 carried donor alleles for *qmeC* as well as *aroE*, establishing that these two markers can be cotransduced. However, among 49 $aroE^+$ transductants, the *qmeC* donor allele was absent. Probably differences in viability of different recombinant types are involved, since the frequency of cotransduction of *aroE* and *str* is much lower when *aroE* is the selected marker (12/49 = 24%) than when *str* is selected (29/40 = 72%).

The gene *qmeD*, which can be transferred as an early marker by Hfr KL16, can be cotransduced with *thy*. A three-point cross involving the *lys* marker was carried out. Since most of the thy^+qmeD^+ recombinants carry the donor allele lys^- (27/29), the gene order must be *qmeD-lys-thy* (Fig. 5), and the location of *qmeD* at about 56 min.

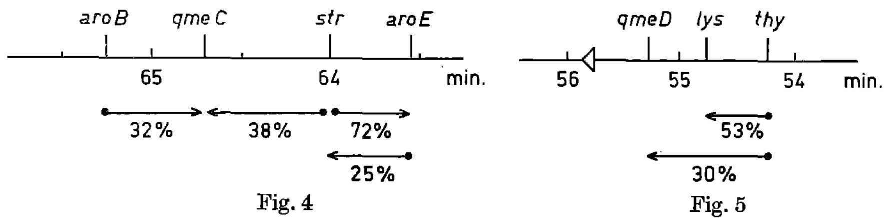

Fig. 4. Location of *qmeC* on the standard genetic map in the 63/65 min region (Taylor and Trotter, 1972). The arrows indicate cotransduction, figures are cotransduction frequencies as given in Table 4. At each arrow the knob is placed under the selected marker and the head under the unselected marker

Fig. 5. Location of *qmeD* on the standard genetic map in the 54-56 min region (Taylor and Trotter, 1972). The arrows indicate cotransduction, figures are cotransduction frequencies as given in Table 4. At each arrow the knob is placed under the selected marker and the head under the unselected marker

Table 4. Cotransduction analysis of *qme* markers

Locus	Donor	Recipient	Selected marker	No. of transductants tested	Unselected markers		
					Gene(s)	No.	%
qmeC	GIA58 ($aroB^{+}$ $qmeC^{-}$ str^{-} $aroE^{+}$)	AB2826 ($aroB^{-}$ $qmeC^{+}$ str^{+})	$aroB^{+}$	127	$qmeC^{-}$	41	32
					str^{-}	0	<1
		RE113 ($qmeC^{+}$ str^{+} $aroE^{-}$)	str^{-}	66	$qmeC^{-}$	25	38
				40	$aroE^{+}$	29	72
			$aroE^{+}$	49	$qmeC^{-}$	0	<2
					str^{-}	12	25
qmeD	KMBL171 (thy^{+} $qmeD^{+}$)	GIA63 (thy^{-} lys^{+} $qmeD^{-}$)	thy^{+}	55	$qmeD^{+}$	20	36
	GIA58 (thy^{+} $qmeD^{+}$)		thy^{+}	60	$qmeD^{+}$	16	27
	GIA44 (thy^{+} lys^{-} $qmeD^{+}$)		thy^{+}	104	lys^{-} $qmeD^{+}$	27	
					lys^{-} $qmeD^{-}$	23	
					lys^{+} $qmeD^{-}$	52	
					lys^{+} $qmeD^{+}$	2	
				104	lys^{-}	54	53
					$qmeD^{+}$	29	28
qmeE	GIA65 ($aroD^{+}$ $qmeE^{-}$)	GIA12 ($aroD^{-}$ $qmeE^{+}$)	$aroD^{+}$	48	$qmeE^{-}$	28	58

The gene *qmeE* (in GIA65) can be cotransduced with *aroD* (58%), but the orientation of *aroD* and *qmeE* on the standard genetic map has not been determined.

Strain GIA64 (carrying *qmeF*) was crossed to several donor strains. From the clockwise transferring Hfr strains B11 and KL19 only the former could transfer

qmeF as an early marker, as judged from the frequency of *qme*$^+$ among *pyrF*$^+$ recombinants (50% and 4%, respectively). In crosses with the anticlockwise transferring strains Hfr C and F′8, *leu*$^+$ recombinants were selected; among these the frequency of the unselected *lac*$^+$ marker was considerable (58% and 55%), but this was not the case with *qme*$^+$ (10% and 11%). It was concluded that *qmeF* is located between the origins of transfer of F′8 and KL19, that is between minutes 17 and 22 on the standard *E. coli* map.

In some of the crosses it has been attempted to select directly for glycine resistant recombinants. As is often a property of resistance markers, expression takes some generations, but compared with streptomycin resistance the time involved is unusually long, in the same way as the isolation procedure was necessarily a very slow one. These observations are in good accordance with a role of the gene products as structural components of the cell, which are only gradually replaced.

Discussion

The isolation procedure described offers a way for selecting pleiotropic mutants with properties indicative of changes in the cell's permeability barriers. The combination of increased tolerance to glycine with decreased tolerance to penicillin points to a change in some particular structural component rather than to partial inactivation of the cell with passive leakage as a result. In this respect the strains resemble other cell surface mutants (e.g., Nagel de Zwaig and Luria, 1967; Normark, 1970; Ennis, 1971; Tamaki, Sato and Matsuhashi, 1971; Lopes, Gottfried and Rothfield, 1972; Wijsman, 1972a; Egan and Russell, 1973).

The nature of this pleiotropy may be attributed to interaction of the different surface components, which may influence their protein tertiary structure and in that way their function.

Defining biochemically the proper enzymatic function of the protein affected will be very difficult. The permeability barrier to penicillin has been shown to reside in the "outer membrane" layers (Leive, 1968; Normark, 1970), but whether the primary change has occurred in the lipopolysaccharide layers or in the cytoplasmic membrane, with a secondary effect in the outer membrane, is not clear. This is the reason why the symbol *qme* (unspecified membrane mutant) has been chosen. It has been proposed that the temperature-sensitive mutation provisonally called *gts-49* (Wijsman, 1972a) should be renamed *qmeA*, and another temperature-sensitive mutation *qmeB* (Wijsman, 1974). In the case of *qmeA* and *qmeB* it is difficult to demonstrate that they should be more resistant to glycine under restrictive conditions because they don't grow (*qmeA*) or even lyse (*qmeB*). However, in the case of *qmeB* it could be demonstrated (Wijsman, 1974) that it is more sensitive to penicillin even under permissive conditions (28° C). It may be anticipated that *qme* genes code for essential proteins, in which nonsense mutations would be lethal, and that mutations found are necessarily of the missense type; this means that even by separation of the different proteins of outer and inner membrane it will be difficult to demonstrate a change in the physico-chemical properties of the mutant protein.

The loci presently described do not seem to be occupied by a gene on the recent *E. coli* map (Taylor and Trotter, 1972) that is already known to code for

an evelope component. The fact that the first four mutants isolated represent four different genes mutated indicates the likelyhood that further loci forglycine tolerance may be revealed in new mutants. Even if the procedure of their isolation is tedious, it is another and useful method for obtaining envelope mutants which may help to understand the problem of cell envelope biosynthesis and its genetic control.

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Communicated by H. G. Wittmann

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