Inducible Resistance to d-Cycloserine in *Bacillus subtilis* 168

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Resistance to d-cycloserine could be induced in *Bacillus subtilis* 168 by sublethal concentrations of d-cycloserine. Sensitivity to the antibiotic could be regained by growth in the absence of d-cycloserine. The bactericidal activity of d-cycloserine apparently was not altered by resistant cells, and peptidoglycan synthesis was still inhibited by d-cycloserine in resistant cells. The d-cycloserine resistance apparently resulted from a decreased uptake of the antibiotic. The decrease in d-cycloserine transport could be prevented by simultaneous treatment of the cells with rifampin and d-cycloserine. d-Cycloserine was transported by the same system as glycine in *B. subtilis*. d-Cycloserine was able to exchange for intracellular glycine in both sensitive and resistant cells, suggesting that d-cycloserine is not excluded from the cell in resistant cultures.

Resistance to antibiotics can be achieved by a variety of mechanisms: destruction or chemical modification of the antibiotic (1, 3), alteration of the target of the antibiotic (1), or decreased intracellular accumulation of the antibiotic (1, 4). Tetracycline, an inhibitor of protein synthesis, is actively transported into the cell. Bacteria resistant to this antibiotic have a markedly reduced rate of uptake of tetracycline (1, 4). The induction of tetracycline resistance by sublethal concentrations of the antibiotic can be prevented by inhibitors of ribonucleic acid or protein synthesis (10). Resistant cells contain a protein not found in sensitive strains (14–16), and this protein can be released from *Escherichia coli* by osmotic shock (4).

D-Cycloserine is an analogue of d-alanine and is therefore an inhibitor of dL-alanine racemase (EC 5.1.1.1) (6) and d-alanine:d-alanine ligase (EC 6.3.2.4) (8). Because of its structural similarity, it is actively transported into the cell by the same system(s) that is responsible for d-alanine, L-alanine, and glycine transport (5, 11) in a variety of bacterial species. Previous reports (12, 13) indicate that d-cycloserine resistance in *E. coli* results from a loss, due to mutation, of the active transport of the antibiotic. Sensitivity to the antibiotic is regained only by reversion of the mutation(s). In this study we shall present evidence for an inducible resistance to d-cycloserine in *Bacillus subtilis* 168. This resistance is more similar to the inducible tetracycline resistance (4) than it is to the d-cycloserine resistance observed in *E. coli* (13).

**MATERIALS AND METHODS**

**Materials.** The d-[U-14C]cycloserine (specific activity, 51 μCi/mmol) was a generous gift from Roger Harned of Commercial Solvents Corp., Terre Haute, Ind. The [U-14C]glycine (specific activity, 92 mCi/mmol) and Omnifluor were purchased from New England Nuclear. Rifampin and d-alanine were purchased from Sigma Chemical Co., and acid-hydrolyzed casein was from Nutritional Biochemicals Corp. All other chemicals were of reagent-grade purity.

**Strains.** The strains of *B. subtilis* 168 used in this study were BR151 (lys-3 trpC2 metB10) and RUB1402 (dal hisA1 leu-8 metB10).

**Growth conditions.** Cells were grown aerobically on a New Brunswick gyratory shaker at 240 rpm at 37°C in Spizizen minimal salts medium (10) supplemented with 22 mM glucose and 0.5% casein hydrolysate. Additional supplements included 0.5 mM p-alanine for RUB1402 and 40 μg of tryptophan per ml for BR151. Turbidity was determined with a Klett-Summerson colorimeter at 620 nm. The medium was inoculated from an overnight culture of cells grown in the same medium to give an initial turbidity reading of 10 Klett units.

**Determination of d-cycloserine resistance.** Cells whose d-cycloserine resistance was to be determined were used to inoculate a 10-ml culture to give a final concentration of about 4 × 10⁷ cells per ml. The volume of inoculating culture required was estimated from the Klett reading, with a Klett reading of 40 corresponding to 4 × 10⁷ cells per ml. The viable count was determined immediately after the inoculation of the culture (treated cells). d-Cycloserine was added to a final concentration of 0.5 mM, and the culture was grown aerobically at 37°C for 2
h, at which time the viable count was again determined (survivors). Cycloserine resistance was expressed as survivors per treated cell.

Preparation of cells for transport. In most cases, late-exponential-phase cells (about 150 Klett units) were harvested by filtration (Millipore Corp., 0.45-μm pore size, 45-mm diameter), washed with Spizizen minimal salts, and suspended in a volume of Spizizen minimal salts equal to three times the culture volume. This gave a final cell protein concentration (7) of about 100 μg/ml. Additions were made to this incubation mixture as indicated, and the cell suspension was incubated aerobically at 37°C for the time indicated before transport was measured.

Measurement of transport. Uptake was initiated by the addition of 2.0 ml of cell suspension to 0.5 ml of minimal salts containing the 14C-labeled compound to be transported plus any additions as indicated (uptake mixture). After incubation of the cells at 37°C for the indicated time, transport was terminated by membrane filtration (Millipore Corp., 0.45-μm pore size, 24-mm diameter). The filters were washed with 2 ml of Spizizen minimal salts, placed in scintillation vials, and dried overnight at 45°C; 5 ml of nonaqueous scintillation fluid was added, and the radioactivity was determined in a Beckman LS-230 liquid scintillation spectrometer. The filtration and washing procedures took about 5 s. The sensitivity of transport to inhibition by azide was determined with cells incubated for 30 min at 37°C in Spizizen minimal salts plus 10 mM sodium azide.

Analytical procedures. Protein was determined by the method of Lowry et al. (7) for trichloroacetic acid precipitates, with bovine serum albumin as a standard. Peptidoglycan biosynthesis was measured by incubating cells with 0.09 μmol of N-acetyl-D-1-[14C]glucosamine for 2 min at 37°C in complete growth medium. Peptidoglycan was isolated by the fractionation procedure of Park and Hancock (9), and the radioactivity was determined as discussed below.

Determination of radioactivity. The radioactivity of nonaqueous samples was determined with the use of a scintillation fluid consisting of 4 g of Omnifluor per liter of toluene.

RESULTS

Characterization of D-cycloserine resistance in B. subtilis. During the study of the effects of inhibition of cell wall biosynthesis by D-cycloserine in B. subtilis, we observed that, as a function of time and D-cycloserine concentration, cells were able to recover from inhibition and resume growth. Figure 1 shows the turbidities of cultures treated with various concentrations of D-cycloserine. There was a short time lag before the effect of the antibiotic was evident, which was followed by a decrease in turbidity due to lysis of cells (as determined by phase microscopy and viable count). The cultures were able to recover and resume growth, as a function of the initial concentration of D-cycloserine. This resumption of growth proceeded even if fresh D-cycloserine (0.5 mM) was added (data not shown).

A method of measuring D-cycloserine resistance was devised that was more convenient than monitoring turbidity (see Materials and Methods). The principle is the measurement of viable count before and 2 h after the addition of D-cycloserine. Resistance is then expressed as survivors per treated cell. Figure 2 compares the D-cycloserine resistance of cells not exposed to the antibiotic with that of cells grown in the presence of D-cycloserine for 90 min. At a concentration of D-cycloserine of 0.5 mM, there was almost a 1,000-fold difference in the survival rate of exposed cells.

The resistance to and the transport of D-cycloserine was measured as a function of growth (Fig. 3). Early-logarithmic-phase cells were highly sensitive to killing by the antibiotic, but there was an increase in resistance as growth progressed. The transport of D-cycloserine also
D-cycloserine for resistant cells.

Methods. Induction of D-cycloserine resistance. To demonstrate that D-cycloserine can induce D-cycloserine resistance, early-logarithmic-phase cells were suspended in Spizizen minimal salts medium containing 1 mM glucose, and D-cycloserine resistance was monitored (Fig. 5). After 5 min of incubation, 0.1 mM D-cycloserine was added to one of the two cultures. It can be seen that D-cycloserine resistance in the treated culture increased rapidly compared with the control culture. Under these conditions it is clear that D-cycloserine resistance can be induced by treatment of the cells with D-cycloserine.

Reversibility of D-cycloserine resistance. The acquisition of D-cycloserine resistance was reversible (Fig. 6). D-Cycloserine resistance was induced as described in Fig. 5 for 15 min, and then cells were resuspended in fresh complete growth medium with no antibiotic. The cells were kept in the early logarithmic phase of growth by repeated dilution into fresh medium. The D-cycloserine resistance was monitored as a function of time, and it can be seen that, after a short time lag, sensitivity to the antibiotic was regained.

D-Cycloserine inhibition of peptidoglycan. D-Cycloserine resistance could be attained by the formation of enzymes that would destroy or modify the antibiotic, by the alteration of the target site of the antibiotic such that it is no longer inhibited by the antibiotic, or by a decreased accumulation of the antibiotic. To de-

![Figure 2](http://aac.asm.org/Downloaded from)

![Figure 3](http://aac.asm.org/Downloaded from)
Fig. 4. Effect of D-cycloserine incubation on the transport of D-cycloserine. 0.1 mM D-cycloserine (●) or 0.1 mM D-cycloserine plus 5 μg of rifampin per ml (○) was added to growing BR151 cells at zero time, and the transport of D-cycloserine was measured in washed cell samples at the indicated times. The control uptake rate was that observed at zero time. Transport was measured at a D-[14C]cycloserine concentration of 0.1 mM.

Fig. 5. Induction of D-cycloserine resistance. Strain BR151 was suspended in Spizizen minimal salts plus 1 mM glucose. After 5 min of incubation, D-cycloserine was added to one portion of cells and the D-cycloserine resistance was determined. Symbols: (●) No addition; (○) plus 0.1 mM D-cycloserine.

Fig. 6. Recovery of D-cycloserine sensitivity. Strain BR151 was induced for D-cycloserine resistance by incubation in Spizizen minimal salts containing 1 mM glucose and 0.1 mM D-cycloserine for 10 min. The D-cycloserine was removed by membrane filtration (0.65-μm pore size), and cells were suspended in glucose-minimal salts growth medium. Samples were removed at 20-min intervals, and D-cycloserine resistance was determined as described in Materials and Methods. Cells were periodically diluted into fresh medium to maintain them in the early logarithmic phase of growth.

terminate whether the D-cycloserine is inactivated, the culture supernatant from resistant cells containing D-cycloserine was obtained by membrane filtration (0.45-μm pore size). When cells unexposed to the antibiotic were added to this culture medium, they were killed, indicating that resistant cells do not inactivate significant quantities of the D-cycloserine (data not shown). To determine whether the target site of the antibiotic is altered, peptidoglycan synthesis in the presence or absence of D-cycloserine was measured in uninduced and induced cells (Table 1). At the concentration of D-cycloserine used, peptidoglycan synthesis was inhibited by 98% in uninduced cells and by 95% in induced cells. The actual rate of synthesis in the presence of the antibiotic was lower in the induced cells than in the uninduced cells, in-
Table 1. Inhibition of peptidoglycan synthesis by \( \text{d-}
\)cycloserine in uninduced and induced cells of \( B. \) subtilis 168

| Incubation conditions | Assay conditions | Peptidoglycan synthesis | Inhibition of peptidoglycan synthesis (%) |
|-----------------------|------------------|-------------------------|------------------------------------------|
| No addition           | No addition      | 0.371                   | 98                                       |
|                       | + d-Cycloserine  | 0.0088                  |                                          |
| + d-Cycloserine        | No addition      | 0.113                   | 95                                       |
|                       | + d-Cycloserine  | 0.0051                  |                                          |

* A sample of cells was induced for \( \text{d-}
\)cycloserine resistance by incubation in 0.1 mM \( \text{d-}
\)cycloserine for 10 min as previously described. The \( \text{d-}
\)cycloserine was removed by membrane filtration (0.65-\( \mu \)m pore size). Peptidoglycan synthesis was assayed by incubation of uninduced or induced cells with 0.09 \( \mu \)mol of \( N \)-acetyl-\( \text{d-}[1.14C] \)glucosamine for 2 min at 37°C in complete growth medium containing 5 \( \mu \)g of rifampin per ml plus or minus 2 mM \( \text{d-} \)cycloserine. Peptidoglycan was purified by Park-Hancock fractionation. Peptidoglycan synthesis is expressed as nanomoles of \( N \)-acetylglucosamine incorporated per minute per milligram of protein.

indicating that the target site of \( \text{d-}
\)cycloserine is not more resistant to inhibition in resistant cells. The lower rate of synthesis in induced cells may have been due to residual \( \text{d-} \)cycloserine that was not removed during the washing procedure.

Decrease in \( \text{d-} \)cycloserine transport induced by various concentrations of \( \text{d-} \)cycloserine. To determine the effect of \( \text{d-} \)cycloserine on transport, cells were treated with various concentrations of \( \text{d-} \)cycloserine for 10 min, after which the rate of uptake of \( \text{d-} \)cycloserine was determined. The apparent mode of \( \text{d-} \)cycloserine resistance was a reduction of \( \text{d-} \)cycloserine transport (Fig. 7). The decrease in antibiotic transport was dependent on the incubation concentration of \( \text{d-} \)cycloserine. Concentrations of \( \text{d-} \)cycloserine that were subinhibitory (0.1 mM) induced a considerable loss of transport.

Exchange of \( \text{d-} \)cycloserine for intracellular glycine. It has been proposed (4) that tetracycline resistance is due to the synthesis of a protein that prevents the entry of the antibiotic into the cell. To determine whether \( \text{d-} \)cycloserine is able to enter \( \text{d-} \)cycloserine-resistant cells, we investigated the ability of the antibiotic to exchange for preloaded \( \text{[14C]} \)glycine (Fig. 8). Unexposed and induced cells were allowed to take up \( \text{[14C]} \)glycine for 8 min, at which time excess glycine or \( \text{d-} \)cycloserine was added. If \( \text{d-} \)cycloserine is excluded from the cell in induced cells, \( \text{d-} \)cycloserine should not be able to cause the loss of \( \text{[14C]} \)glycine from the cells. However, if it is not excluded in induced cells, the antibiotic should be effective in causing the loss of intracellular \( \text{[14C]} \)glycine. As can be seen, the latter alternative occurred. It therefore appears that the reduction in \( \text{d-} \)cycloserine accumula-
tation may not be due to an exclusion of the antibiotic from the cell.

**DISCUSSION**

In this study we have described a type of D-cycloserine resistance that is more similar to tetracycline resistance (4) than it is to previously described types of D-cycloserine resistance (12, 13). In this case D-cycloserine resistance is inducible by subinhibitory concentrations of the antibiotic, and sensitivity to D-cycloserine can be regained by allowing the cells to grow in the absence of the antibiotic. This recovery of sensitivity to D-cycloserine is not observed in *E. coli* cells resistant to the antibiotic (13), since resistance is due to a mutation(s) in the gene(s) responsible for transport of D-cycloserine, D-alanine, and glycine.

The apparent mode of the inducible resistance is a marked decrease in the accumulation of D-cycloserine. The antibiotic probably is not chemically altered, since the culture supernatant from cells that have become resistant is still able to kill sensitive cells. The target of the antibiotic, cell wall biosynthesis, is not less sensitive to inhibition by D-cycloserine. Cells induced for D-cycloserine transport rapidly lose the ability to accumulate the antibiotic, and the loss of D-cycloserine transport is dependent upon the concentration of antibiotic used for induction.

Although it is known that tetracycline-resistant cells have decreased rates of accumulation of the antibiotic, it is not established whether this is due to an exclusion of uptake or to an increased rate of efflux (4). This alternative can be investigated for D-cycloserine transport, taking advantage of the fact that the system responsible for the transport of D-cycloserine also transports D-alanine, L-alanine, and glycine (2, 5). If D-cycloserine uptake were decreased, the antibiotic would enter the cell at a slower rate and would be less efficient in exchanging for preloaded (14C)glycine. If efflux is increased, then the D-cycloserine will cause the loss of preloaded (14C)glycine from the cell. The latter case was found to occur, indicating that the decreased accumulation of D-cycloserine may be caused by an active efflux of the antibiotic from the cell.

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