Biochemical Characterization of Lysine Auxotrophs of *Staphylococcus aureus*¹

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Lysine biosynthesis in *Staphylococcus aureus* has been studied by use of a series of lysine auxotrophs. The strains were isolated after chemical mutagenesis. The majority of these mutant strains were classified according to the enzymatic step found to be deficient. Specific enzyme assays as well as nutritional tests were used to group the organisms. The enzymes included were dihydrodipicolinate synthetase, dihydrodipicolinate reductase, diaminopimelate epimerase, and diaminopimelate decarboxylase. The accumulation of diaminopimelate in certain mutants and the demonstration of dihydrodipicolinate synthetase and reductase provide the first detailed evidence that *S. aureus* utilizes the diaminopimelate pathway for lysine biosynthesis. A cell-free system was used to study the regulation of these enzymes with the exception of diaminopimelate epimerase. Lysine repressed all of the enzymes tested. The repression appeared to be coordinate in nature. The data presented provide suggestive evidence that the lysine biosynthetic region in *S. aureus* constitutes an operon.

Microorganisms synthesize lysine by two distinctly different biochemical pathways. Yeasts and fungi utilize a pathway in which α -aminoadipic acid is an intermediate (15). Bacteria and algae form lysine in a series of reactions terminating in the decarboxylation of *meso*-diaminopimelic acid (DAP). Part of the carbon skeleton is derived from aspartic acid after reduction to aspartic- β -semialdehyde (20). The pathway from aspartic semialdehyde to lysine as found in *Escherichia coli* (2, 7, 11, 16, 25) is given in Fig. 1. Work and her associates have detected the presence of DAP epimerase and DAP decarboxylase (2) in cells of *Staphylococcus aureus*. The activity was found to be low and dependent upon the composition of the medium.

In the present study, lysine auxotrophs have been used to elucidate the lysine biosynthetic pathway in these organisms. The ability of DAP to alleviate the lysine requirement and the detection of DAP within mutant cells is of interest, since Antia et al. (2) were unable to detect this amino acid in the Oxford strain of *S. aureus*. The enzymes involved in lysine biosynthesis beyond

aspartic semialdehyde and their regulation have been investigated in detail.

MATERIALS AND METHODS

Bacterial strains. The parental lysine prototroph was *S. aureus* H.H. 9196 from the Hahnemann Medical College stock culture collection. Lysine auxotrophs were isolated after exposure to diethyl sulfate (Fisher Scientific Co., King of Prussia, Pa.) by the procedure of Ronen (18) or after exposure to *N*-nitro-*N*-methyl-*N'*-nitrosoguanidine (K & K Laboratories, Plainview, N.Y.) by the method of Adelberg et al. (1). The mutagen-treated cells were suitably diluted and surface-plated on Trypticase Soy Agar (BBL). Lysine auxotrophs were selected by the replica-plating technique (13). The mutant stock cultures were maintained on Brain Heart Infusion (BHI) Agar slants (Difco), which were stored at 4 C and transferred at monthly intervals. All incubation periods were at 37 C. Prior to use, mutants were phage-typed by use of the typing procedure recommended by Blair and Williams (4).

Media and growth studies. The defined medium (DM) utilized was that of Bondi et al. (5) supplemented as required with lysine or DAP. When a solid medium was needed, Noble agar (Difco) was added to a concentration of 2%.

In growth studies, the inoculation procedure of Bondi et al. (5) was followed. Each tube of 10 ml of DM received 70,000 organisms per ml. Growth was followed turbidimetrically with a Coleman Junior colorimeter at 525 nm.

Accumulation studies. A flask containing 50 ml of

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DM with 14.5 μM lysine was inoculated with 1 ml of organisms grown in DM containing 145 μM lysine. After 72 hr of incubation with shaking, the cells were removed, washed with water, and resuspended in 10 ml of water. This cell suspension was boiled for 10 min and centrifuged. The extract was evaporated to dryness and redissolved in 0.2 ml of water. The DAP present was detected by cellulose thin-layer chromatography (TLC). Each plate (20 cm square) was poured by use of a slurry containing 3 g of cellulose for TLC (Schleicher & Schuell Co., Keene, N.H.) and 18 ml of distilled water. After drying to a matte finish at room temperature, plates were heated at 100 C for 10 min. A series of 10 μliter samples were spotted along a line 2 cm from the origin. The plates were developed in a small chamber containing a methanol-pyridine-HCl-water solvent (17). The solvent was allowed to ascend for 15 cm beyond the spots. The plates were sprayed with 0.2% ninhydrin in acetone and heated at 100 C for 5 min. DAP gives a characteristic green color with ninhydrin in the presence of pyridine and HCl.

Enzyme studies. Cells from a 12-hr BHI slant were washed into 1 ml of saline and used to inoculate DM containing 145 μM lysine in the proportion of 0.2 ml of cells to 40 ml of DM. After 12 hr of incubation with shaking, the cells were removed by centrifugation and washed. The cells were added to a volume of DM equal to that of the first culture but with a concentration of lysine ranging from 0 to 290 μM . The cultures were shaken for 4 hr prior to use in the whole-cell system and for 6 hr prior to the preparation of cell-free systems. The whole-cell suspensions consisted of washed cells from 40 ml of DM suspended in a volume of 2 ml. Cell-free systems were prepared with washed cells harvested from 200 ml of DM resuspended to a volume of 5 ml in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer containing 0.145 M NaCl at pH 7.5. The lysostaphin lytic procedure of Shaw and Brodsky (19) was followed. The lysostaphin was supplied by P. Tavormina (Mead Johnson, Evansville, Ind.). The protein present was determined by the procedure of Lowry et al. (14).

The 270-nm absorption assay of Yugari and Gilvarg (25) was used for the detection of dihydrodipicolinic acid (DHP) synthetase. The buffer used was 1 M imidazole at pH 7.4. The DL-aspartic- β -semialdehyde was synthesized by the procedure of Black and Wright (3) and was generously supplied by A. A. Yousten. DHP reductase activity was measured by the production of a colored adduct with *o*-aminobenzaldehyde (K & K Laboratories). The assay procedure is that of Farkas and Gilvarg (7) modified by the use of 1 M Tris-hydrochloride buffer at pH 7.4.

The assay for DAP decarboxylase activity was that of Work (24). The *meso*-DAP used was isolated as described below. The disappearance of DAP was measured by Work's acidic ninhydrin test for use in the presence of lysine (22). The assays described were used in cell-free systems. For use with a whole-cell suspension, the volumes of all the reagents were tripled. To these were added 0.5 ml of cells, 0.5 mg of

cetyltrimethylammonium bromide in a volume of 0.05 ml, and water to a final volume of 3 ml.

The test for DAP epimerase, a qualitative test, was that described by Work (23). The final reaction mixture was assayed by use of descending paper chromatography and Rhuland's solvent (17).

Isolation of DAP isomers. The defined medium and growth conditions of Knox et al. (12) were used to obtain DAP accumulation by *E. coli* ATCC 12408. When a plateau of DAP accumulation was reached, the spent medium was clarified by centrifugation and the procedures of Gilvarg (9) were followed to isolate and separate the *L* and *meso* isomers.

Designation of mutants. The numbering system used was suggested by the Subcommittee on Bacteria and Bacteriophage of the Committee on Maintenance of Genetic Stocks of the Genetics Society of America. Each number was prefaced with AB-HH to indicate the laboratory of origin. The parental strain was renumbered as AB-HH 69:lys⁺. The lysine mutants of *S. aureus* were grouped on the basis of nutritional and biochemical studies (see Results). Reference to Fig. 1 shows that lysA mutants lacked DHP synthetase, lysB strains were deficient in DHP reductase, lysF mutants were deficient in DAP epimerase, lysG strains lacked DAP decarboxylase activity, and lysO is a mutant strain which lacks activity of all the enzymes tested. Those mutants which are not designated by a letter possess activity of all enzymes tested but require lysine for growth.

RESULTS

Isolation and characterization of lysine auxotrophs. A total of 63 lysine auxotrophs were isolated. These were shown to have originated from the parental strain by means of phage-typing. As expected, the growth of the strains in DM was proportional to the amount of lysine added. None of the mutants used in the study grew in the absence of lysine.

Lysine-requiring mutants were tested to see whether compounds other than lysine would satisfy the growth requirements. Table 1 shows that DM supplemented with 145 μM DAP will permit a low level of growth of 19 mutants. DM supplemented with DAP and 2 μg of pyridoxal HCl per ml was tested to see whether the inability to utilize DAP was due to a lack of pyridoxal within the cell. To rule out the presence of tricarboxylic acid cycle mutants resulting in amino acid requirements (21), the strains were tested in DM lacking methionine, supplemented with 10 μM sodium acetate, 10 μM sodium citrate, 1 μM lipoic acid, or 145 μM lysine, or unsupplemented. In no instance did the strains tested grow in the absence of lysine. The omission of methionine did not appreciably change the amount of growth.

Accumulation of DAP was detected by TLC of cell extracts. These data are also presented in

FIG. 1. Structures and enzymes involved in the synthesis of lysine by bacteria. The enzymes are represented as follows: (1) dihydropyridine synthetase, (2) dihydropyridine reductase, (3) tetrahydropyridine acylase, (4) *N*-succinyl-diaminopimelic acid-glutamic acid transaminase, (5) succinyl-diaminopimelate deacylase, (6) diaminopimelate epimerase, (7) diaminopimelate decarboxylase.

Table 1. It is of interest to note that all but one of the organisms which accumulate DAP obligately require lysine. This indicates that the mutational block is between DAP and lysine. The single exception, AB-HH 21:lysF, is able to utilize DAP for growth as well as to accumulate this amino acid. Of the 35 DAP nonaccumulating organisms, 18 were able to utilize DAP to satisfy the growth requirement. This indicates that the block is probably prior to the utilization of DAP. The nature of the lesion of the remaining 17 organisms cannot be determined from the nutritional studies.

Biochemical classification of lysine mutants. The auxotrophs were grouped after the assay of whole-cell suspensions for the various enzymes involved in the synthesis of lysine. The average enzyme activities for each mutant group are given in Table 1. With the exception of AB-HH 17:lysO and AB-HH 21:lysF, all organisms tested had DAP epimerase activity. The strains which were deficient in DAP decarboxylase accumulated primarily *meso*-DAP, indicating that they were decarboxylase mutants. Paper chromatography demonstrated that the compound accumulated during the growth of AB-HH 21:lysF was L-DAP, a finding consistent with the strain's being an epimerase mutant.

The auxotrophs which possessed both DAP epimerase and DAP decarboxylase activity and selected lysG mutants were assayed for DHP synthetase and reductase activity. It can be seen that six of the organisms tested lack DHP synthetase activity, and an additional five have a deficient DHP reductase. AB-HH 17:lysO lacks synthetase activity. Reductase activity in the synthetase mutants could not be determined owing to lack of substrate. The remaining strains are probably blocked somewhere between tetrahydropyridine acid and L-DAP, but these enzymes were not examined.

Control of lysine biosynthesis. With the use of lysostaphin, cell-free extracts could be prepared quite readily, making it possible to examine the regulation of this system. Cell-free preparations from cells shaken in DM without lysine for 6 hr were assayed for DHP synthetase and DHP reductase. Lysine was incorporated into the reaction mixture to detect end-product inhibition of these enzymes (Table 2). Neither of the strains showed inhibition of activity; in fact, greater activity was found in the presence of lysine.

An experiment was designed to show repression of this system. Cells were grown in the usual manner and derepressed in various amounts of lysine. The variation of DHP synthetase with

TABLE 1. *Classification of lysine auxotrophs*

Mutant group	Strain AB-HH	Growth ^a on DAP	DAP accumulation	Avg enzyme activity			
				DAP ^b de-carboxylase	DAP epimerase	DHP ^c synthetase	DHP ^d reductase
Lys A	11, 15, 24, 35, 61 12	0 0.05	—	0.0061	+	0	NT ^e
Lys B	29, 33, 46 5, 20	0 0.12	—	0.0076	+	0.0028	0
LysF	21	0.13	+	0.0093	0	ND ^f	ND
LysG	2, 7, 8, 9, 13, 16, 18, 22, 23, 25, 26, 27, 28, 31, 32, 37, 38, 39, 40, 43, 47, 51, 54, 57, 58, 63	0	+	0	+	0.0029	0.081
LysO	17	0	—	0	0	0	NT
Lys	6, 10, 19, 42, 44, 45, 53, 56, 62 1, 3, 4, 11, 14, 30, 34, 36, 41, 48, 49, 50, 52, 55, 59, 60	0 0.09	—	0.0065	+	0.0102	0.081
Lys ⁺	69	0.30	—	0.0056	+	0.0045	0.099

^a Growth in DM with 145 μ M DAP measured at 72 hr and given as optical density at 525 nm.^b Activity is given as micromoles of DAP destroyed per milligram (dry weight) of cells per minute.^c Activity in terms of change of A_{270} per milligram (dry weight) of cells per minute.^d Activity in terms of change of A_{440} per milligram (dry weight) of cells.^e NT = not tested due to unavailability of substrate.^f ND = not determined.TABLE 2. *Effect of lysine on enzyme activity*

AB-HH	Lysine	Synthetase ^a	Reductase ^b
	<i>mM</i>		
21:lysF	0	0.018	0.219
	1	0.027	0.400
69:lys ⁺	0	0.0142	0.158
	1	0.0183	0.200
	10	0.0167	—

^a Activity given as change in A_{270} per milligram of protein per minute.^b Activity given as change in A_{440} per milligram of protein.

increase in lysine is shown in Fig. 2. Two things are immediately obvious. The first is the increase in the activity at low concentrations of lysine. This elevation of activity was found in both the wild type and mutants. In addition, lysine repression of DHP synthetase activity can be shown by the decrease in enzyme activity at higher lysine concentrations.

Similar assays were done for DHP reductase

and DAP decarboxylase. Figure 3 presents a comparison of the per cent activity for each of the three enzymes within a given strain. The per cent activity represents the activity of cells held at a given lysine concentration compared with that when cells were held in the absence of lysine.

FIG. 2. *Effect of lysine on the formation of dihydrodipicolinate synthetase in S. aureus.*

FIG. 3. Comparison of the repression of DHP synthetase, DHP reductase, and DAP decarboxylase of the parent and selected mutants. Symbols: ▲, decarboxylase; ●, synthetase; ■, reductase.

DISCUSSION

Although the enzymes DAP decarboxylase and DAP epimerase were previously detected in *S. aureus* (3), DAP could not be detected either in the whole cells (2) or in cell wall preparations (6). The inability to detect DAP raised the question of the role of this amino acid in lysine biosynthesis by *S. aureus*. The observation that DAP permits growth of some lysine auxotrophs in the absence of lysine and that DAP accumulates within other mutants indicates that DAP plays an important role in the synthesis of lysine by *S. aureus*.

Demonstration of DHP synthetase, DHP reductase, DAP epimerase, and DAP decarboxylase activity in *S. aureus* indicates that this organism follows a similar if not identical pathway to that observed in *E. coli*. Groups of mutants, each deficient in one of the above mentioned enzymes, have been isolated. Two additional groups were also isolated. The first is represented by a single strain, AB-HH 17:lysO. This strain lacks activity in the three enzymes tested; yet the addition of lysine alone to the DM permits growth. This organism has arisen from either an extremely polar mutation in the area of the lysine locus near the operator or a lesion of the O^o class. According to Jacob et al. (10), this class of operator mutant is similar to a polar mutant but can synthesize small quantities of each enzyme, possibly too small to be detected by our assay procedures. The characterization of the mutants within the last group of lysine requirers poses a problem. These organisms possess all of the enzymes included in this survey but still require lysine for growth.

The addition of succinate, acetate, or lipoate to the medium does not permit growth, eliminating the possibility that the strains are the type of tricarboxylic acid mutants described by Stouthamer in *Aerobacter aerogenes* (21). Thus, one can speculate that one of the three enzymes not included in this study is mutant. Of the 22 mutants in this group, 14 are able to utilize either DAP or lysine for growth. The remainder, e.g., AB-HH 6:lys, are unable to utilize DAP for growth although they have the enzymatic potential to do so. It is possible that, in addition to a lesion in the lysine biosynthetic pathway, a second lesion affecting a DAP permease is present.

The recent report by Shaw and Brodsky (19), which dealt with the preparation of cell-free extracts by the action of lysostaphin, provided a rapid, gentle procedure which could be used with a relatively small number of cells. In our hands, there has been no loss of activity associated with cell breakage. The cell-free extracts were used to study the regulation of the lysine pathway.

In his review, Stadtman (20) stated that the DHP synthetase and DAP decarboxylase in *E. coli* were repressed by lysine. In addition, DHP synthetase was sensitive to end-product inhibition by lysine. Gilboe et al. (8), in their study of *Streptococcus faecalis*, were unable to show any evidence of end-product inhibition of DHP synthetase. They were able to demonstrate repression of DHP synthetase and DAP decarboxylase. In the present study, no evidence of end-product inhibition is shown, even when 10 mM lysine was incorporated into the reaction mixture. It is noted that the addi-

tion of lysine to the assay mixture slightly increased the enzyme activity.

DHP synthetase, DHP reductase, and DAP decarboxylase have been shown to be repressed by lysine in *S. aureus*. At higher levels of lysine, a plateau of activity is reached with 60 to 50% of the original activity remaining. As shown in Fig. 3, the activity curves for the three enzymes are essentially parallel for any given strain. It appears that these enzymes are coordinately repressed, indicating that they are under the control of a single operator. This assumption is strengthened by the isolation of AB-HH 17:lysO in which the loss of several enzymes occurred after treatment with diethyl sulfate, a mutagen inducing point mutations.

This evidence is consistent with the concept of an operon (10) in which a specific operator locus adjacent to a group of genes controls the activity of the group and is sensitive to repression, thereby decreasing the activity of the genes under its control. The biochemical evidence presented supports the hypothesis that there is a lysine operon in *S. aureus*. A weakness in the argument is the failure to show that the lysine biosynthetic genes occupy adjacent sites on the chromosome. Preliminary transduction studies (in progress) with standard staphylococcal typing phage 53 have shown a higher transduction frequency for any wild-mutant cross than for any mutant-mutant combination tested (12 tested). This indicates that the lysine loci are closely linked and represent a gene cluster. Thus, it is thought that lysine biosynthesis in *S. aureus* from the aspartic- β -semialdehyde branch point exists as an operon.

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