Inhibition of microbial growth by interference with siderophore biosynthesis. Oxidation of primary amino groups in aerobactin synthesis by *Escherichia coli*.

(Oxidation of lysine; aerobactin; iron supply; *E. coli*)

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1. SUMMARY

The first step of aerobactin biosynthesis, oxidation of an aliphatic primary amino group to an N-hydroxy-amino compound seems to be involved in the biosynthesis of most of the hydroxamate-type siderophores which are widely distributed among bacteria and fungi. Therefore, the first step of aerobactin biosynthesis, oxidation of lysine to N6-hydroxylysine was studied as a model reaction using a strain of *Escherichia coli* that contains the first gene *aerA* of aerobactin synthesis on a multicopy plasmid and which is lacking the gene for the subsequent step in the pathway. In addition, culture conditions are described which lead to the secretion of N6-hydroxylysine into the medium in amounts that can easily be quantitatively determined by a simple, reliable chemical assay. This assay can be used for screening inhibitors of the oxidation of α-amino groups, which should interfere with the biosynthesis of siderophore hydroxamates and thus should create bacteriostatic conditions.

2. INTRODUCTION

Siderophores are synthesized by bacteria and fungi and supply microorganisms with iron. They are secreted into the culture medium, bind Fe$^{3+}$ with extremely high affinity, and then the iron compounds are transported into the cells. Siderophores bearing hydroxamate groups are most commonly found. Although their structures differ widely they contain oxidized aliphatic α-amino groups which are acylated [1]. An example is aerobactin, which consists of two residues of 6(N-acetyl-N-hydroxy)lysine linked to the terminal carboxyl groups of citrate [2]. We have elucidated the biosynthesis of aerobactin determined by the plasmid ColV-K311 which was isolated from a human strain of *E. coli*. The genes termed *aerA, aerB, aerD* and *aerC* specified in this order functions for the oxidation of lysine, acetylation of 6-hydroxyaminolysine and the stepwise addition of two residues of N6-acetyl-N6-hydroxylysine to citrate [3,4].

Here we report on the oxidation of lysine. The oxidation reaction is the most difficult to study since cell-free fractions contain very low, if any, activity [5]. Therefore, we used strains that con-
tained the oxidase gene aerA on a multicopy vector, and which, in addition, were lacking the aerB gene that determines the acetylation of N^ε-hydroxylysine. By this means, N^ε-hydroxylysine was secreted from the cells into the culture medium in amounts that were easily detectable by a simple quantitative chemical assay.

3. MATERIALS AND METHODS

3.1. Strains and culture conditions
The following strains of E. coli K-12 were used: EN39 is a derivative of H1443 arOB rpsL lac araD that contains the plasmid ColV-K311 with a Tn10 transposon insertion and which contains a mutation in the aerB gene [3]; GR111 is a derivative of H1443 that contains the aer operon on pBR322 with a deletion of the aerB and aerC genes (pGRG111) [3]; EN222 is a lys mutant of E. coli K-12 that was transformed with pRG111. ZI346, ZI349, ZI350 contain MudI(Ap lac) operon fusions in the aerobactin (aer) gene region [6]. The strains were grown in M56 salt solution that consisted of 13.6 g KH2PO4, 2 g (NH4)2SO4, 0.3 g MgSO4 · 6 H2O, 0.01 g CaCl2 per liter, adjusted to pH 7.4 with KOH. For growing cells 100 mg shikimic acid and 4 g glucose per liter were added. Either ampicillin (50 μg/ml) or tetracycline (15 μg/ml) were added to maintain the plasmids.

3.2. Determination of N-hydroxylysine formation
Strains to be tested were grown in 400 ml M56 medium. Samples of 40 ml were withdrawn, centrifuged and resuspended in 10 ml M56 salt solution. To 5 ml of the cell suspension 1 ml 10 mM lysine, or 1 ml M56 salt solution was added as control. The suspension was shaken for 45 min at 37°C, centrifuged and the supernatant taken for the determination of N-hydroxylysine. One ml was incubated with 0.5 ml of 1% sulfanilic acid in 25% acetic acid and 0.2 ml 1.3% iodine in glacial acetic acid for 5 min at room temperature. Then, excess iodine was destroyed by addition of 0.2 ml 0.2 M sodium thiosulfate. The addition of 0.2 ml of 0.6% α-naphthylamine in 30% acetic acid yielded a red color that was measured spectrophotometrically at 520 nm after incubation for 30 min [3,7]. Samples (2 ml) to be tested were also taken from a growing culture, centrifuged and then N-hydroxylysine was determined directly in the culture supernatant.

Formation of N-hydroxylysine in microtiter plates was performed with logarithmically growing cells in 100 ml M56 salt solution supplemented with 0.4% glucose, 0.5 mM l-lysine and 50 μM ampicillin. Cells were harvested at an absorbance of 1.3 at 578 nm, washed once with M56 salt solution, and then suspended in 20 ml M56 salt solution containing 0.5 mM lysine, 100, 400 and 800 μl of the cell suspension were added to microtiter plates containing 96, 48 and 24 wells, respectively. The plates were shaken for 60 min at 37°C in a water bath. Then, 50 μl sulfanilic acid solution, 20 μl iodide solution, 20 μl thiosulfate and 20 μl naphthylamine, as described above, were added to the 96-well plate, and 4 and 8 times the volumes to the plates with 48 and 24 wells, respectively.

3.3. β-Galactosidase assay
Cells with the MudI(Ap lac) operon fusions were grown in the M56 medium supplemented with 15 μg/ml tetracycline at 27°C. In parallel cultures, containing in addition 10 mg FeCl3 per liter, no β-galactosidase was formed. Samples of 0.5 ml were withdrawn from logarithmically growing cells at 90-min time intervals. β-Galactosidase was determined as described previously [6,8].

4. RESULTS

4.1. N-oxidation of lysine
To determine N-oxidation of lysine during the growth of cells, strain EN39 pColV with a defective aerB gene was cultivated in the iron-limited medium M56. Although the cells had been pregrown overnight in the same medium, N-hydroxylsine was only detectable in the culture supernatant at the end of the logarithmic growth phase (Fig. 1). When the cells were grown for a certain time, harvested, resuspended in M56 salts with lysine without addition of glucose and shikimate acid and then incubated at 37°C, the amount of hydroxlysine formed during 45 min rose to a certain concentration and decreased again at the stationary growth phase (Fig. 1). The same result
was obtained with the strain GR111 which contained a fragment of the aer region of the ColV plasmid comprising the aerA and aerD genes cloned on pBR322 (Fig. 2). The amount of N-hydroxylysine determined by the multicopy pBR322 derivative was about 3 times higher than the amount specified by the singlecopy ColV plasmid. These data demonstrate that synthesis of N-hydroxylysine followed the frequently observed time course of the formation of secondary metabolites, although aerobactin is of primary importance for the iron supply.

To see whether the time course observed reflected synthesis of the lysine oxidase, we measured transcription of Mudl(Aplac) insertions at different sites of the aer operon [6]. They were not precisely mapped with regard to the various biosynthetic genes. But regardless of where they were located, all three strains tested, ZI346, ZI349, ZI350, exhibited exactly the same time course of fl-galactosidase activity as shown in Figs. 1 and 2 for the N-oxidation activity (not shown). As described previously, fl-galactosidase was only synthesized under iron-limiting conditions [6,8].

The dependence of the amount of N-hydroxylysine formed on the available lysine concentration was tested with strain GR111 grown to the late logarithmic growth phase. Cells (10⁹ per ml) were incubated for 180 min in M56 salt solution supplemented with various concentrations of L-lysine. Under these conditions the concentration of N-hydroxylysine in the medium increased with increasing concentrations of lysine up to 75 μmol per liter (Fig. 3). A linear dependence on the concentration of cells in the assay was observed (Fig. 4). In the cultures to which 10 and 25 μM lysine were added, 75% was converted to N-hydroxylysine.

The above results demonstrate the reliability of the lysine oxidation test. To control the assay conditions more precisely we transformed a lysine auxotrophic E. coli K-12 strain with the plasmid pRG111. The formation of N-hydroxylysine was now dependent on the amount of lysine added to the medium. The result obtained with strain EN222 (not shown) coincided with those shown in Fig. 3. A linear dependence on the concentration of cells in the assay was observed (Fig. 4). In the cultures to which 10 and 25 μM lysine were added, 75% was converted to N-hydroxylysine.

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could be oxidized. The assay using the untransformed lysine auxotroph lacking pRG111 was completely colorless.

To see whether the conditions described could be used to screen for inhibitors of the N-oxidation reaction, the experiments with strain EN222 pRG111 were repeated in microtiter plates containing 24, 48 and 96 wells. After 60 min of slight shaking in M56 salt solution supplemented with 0.5 mM lysine, the Tomlinson test [7] was performed. The red color of the azo-dye could easily be seen, even in the smallest well. Spectrophotometric measurements of a sample taken from the microtiter plate with 24 wells yielded an absorbance of 0.84, whereas the control well without added lysine gave an absorbance of 0.23.

5. DISCUSSION

The simple determination of N-hydroxylysine relies on the following measurements: (a) usage of a strain that contains the aerA gene which encodes the oxidase on a multicopy plasmid; (b) inhibition of further metabolism of N-hydroxylysine by deleting the acetylase gene aerB; (c) addition of lysine to a lysine auxotrophic strain which allows definition of the lysine supply for the oxidation reaction; (d) growth of cells in the iron-limiting M56 medium that derepresses transcription of the aer operon; (e) performance of the oxidation reaction in a salt solution with the oxidase formed during the growth phase. The latter point is important for screening inhibitors that interfere with the oxidation, since inhibitors of the cell metabolism, for example of protein biosynthesis, should be without major effect.

Hydroxamates are important for the iron supply under natural conditions, as a number of reports clearly demonstrate [9–11]. Inhibition of their biosynthesis by inhibitors that inactivate the oxidase seems worth being tried under two premises. First,
the oxidases of the various microbes contain similar active centers, since they all transfer oxygen to aliphatic α-amine groups. Second, such a reaction does not occur in the human and animal hosts to avoid toxic side effects. This seems to hold true, since, to the best of our knowledge, oxidation of secondary and aromatic amines but not of aliphatic amino groups have been described in man. Lack of permeation of inhibitors into cells could be overcome by using a cell-free system, for example as described by Jackson et al. [12]. The development of an in vitro assay should be facilitated by using our strains which overproduce the oxidase.

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REFERENCES