ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1988, p. 1680–1683
0066-4804/88/111680-04$02.00/0

Vol. 32, No. 11

# Sensitization by Heat Treatment of *Escherichia coli* K-12 Cells to Hydrophobic Antibacterial Compounds

TETSUAKI TSUCHIDO* AND MITSUO TAKANO

*Department of Fermentation Technology, Osaka University, Suita, Osaka 565, Japan*

Received 2 May 1988/Accepted 31 August 1988

**The sensitivities of intact and heat-injured cells of *Escherichia coli* K-12 to several antibacterial compounds were measured by the prolongation of growth delay. Cells exposed to sublethal heat became more sensitive to various hydrophobic compounds, such as medium-chain fatty acids, alkyl esters of *p*-hydroxybenzoic acid, and some kinds of antibiotics or dyes, than unheated cells; but there was a smaller or no increase in sensitivity to short-chain fatty acids, chloramphenicol, and vancomycin. The destruction by heat of a permeability barrier of the outer membrane may have sensitized the cells to hydrophobic compounds. The sensitization was much lower for a strain defective in lipopolysaccharide, which is important as a barrier against hydrophobic compounds.**

Gram-negative bacteria, including *Escherichia coli* and *Salmonella typhimurium*, are generally more resistant to growth inhibition by a variety of hydrophobic antibacterial compounds than are gram-positive bacteria. The resistance of gram-negative bacteria is attributed to the presence of the outer membrane, which acts as a permeability barrier (10). If the outer membrane is disrupted, therefore, gram-negative bacteria should become sensitive to hydrophobic antibacterial compounds. This situation was proven for cells treated with EDTA, which destabilizes the outer-membrane structure by chelating divalent cations (8, 9).

A similar situation may also occur with heat-treated cells of gram-negative bacteria. We have already reported that sublethal heat treatment of *E. coli* cells induces blebbing and vesiculation of the outer membrane from the cells, accompanied by the release of lipopolysaccharide from the outer membrane into the menstruum (7), indicating substantial destruction of the outer membrane structure. Such cells are permeable to hydrophobic dyes, such as crystal violet (17) and 1-phenylnaphthylamine (T. Tsuchido, I. Aoki, and M. Takano, unpublished observations).

Although different mechanisms are possible for the heat-induced sensitization of bacterial cells to chemical agents (20), the sensitization of gram-negative bacteria to growth inhibition by hydrophobic compounds may be generally explained by destruction of the permeability barrier. In fact, *E. coli* cells heated at 52°C for 5 min became sensitive and permeable to a hydrophobic antibiotic, tylosin (18).

In this study, we investigated the sensitivity of *E. coli* cells to a series of fatty acids and their esters, which have been our recent concern (15, 16), and to hydrophobic antibiotics and dyes by evaluation of the growth delay caused by their inhibitory action.

## MATERIALS AND METHODS

**Microorganisms.** An *Escherichia coli* K-12 strain, W3110, was generally used. In one experiment, *E. coli* JE1011 $F^-$ *thr leu trp his thy thi ara lac gal xyl mtl str* and its lipopolysaccharide-defective mutant, NS3, which were generous gifts from M. Matsuhashi (14), were also used. This mutant lacks outer-core oligosaccharides because of the loss of phosphate diester bridges in the lipopolysaccharide backbone (14).

**Culture conditions.** Cells were grown at 37°C in EM9 medium (17) supplemented with 0.2% glucose for strain W3110 or with 0.2% glucose, 50 μg of thymidine per ml, 2 μg of thiamine per ml, 0.1 mM tryptophan, and 0.2% yeast extract (Nihon Pharmaceutical Co., Ltd., Osaka, Japan) for strains JE1011 and NS3. The specific growth rates for the latter two strains were 0.70 and 0.69/h, respectively. Cells at the logarithmic growth phase, in which the optical density at 650 nm ($OD_{650}$) of the culture was about 0.4, were harvested and washed twice with 50 mM Tris hydrochloride buffer containing 10 mM magnesium sulfate (TM buffer) at pH 8.0.

**Heat treatment.** Cells kept at 0°C were heat treated at 55°C for 15 s, unless otherwise stated, in TM buffer as described previously (17).

**Measurement of growth-delay time.** Portions (0.2 ml) of heated and unheated samples were transferred into L-shape glass tubes containing 3.8 ml of fresh medium. The tubes were shaken at 37°C in a Bioscanner OT-BS-48 (Ohtake Works, Ltd., Tokyo, Japan) (13). The solutions of various antibacterial compounds were added to the medium described above before inoculation. The growth time, the time required for a culture to grow from inoculation to an $OD_{650}$ level of 0.1, and the growth-delay time were calculated by a programmed computer and printed. The degree of sensitization was expressed as the sensitization index by the ratio of the delay time for heated cells ($\Delta t_h$) to that for unheated cells ($\Delta t_c$), which was estimated at an $OD_{650}$ of 0.1 (see Fig. 1). If heated cells have the same sensitivity to an inhibitor as unheated cells, the sensitization index should be 1. In this paper, as a standard criterion, the sensitization index was calculated when $\Delta t_c$ was 1 h.

**Chemicals.** All fatty acids were used as sodium salts (Tokyo Chemical Industries, Tokyo, Japan). Glycerol monododecanoate and sucrose monohexadecanoate were purchased from Riken Vitamin Co., Ltd., Tokyo, Japan, and Mitsubishi Kasei Corp., Tokyo, Japan, respectively. The purity of these compounds was 99% or more. Alkyl esters of *p*-hydroxybenzoic acid were obtained from Tokyo Chemical Industries. Antibiotics, such as tylosin tartrate, vancomycin, rifampin, and chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), and dyes, such as crystal violet and malachite

* Corresponding author.

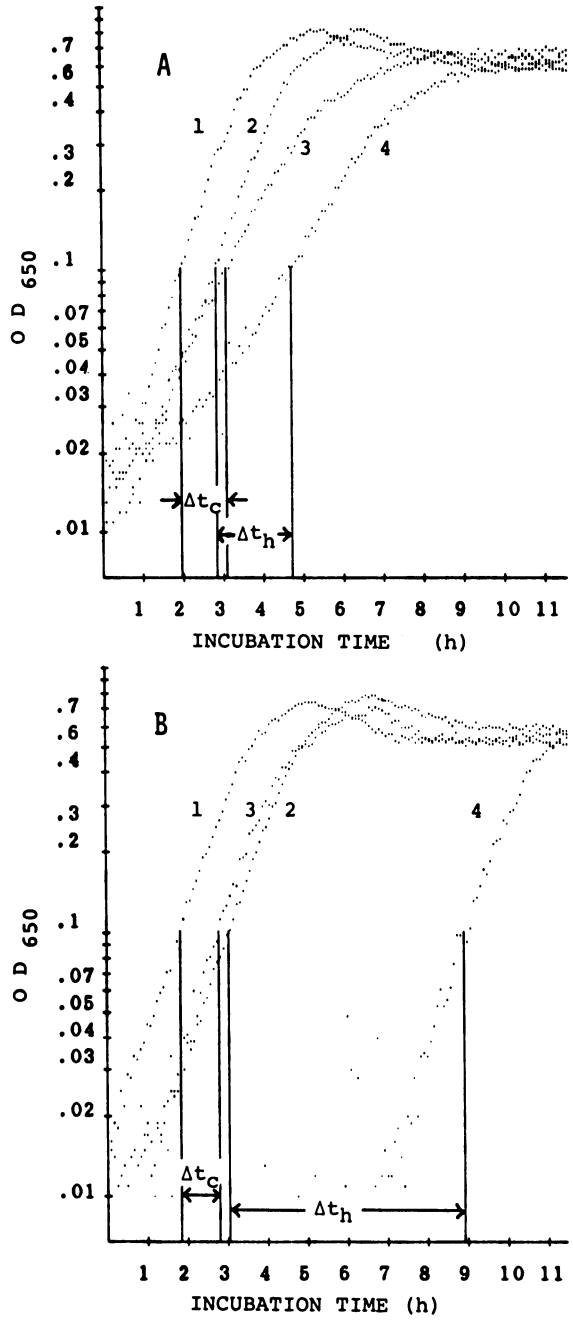


FIG. 1. Bioscanner chart for growth of *E. coli* cells in the presence of fatty acids after heat treatment at 55°C for 15 s. (A) Hexanoic acid at 60 mM. (B) Dodecanoic acid at 2 mM. The vertical lines indicate the times when the $OD_{650}$ of culture reached 0.1. $\Delta t_c$ and $\Delta t_h$ indicate the growth delay caused by either fatty acid in unheated cells and heated cells, respectively. Dotted lines: 1, unheated cells; 2, heated cells; 3, cells treated with fatty acid; 4, cells heated and then treated with fatty acid.

green oxalate (Wako Pure Chemical Industries, Osaka, Japan), were also of reagent grade.

## RESULTS

**Sensitization to fatty acids and their esters by heat.** After heat treatment at 55°C for 15 s of logarithmically growing cells of *E. coli* W3110, the cells were cultivated at 37°C in a Bioscanner tube containing fresh medium with or without either hexanoic acid or dodecanoic acid at a concentration of 60 or 2 mM, respectively (Fig. 1). Heated cells had a growth delay of about 1 h in the absence of fatty acid. The delay in growth at an $OD_{650}$ of 0.1 ($\Delta t_c$) was about 1 h for either fatty acid at the indicated concentrations. Hexanoic acid affected the growth rate, but dodecanoic acid prolonged the apparent lag period. Heat treatment sensitized cells more to dodecanoic acid than to hexanoic acid when the growth delays were simply compared (Fig. 1).

The situation described above is obvious from the delay times with either fatty acid at different concentrations (Fig. 2). The concentrations at which there was a growth delay of 1 h for unheated cells were calculated from Fig. 2 to be 51 mM for hexanoic acid and 2.3 mM for dodecanoic acid. At these concentrations, the values of the sensitization index were calculated graphically to be 1.9 and 6.0, respectively (Fig. 2; Table 1).

The effect of the alkyl-chain length of fatty acids on the degree of heat-induced sensitization was further examined. Table 1 indicates that medium-chain fatty acids, such as octanoic acid and decanoic acid, had the greatest effects, with the sensitization index above 10; next was dodecanoic acid, although the concentration causing a growth delay of 1 h decreased with the increase in the alkyl-chain length of fatty acids tested. However, hydrophilic acids such as butanoic acid and hexanoic acid with partition coefficients of less than 0.01 had low sensitization indexes.

To analyze the heat-induced sensitization by a theory presented previously (13; T. Tsuchido, T. Koike, and M. Takano, submitted for publication), we plotted the logarithm of relative inoculum size against the growth times described above for untreated cells and cells treated with hexanoic acid or dodecanoic acid after heat treatment. The $G_{10}$ values, which are the increments of the growth-delay time when the inoculum size decreases one-tenth and which were obtained from the slope of the line in Fig. 3 (13), were almost identical for untreated cells and heated cells, as previously reported (13), being 1.8 and 1.9 h, respectively. In the presence of 50 mM hexanoic acid, the $G_{10}$ increased to 3.7 h for unheated cells and 4.8 h for heated cells; and in the presence of 1 mM dodecanoic acid, they were 2.0 h and 2.1 h, respectively, almost identical to those for the control cells. These results

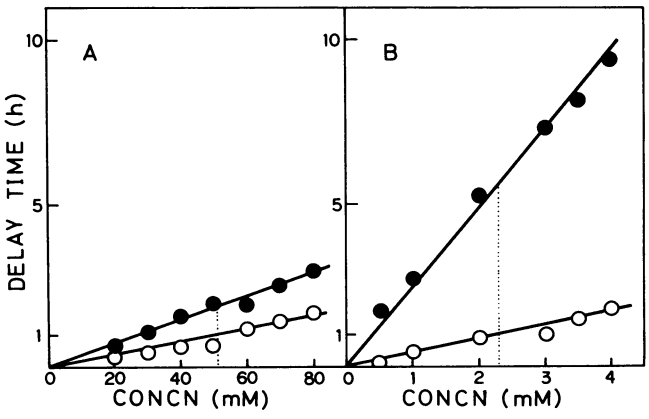


FIG. 2. Growth delays of unheated cells and heated cells caused by treatment with hexanoic acid (A) or dodecanoic acid (B) at different concentrations. Symbols: ○, unheated cells ($\Delta t_c$); ●, heated cells ($\Delta t_h$). The vertical dotted lines indicate the concentration when $\Delta t_c$ is 1 h.

TABLE 1. Effect of carbon-chain length of fatty acid on sensitization of cells by heat treatment at 55°C for 15 s

| Fatty acid | Partition coefficient[a] | Concn (mM)[b] | Sensitization index[c] |
|---|---|---|---|
| Butanoic | <0.01 | 165 | 1.9 |
| Hexanoic | <0.01 | 51 | 1.9 |
| Octanoic | 0.06 | 37 | 12.0 |
| Decanoic | 0.63 | 9.8 | 10.8 |
| Dodecanoic | 6.3 | 2.3 | 6.0 |

[a] Reference 3.
[b] Concentrations at which $\Delta t_c$ equals 1 h. See Fig. 2 for details.
[c] See the text. Indicated values are $\Delta t_h$ when $\Delta t_c$ is 1 h.

TABLE 2. Sensitivity to antibiotics and dyes of cells heated at 55°C for 15 s

| Compound | Partition coefficient[a] | Concn (mM)[b] | Sensitization index[c] |
|---|---|---|---|
| Crystal violet | 14.4 | 2.1 | 3.8 |
| Chloramphenicol | 12.4 | 0.0041 | 1.3 |
| Rifampin | 8.8 | 0.006 | 4.0 |
| Malachite green | 4.2 | 3.6 | 3.5 |
| Tylosin | 0.57 | 0.07 | 5.2 |
| Vancomycin | <0.01 | 0.017 | 1.0 |

[a,b] See footnotes *a* and *b* to Table 1.
[c] Data are from reference 10, except for tylosin, for which the value was obtained in this study with an *n*-octanol-water system.

also reflect the fact that heated cells became sensitive to hexanoic acid because of inhibition of the growth rate; this is in contrast to dodecanoic acid, which affected the apparent lag period. Heat treatment also sensitized cells to glycerol monododecanoate at 2 mM but not to sucrose monohexadecanoate even at 10 mM (data not shown).

**Sensitivity of heated cells to antibiotics and dyes.** Several antibiotics and dyes are ineffective against gram-negative bacteria (9–11, 22). We examined the effects of heat treatment on sensitivity to such compounds. Heated cells became sensitive to hydrophobic antibiotics and dyes that have partition coefficients of over 0.01, except chloramphenicol; whereas they did not become sensitive to a hydrophilic antibiotic, vancomycin (Table 2). The degree of sensitization by heat was not parallel with the partition coefficient of each compound, as is also shown by the results obtained with fatty acids (Table 1). The reason for the much lower sensitization to chloramphenicol is not known (10).

**Sensitivity of a lipopolysaccharide-defective strain to hydrophobic antibacterial compounds.** Nikaido (10) and Sheu and Freese (12) reported that lipopolysaccharide-defective strains of gram-negative bacteria are more susceptible to growth inhibition by several hydrophobic compounds than are wild-type strains. In fact, such a strain of *E. coli*, NS3, was more sensitive to dodecanoic acid and tylosin than was the wild-type strain, JE1011, when evaluated at concentrations causing a growth delay of 1 h for unheated cells (Table 3).

On the other hand, since heat treatment releases the lipopolysaccharide molecules from the outer membrane (4, 7), the degree of heat-induced sensitization for a lipopolysaccharide-defective strain should be lower than that for the wild-type strain because of inherent permeability. Table 3 indicates that the sensitization indices for NS3 for dodecanoic acid, alkyl esters of *p*-hydroxybenzoic acid, and tylosin were lower than those for wild-type strain JE1011 under the heating conditions (55°C, 5 s) used. The sensitizations to hexanoic acid were almost identical for the two strains.

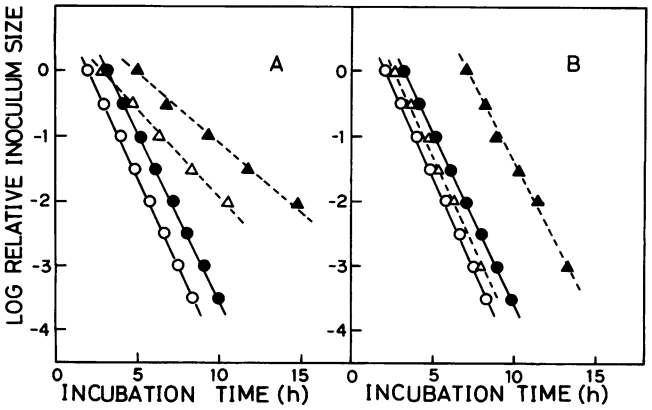


FIG. 3. Growth times of cells at various inoculum sizes in the presence of hexanoic acid at 50 mM (A) or dodecanoic acid at 1 mM (B). Symbols ($G_{10}$ values) (see the text): ○, untreated cells (1.8 h); ●, cells heated at 55°C for 15 s (1.9 h); △, cells treated with hexanoic acid (3.7 h) or dodecanoic acid (2.0 h); ▲, cells heated and then treated with hexanoic acid (4.8 h) or dodecanoic acid (2.1 h).

## DISCUSSION

*E. coli* cells were sensitized by sublethal heat treatment to various hydrophobic antibacterial compounds, similar to treatment with EDTA (2, 8, 9) or to the mutation causing lipopolysaccharide deficiency (1, 9, 11, 14). According to the model of Nikaido and Vaara (11), in lipopolysaccharide-defective cells or cells treated with EDTA, the outer leaflet of the outer membrane contains more lipid molecules than intact cells of the wild type, which may be almost excluded from the outer leaflet of the outer membrane in normal cells of the wild type because of the loss of or structural defects in the lipopolysaccharide molecule. We previously reported that heat treatment at 55°C caused blebbing and vesiculation of the outer membrane of *E. coli* cells (7), thereby causing the release of lipopolysaccharide, the penetration of crystal violet into the cells, and an increase in cell-surface hydrophobicity (17). Therefore, in heat-treated cells it is likely that, as a simple model, lipid molecules also appear in the outer leaflet of the outer membrane as a result of rapid structural disturbance, as suggested by the finding that the phospholipids in heated cells are hydrolyzed by external phospholipase C (17). The molecules of hydrophobic compounds added externally, such as medium-chain fatty acids,

TABLE 3. Sensitivities to antibacterial compounds of *E. coli* JE1011 and NS3 cells heat treated at 55°C for 5 s

| Strain | Compound | Concn (mM)[a] | Sensitization index[b] |
|---|---|---|---|
| JE1011 | Hexanoic acid | 43 | 1.5 |
| | Dodecanoic acid | 0.55 | 4.7 |
| | Methyl *p*-hydroxybenzoic acid | 6.1 | 4.7 |
| | Butyl *p*-hydroxybenzoic acid | 3.2 | 10.4 |
| | Tylosin | 0.024 | 6.0 |
| NS3 | Hexanoic acid | 18 | 1.4 |
| | Dodecanoic acid | 0.32 | 3.5 |
| | Methyl *p*-hydroxybenzoic acid | 3.4 | 2.4 |
| | Butyl *p*-hydroxybenzoic acid | 1.4 | 4.4 |
| | Tylosin | 0.0012 | 1.5 |

[a,b] See footnotes *b* and *c* to Table 1.

alkyl esters of *p*-hydroxybenzoic acid, antibiotics, and dyes, would penetrate into the cells through the lipid-rich area of the outer membrane or would simply be incorporated into such an area. On the other hand, hexanoic acid may penetrate into the cells through aqueous pores in the outer membrane because of being hydrophilic. It is unknown whether heat-induced permeabilization of hydrophobic compounds requires the release of outer membrane vesicles. Hancock (2) proposed other possibilities for the permeabilization mechanism which are different types of structural discontinuities of the outer membrane; for example, the blebbing itself, which was also seen with heated cells (7).

Fatty acids and their esters have a combined effect with heat in growth inhibition or death of cells (6, 19–21). That heat-treated cells became sensitive to medium-chain fatty acids but not to a short-chain fatty acid, hexanoic acid, coincides with the results of Freese et al. (1), who found that the growth, amino acid transport, and oxygen consumption of *E. coli* are inhibited by the latter but not by the former at concentrations that cause complete inhibition of those activities in *Bacillus subtilis* (1, 12). Freese et al. (1) found evidence that this difference resulted from the impermeability of the outer membrane to medium-chain fatty acids.

Although the lines indicating the relationship between growth-delay time and concentration of fatty acid appeared to be linear (Fig. 2), this was not so for alkyl esters of *p*-hydroxybenzoic acid and other antibiotics. For these, the lines were downward-convex curves (data not shown), indicating that the sensitization index depended upon the concentration of these compounds, unlike those for fatty acids.

In general, the biological activity of a homologous series of antibacterial compounds with an alkyl chain is known to be parabolic against their partition coefficients (3). Of the fatty acids, dodecanoic acid is the most active (5). However, the sensitization index was maximum for a fatty acid with a shorter alkyl chain, suggesting that factors other than outer-membrane permeability also contribute to the sensitization mechanism; for example, the sensitization of another thermally injured site (20).

## ACKNOWLEDGMENT

We thank Heon-Young Cho for his technical assistance with some of the experiments.

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