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Mutation Affecting Resistance of *Escherichia coli* K12 to Nalidixic Acid

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A new mutation, *nalD*, determining resistance of *Escherichia coli* to nalidixic acid (NAL) is reported. The *nalD* mutant described is resistant to NAL at 37 °C but sensitive at 30 °C. It is defective in penetration of NAL and glycerol through the outer membrane at 37 °C. The *nalD* mutation is located half-way between 89 and 89·5 min on the *E. coli* genetic map.

INTRODUCTION

Nalidixic acid (NAL) is an inhibitor of chromosomal DNA replication in *Escherichia coli* (Goss *et al.*, 1965; Engle *et al.*, 1982). The drug inactivates subunit A of DNA gyrase, an enzyme catalysing the introduction of a negative superhelical turn into duplex DNA in reactions requiring ATP (Sugino *et al.*, 1977; Cozzarelli, 1980). Different classes of NAL-resistant *E. coli* mutants are defective in the genes encoding functionally different proteins. Resistance to high NAL concentrations is connected with mutation in the structural gene for DNA gyrase subunit A (*gyrA*). The only NAL-transport defective strain reported so far is the mutant *nalB* (Hane & Wood, 1969). This strain is resistant to very small doses of the drug, and is thus not very useful in studies of NAL transport *in vivo*. Here we report another permeability mutant which is resistant to much higher concentrations of nalidixic acid than the *nalB* mutant.

METHODS

Organisms. The strains used (Table 1) were derivatives of *E. coli* K12. Strains AB1157-25 and AB1157-81 are spontaneous NAL-resistant (Nal^r) mutants isolated from agar plates containing NAL (10 μg ml^{-1}). Colonies formed on this medium at 37 °C were passaged several times in the presence of NAL at the same temperature. Then the growth of the bacteria was tested at 30 °C, 37 °C and 42 °C.

The following phages were used: TuIa, TuIb, Tc45, T_6, λ vir and P1 vir.

Media. The following media were used: minimal medium (Davis & Mingioli, 1965); M-9 (Miller, 1972); basic Tris/HCl (Levinthal *et al.*, 1962) with glucose added (0·4%, w/v), and, when necessary, amino acids (10 μg ml^{-1}) and thiamin (1 μg ml^{-1}); LB medium (Miller, 1972) and nutrient broth. Solid media were prepared by addition of 1·5% or 0·5% (w/v) agar to the liquid medium. Differentiating medium (Miller, 1972) contained 1·5% (w/v) agar, 4% (w/v) maltose and 0·005% (w/v) tetrazolium.

NAL resistance. This was tested on nutrient agar containing 20 μg NAL ml^{-1}. In genetic experiments the following method was employed: colonies from selective medium were transferred to 1 ml 0·85% (w/v) NaCl solution. The suspension was then diluted (usually 10^{-2}) to give a suitable concentration of cells and 0·1 ml was plated on medium containing NAL.

Mapping of nalD mutation. (i) Conjugation method. Overnight cultures of Hfr strains and AB1157-81-1 in minimal medium were diluted 10 times in the same medium and incubated in a water bath for 3 h at 37 °C with shaking. The mixture of donor (0·5 ml) and recipient (4·5 ml) cells was incubated for 20 min to 1 h at 37 °C depending on the Hfr strain used and the recombinants required. DNA transfer from the donor was mechanically interrupted by dilution and strong pipetting in streptomycin solution (1 mg ml^{-1}). Suitable dilutions were spread on selective media.

(ii) Transduction method. The lysates were prepared according to Miller (1972). Rifampicin-resistant (Rif^r) strains F$^-$ AB1157-81-1 and HfrC were isolated through selection of spontaneous mutants formed on media

Abbreviation: NAL, nalidixic acid.

Table 1. *Bacterial strains*

Strain	Mating type	Relevant genotype or phenotype and comments	Source or reference
AB1157*	F⁻	Thr⁻ Leu⁻ Ara⁻ *proA* Tsx⁻ Gal⁻ His⁻ Mtl⁻ Xyl⁻ *argE3* Thi⁻ Str^r	Howard-Flanders *et al.* (1966)
AB1157-25	F⁻	LamB⁻ *nalA* spontaneous mutant, derivative of AB1157	This paper
AB1157-81	F⁻	LamB⁻ Nal^r derivative of AB1157	This paper
AB1157-81-1	F⁻	LamB⁺, P1 transductant, donor HfrC; derivative of AB1157-81	This paper
E101	F⁻	*nrdA101* Thr⁻ *leuG* Thi⁻ *thyA6 deoC1 supE44* Ton⁻ Azi⁻ Str^r	Phabagen*
CSH70	Hfr	*metB argE* Thi⁻	Phabagen* (Miller, 1972)
H	Hfr	Thi⁻ *galK* Phx⁻ Rel⁻	Phabagen*
H292	Hfr	Lam⁻ *tonA* Phx⁻	Phabagen*
KL209	Hfr	Thi⁻ *malB* Phx⁻ Lam⁻ *supE*	Phabagen* (Low, 1973)
C†	Hfr		Cold Spring Harbor CHS61 = D7011

* Phabagen Collection, Laboratory for Microbiology, Utrecht, The Netherlands.
† From Max-Planck Institut für Virusforschung, Tübingen, FRG.

containing 100 μg rifampicin ml^{-1}. Rif^r mutants of strain AB1157-81-1 were tested on agar medium containing NAL at 37 °C and 30 °C; growth was observed only at 37 °C. Rif^r mutants of strain HfrC remained Nal^s. Rifampicin resistance was mapped in both strains at 90 min of the genetic map, therefore they are mutants in gene *rpoB* (Bachmann, 1983).

Minimal inhibitory concentration (MIC). MIC and the ability to form colonies in the presence of different NAL concentrations were tested as described by Inouye *et al.* (1978).

Inhibitory effect of NAL on DNA synthesis in intact cells. Methods described by Bourguignon *et al.* (1973) were used. The experiments were performed at 37 °C and the final concentration of NAL was 10 μg ml^{-1}. The EDTA concentration in 0·12 M-Tris/HCl buffer (pH 8·0) was 2 × 10^{-4} M. [^{3}H]Thymidine (10 μCi ml^{-1}; 370 kBq ml^{-1}) and deoxyadenosine (250 μg ml^{-1}) were added to the samples at the beginning of the experiments. Precipitation was performed with 10% (w/v) trichloroacetic acid (TCA). Radioactivity was determined in a Beckman L355 liquid scintillation counter.

Permeability assay. [^{3}H]NAL penetration through the *E. coli* outer membrane was tested as described by Nakae & Ishii (1980). [^{3}H]NAL was prepared as described by Płuciennik & Hrebenda (1982); its specific activity was 15 mCi mmol^{-1} (555 MBq mmol^{-1}). The specific activity of [^{3}H]glycerol (Amersham) was 202 mCi mmol^{-1} (7·47 GBq mmol^{-1}).

Electrophoresis of outer membrane proteins. Electrophoresis was performed in 10% (w/v) SDS-polyacrylamide gel according to Laemmli (1970). Protein samples were prepared as described by Lugtenberg *et al.* (1975). Solutions containing Tris/HCl, glycerol, bromothymol blue, 2-mercaptoethanol and protein (150 μg ml^{-1} in a volume of 30 μl), were boiled for 3 min and loaded onto the gel. The gel was stained for 18 h in an aqueous solution of Coomassie blue with acetic acid (9%, v/v) and ethanol (4·5%, v/v).

RESULTS

Characteristics of the Nal^r mutant AB1157-81-1

Nal^r mutants were isolated at a frequency of 1–5 × 10^{-7}. Among 100 strains which grew on NAL-containing plates, tested at 30 °C and 37 °C, only one (AB1157-81) was cold-sensitive. The MIC of nalidixic acid for this strain was 6 μg ml^{-1} at 30 °C and 24 μg ml^{-1} at 37 °C. At 42 °C strain AB1157-81 was resistant to bacteriophage λ and did not synthesize the LamB protein; it was λ^s at 37 °C and 30 °C. The isogenic strain AB1157-81-1 Nal^r LamB⁺ (sensitive to λ at 42 °C and 37 °C) was obtained by transduction. Fig. 1 illustrates the growth of AB1157-81-1 and two control strains (the wild-type, AB1157, MIC at 30 °C and 37 °C 6 μg ml^{-1}, and AB1157-25, a *gyrA* mutant, MIC 345 μg ml^{-1}) on nutrient agar containing various concentrations of NAL. The *gyrA* mutation in AB1157-25 was mapped by P1 transduction. In an analogous experiment with AB1157-81-1 a defect in the *gyrA* gene in this strain was excluded (not shown).

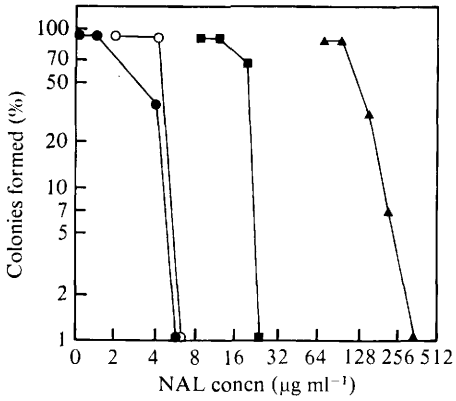

Fig. 1. Growth of AB1157 (a wild-type strain) and mutants AB1157-81-1 and AB1157-25 in the presence of various concentrations of NAL, in terms of percentage of colonies growing at 30 °C or 37 °C on NAL-containing medium in relation to the control. ○, AB1157 at 37 °C; ●, AB1157-81-1 at 30 °C; ■, AB1157-81-1 at 37 °C; ▲, AB1157-25 at 37 °C.

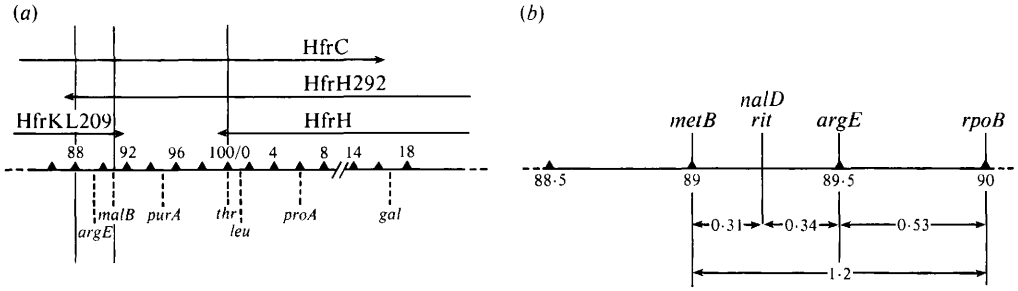

Fig. 2. Part of the *E. coli* genetic map: (*a*) indicating the origin and direction of chromosomal DNA transfer by individual Hfr strains (horizontal lines mark the region of the chromosome where the NAL-resistance mutation was mapped); (*b*) with map distances between individual markers determined by transduction (the NAL-resistance mutation is designated *nalD*).

The electrophoretic profiles of the major outer membrane proteins of strains AB1157 and AB1157-81-1 showed that OmpF, OmpC and LamB were present in the membranes at 30 °C, 37 °C and 42 °C, whereas PhoE and phage T_6 receptor proteins were not found on the gel.

Mapping of NAL-resistance mutation

NAL resistance in AB1157-81-1 was mapped by conjugation and P1 transduction. Fig. 2(*a*) shows part of the *E. coli* map with the origins of transfer from the Hfr donor to the F^- (AB1157-81-1) recipient marked. Nutritive recombinants were selected in the recipient (see Table 2). Recombination rates for proximal markers were 4·5% for HfrC × AB1157-81-1 and 5% for HfrH × AB1157-81-1. Colonies of individual recombinants were purified and plated in parallel onto nutrient agar plates containing NAL (see Methods), and incubated at 30 °C and 37 °C. Since the Hfr strains used in the experiments were sensitive to NAL we were able to assume that AB1157-81-1 recombinants unable to grow on NAL-containing medium at 37 °C contained the wild-type Nal allele (Nal^s). The genetic linkage between Nal^s and the selected genetic marker in the recombinants indicated the region on the *E. coli* chromosome where the mutation to NAL-resistance is located (Table 2). The highest degree of linkage between Nal^s and a selected marker was found for Arg^+ AB1157-81-1 recombinants, regardless of whether they were selected after crossing with HfrC, HfrKL209 or HfrH292.

The origins of DNA transfer of HfrKL209 and HfrH292 are located in the vicinity of *argE*. The distance between their origins is 3 min, and the two strains transfer chromosomal DNA in

Table 2. *Conjugation mapping of the NAL-resistance mutation in AB1157-81-1*

Cross	Selected phenotype	No. of recombinants tested	Unselected phenotype*	Frequency of unselected markers among selected recombinants (%)
HfrH × F⁻ AB1157-81-1	Thr⁺ Leu⁺	100	Nal^s	0
	Pro⁺	100	Nal^s	0
	His⁺	100	Nal^s	0
HfrC × F⁻ AB1157-81-1	Pro⁺	150	Thr⁻ Leu⁻	24·6
			Arg⁻	2·0
			Nal^s	1·3
	Thr⁺ Leu⁺	100	Pro⁻	36
			Arg⁻	14
			Nal^s	10
	Arg⁺	100	Nal^s	20
HfrKL209 × F⁻ AB1157-81-1	Arg⁺	96	Nal^s	70
HfrH292 × F⁻ AB1157-81-1	Arg⁺	150	Nal^s	90·6

* Nal^s, sensitivity to 20 μg NAL ml^{-1}.

Table 3. *Transduction mapping of the NAL-resistance mutation in AB1157-81-1*

Donor	Recipient	Selected marker	Unselected marker	No. of transductants tested	Co-transduction frequency (%)	Map distance* (min)
HfrC	AB1157-81-1	*argE*⁺	Nal^s	100	57	0·34
			Rif^r	100	39	0·53
AB1157-81-1	CSH70	*metB*⁺	Nal^r†	100	60	0·31
			Rif^r	200	7·5	1·2

* Calculated according to Wu (1966).
† Nal^r, resistance to 20 μg NAL ml^{-1}.

opposite directions. Hence the NAL-resistance mutation is located between 88 min and 91 min of the genetic map.

The precise location of the mutation was determined by transduction. Phage P1 was grown on Rif^r derivatives of HfrC (Nal^s Rif^r) and on AB1157-81-1 (Nal^r Rif^r). The P1 lysate obtained from the HfrC donor was used to transduce *argE*⁺ to an AB1157-81-1 ArgE⁻ Nal^r Rif^r recipient strain. The number of Nal^s and Rif^r clones among the ArgE⁺ transductants was determined. The co-transduction rate of these markers is shown in Table 3. The *metB*⁺ allele of AB1157-81-1 Rif^r Nal^r was transduced into strain CSH70 (MetB⁻ Nal^s Rif^s), and Nal^r and Rif^r clones were screened among the MetB⁺ transductants (see Table 3). The MetB⁺ transductants that were Nal^r at 37 °C (60 clones) were Nal^s at 30 °C. Fig. 2(*b*) shows part of the *E. coli* genetic map indicating the location of the NAL-resistance mutation. The distances between the genes were determined by using the equation for three-factor transduction (Wu, 1966), assuming 2·0 min as the maximal size of the transduced DNA. In agreement with this, Nal^r mapped half-way between *metB* and *argE*, i.e. at 89·5 min. The gene affected by the NAL-resistance mutation is designated *nalD* in Fig. 2.

Permeability of the cell envelope for NAL

In studies on the transport of NAL in the mutants EDTA was used to increase the permeability of the outer membrane (Fig. 3). In the case of AB1157-25 (*gyrA*), the addition of NAL to a culture growing at 37 °C did not inhibit DNA synthesis (as measured by increase in

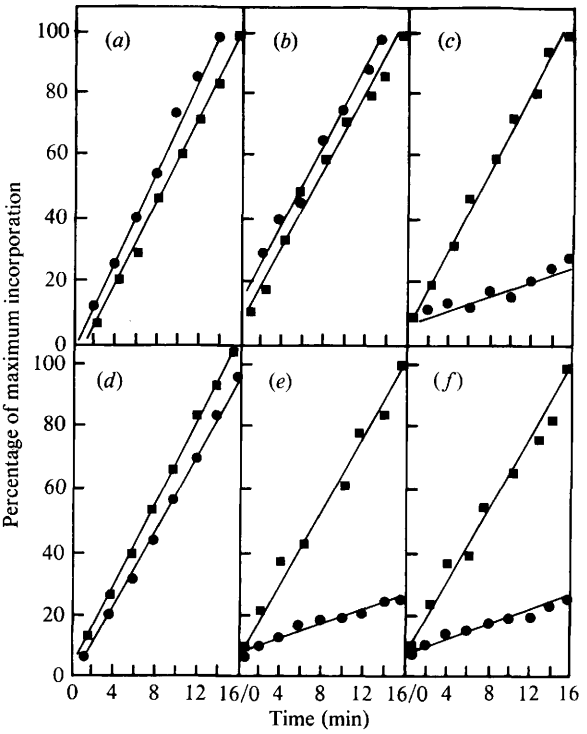

Fig. 3. Effect of NAL on DNA synthesis in cells without treatment with EDTA (*a*,*b*,*c*) and after treatment with EDTA (*d*,*e*,*f*). (*a*,*d*) AB1157-25 (*gyrA*); (*b*,*e*) AB1157-81-1 (Nal^r mutant); (*c*,*f*) AB1157 (a wild-type). ●, Cultures supplemented with NAL (10 μg ml^{-1}); ■, no NAL added. All cultures were grown at 37 °C.

radioactivity in the TCA-insoluble fraction) after treatment with EDTA (Fig. 3*d*). The addition of NAL or EDTA alone under identical conditions did not affect DNA synthesis (Fig. 3*a*,*d*). Resistance to NAL in this strain was thus independent of changes in the permeability of the outer membrane for the drug. DNA synthesis in the wild-type (Nal^s) strain AB1157 was not sensitive to EDTA treatment (Fig. 3*c*) but, as expected, DNA synthesis was inhibited after addition of NAL (Fig. 3*c*,*f*). In strain AB1157-81-1 (*nalD*) the combined use of NAL and EDTA strongly inhibited DNA synthesis (Fig. 3*e*), whereas either compound alone had no effect (Fig. 3*b*,*e*). This suggests a defect in the penetration of NAL in strain AB1157-81-1.

Penetration of [^{3}H]NAL and [^{3}H]glycerol

The outer membrane protein profiles of mutant and wild-type strains showed no differences, suggesting that NAL does not utilize the porins when penetrating this layer. To test the possibility that NAL penetrates directly through the phospholipid bilayer, we used glycerol, which is known to do so. Results of [^{3}H]NAL and [^{3}H]glycerol penetration into untreated AB1157-81-1 and AB1157 cells are shown in Fig. 4. With both [^{3}H]NAL and [^{3}H]glycerol the radioactivity level found in AB1157-81-1 cells at 37 °C was half or less that found at 30 °C. The permeation of both compounds into the wild-type (AB1157) was higher than that into the mutant. In AB1157 the amounts of both [^{3}H]NAL and [^{3}H]glycerol taken up at 30 °C were similar to the amounts taken up at 37 °C. We conclude from these experiments that in AB1157-81-1 a rapid inhibition of NAL and glycerol uptake takes place at 37 °C. Hence the mutation causing NAL-resistance in AB1157-81-1 has been designated *nalD* – nalidixic acid diffusion.

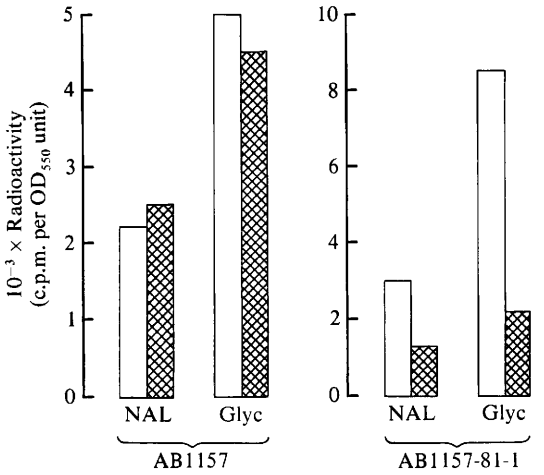

Fig. 4. Penetration of [^{3}H]NAL and [^{3}H]glycerol into cells of AB1157 (Nal^s) and AB1157-81-1 (*nalD*). Cells grown in M-9 medium at 30 °C (white bars) or 37 °C (hatched bars) were incubated with the radioactive compound for 30 s at the same temperature, then washed and the radioactivity measured. The radioactivity measurements were normalized to 1 OD_{550} unit.

DISCUSSION

There are several *E. coli* mutants resistant to NAL. Two of them, i.e. *gyrA* (48 min) and *nalB* (58 min), have been discussed by Hane & Wood (1969). The *gyrA* mutation is responsible for changes in the DNA gyrase subunit A, while *nalB* causes defective transport of NAL into the cells. According to Kumar (1980), mutations in other genes, apart from defects in specified protein functions, may lead to NAL resistance in bacteria. This group includes the following mutations: *cya* (85 min), *crp* (74 min), *ptsH* (52 min), *purB* (25 min) and *icd* (25 min). We describe here another *E. coli* mutant (*nalD*; nalidixic acid diffusion) which is resistant to NAL at 37 °C and sensitive at 30 °C. The *nalD* mutation differs from those reported earlier in that it is located half-way between 89 and 89·5 min of the genetic map of *E. coli* (Bachmann, 1983).

The chromosome region in the vicinity of gene *rpoB* (90 min) constitutes one of the two gene clusters that are involved in ribosomal protein synthesis (Kuwano *et al.*, 1977; Watson *et al.*, 1975). The *rit* mutation (89 min) determining 50S ribosome subunit thermolability is also located here (Ono & Kuwano, 1978). At present we have no data to support the suggestion that *rit* and *nalD* mutations concern the same gene.

Our mutant strain is deficient in penetration of NAL into cells at 37 °C, but not at 30 °C. We have demonstrated the defect in penetration indirectly, by investigating inhibition of DNA replication in EDTA-treated cells, and directly, by testing the penetration of [^{3}H]NAL into the mutant cells at different temperatures. EDTA causes loosening of the outer membrane of Gram-negative bacteria and has been used in experiments to test outer membrane permeability, as for example in *nalA* (*gyrA*) and *nalB* mutants (Bourguignon *et al.*, 1973; Scudamore *et al.*, 1979). To test [^{3}H]NAL and [^{3}H]glycerol penetration we adopted the original method of Szmelcman *et al.* (1976) modified by Nakae & Ishii (1980). Accordingly, investigations of penetration were limited to the measurement of passive transport (diffusion) of water-soluble substances through the outer membrane. EDTA also acts on the cytoplasmic membrane, so we cannot exclude the possibility that it affects the rate of NAL penetration through this layer. However, on the basis of our experiments we suggest that NAL-resistance in strain AB1157-81-1 grown at 37 °C results exclusively from the defect(s) in drug penetration through the outer membrane. This effect of the *nalD* mutation is not specific for NAL, but includes also the inhibition of glycerol diffusion. At present no data are available to suggest that AB1157-81-1 is more generally deficient in transport. It is as sensitive as the wild-type to β-lactam antibiotics (penicillin, ampicillin, methicillin and cephaloridine), tetracycline and chloramphenicol (data not shown).

The mechanism of NAL-transport through the outer membrane is so far unknown. In the case of hydrophilic substances of molecular weight lower than 600, transport takes place mainly through general channels formed by OmpF and OmpC porin proteins (Nakae, 1976; Decad & Nikaido, 1976). The molecular weight of NAL is 232·3, and the compound is easily dissolved in alkalized water and in polar solvents (Pedrini, 1979); it may therefore be a candidate for passive transport mediated by OmpF and OmpC channels. The outer membrane proteins of AB1157-81-1 and the wild-type strain, isolated at 37 °C, include OmpF OmpC and LamB. Both strains were sensitive to the porin-dependent bacteriophages TuIa and TuIb, and resistant to Tc45. These bacteriophages use porins OmpF, OmpC and PhoE, respectively, as receptors. Therefore, the decreased permeability to NAL of the NAL-resistant mutant may be due not to the absence of OmpF or OmpC proteins, but to the relative levels of these proteins in the outer membrane. Earlier studies (Van Alphen & Lugtenberg, 1977; Ozawa & Mizushima, 1983) showed that the ratio of OmpF to OmpC in the *E. coli* outer membrane changes under the influence of differences in medium osmolarity as well as following addition of cAMP. An alternative possibility, favoured by us, is that passive transport of NAL may occur directly through the phospholipid bilayer. Yamaguchi *et al.* (1982) have reported that ampicillin, in contrast to cephaloridine, can diffuse to the periplasm through the outer membrane when it is deprived of OmpF and OmpC channels. Glycerol is one of the compounds which are transported via facilitated diffusion. It is fairly soluble in lipid bilayer membranes (Lin, 1976). The diffusion of glycerol is significantly decreased in our mutant grown at 37 °C as compared with that at 30 °C.

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