Diaminopimelic Acid and Lysine Auxotrophs of *Pseudomonas aeruginosa* 8602

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SUMMARY

Lysine auxotrophs have been isolated from *Pseudomonas aeruginosa* 8602. Three of the mutants were deficient in diaminopimelate decarboxylase and accumulated *meso*- and LL-diaminopimelic acid (DAP) but otherwise were indistinguishable from the parent strain. The fourth mutant required lysine for optimal growth, grew slowly on *meso*- but not LL-DAP, and the DAP which accumulated in large amounts was solely the LL-isomer. This mutant was deficient in diaminopimelate epimerase. No significant differences were detected between its wall composition and that of the parent strain but it was particularly sensitive to carbenicillin.

INTRODUCTION

The role of diaminopimelic acid (DAP) as a key intermediate in the biosynthesis of lysine in bacteria is now well established (see review by Work, 1960). It has also been shown to be present in the mucopeptide component of a number of Gram-positive bacteria and in all the Gram-negative bacteria so far examined (Salton, 1964). Whereas the other dibasic amino acids found in the mucopeptide of Gram-positive bacteria have been shown to exist in the L-configuration, DAP has been found as both LL- and *meso*-isomers (Hoare & Work, 1957), although the latter is the most widely distributed. In Gram-negative bacteria, where the mucopeptide makes up a much smaller proportion of the wall, the DAP-isomer present has been thoroughly investigated in only a few species. The *meso*-isomer has been identified chromatographically in all the Gram-negative bacteria studied and firmly identified in *Aerobacter cloacae* (Anwar, Roy & Watson, 1963) and in *Escherichia coli* (Diringer & Jusic, 1966). There have been no reports of the occurrence of either the LL- or DD-isomers in walls of Gram-negative bacteria. An opportunity to study the role of DAP in pseudomonads arose from the isolation of lysine auxotrophs, one of which was shown to be deficient in diaminopimelate epimerase (EC 5.1.1.7). This paper reports their growth requirements, wall composition and response to antibiotics known to interfere with wall synthesis.

METHODS

Organisms and media. The parent strain was *Pseudomonas aeruginosa* 8602 (Brammar, McFarlane & Clarke, 1966). It was maintained on slopes containing nutrient broth (Oxoid code no. CM 67; Oxo, London; 25 g. and 10 g. agar/l.). This medium without agar is designated complex medium. The minimal medium contained (g./l.): K₂HPO₄, 12.5; KH₂PO₄, 3.8; (NH₄)₂SO₄, 1.0; MgSO₄·7H₂O, 0.1; carbon source, 10; trace element solution, 5 ml. (Kelly & Clarke, 1962). Succinate was normally used as carbon source but under

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some restrictive conditions this induces the lytic phase of the lysogenic phage and it was then replaced by glucose or citrate. L-Lysine (1 mM) was added for growth of the mutants unless otherwise stated. Dilution buffer contained (g./l.): KH_2PO_4 , 3; Na_2HPO_4 , 7; NaCl, 4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. Media were sterilized by autoclaving (121° , 15 min.). All liquid cultures were shaken at 37° .

Isolation of mutants. Lysine auxotrophs were isolated by penicillin enrichment after exposure of the parent strain to a mutagen as follows. The bacteria were grown overnight in 5 ml. of complex medium. They were harvested and resuspended in 4 ml. citrate buffer (0.1 M, pH 6.0). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (0.1 mg./ml. of citrate buffer) was added and after 40 min. at room temperature the bacteria were harvested and resuspended in 5 ml. of dilution buffer. This suspension (0.25 ml.) was used to inoculate 5 ml. of minimal medium + succinate and incubated for 2 h. Carbenicillin (1 mg.) was then added and incubation continued for 90 min. The bacteria were harvested, resuspended in 5 ml. of dilution buffer and 0.1 ml. added to 5 ml. of minimal medium + succinate + lysine before incubating overnight. The bacteria were spread on agar plates of minimal medium + succinate + lysine (2 $\mu\text{g./ml.}$). After incubation for 48 h. pin-point colonies were picked and streaked on plates of minimal medium + succinate with and without lysine to test their lysine requirement.

Growth and inhibition tests. Bacteria from an overnight culture in complex medium were harvested, washed with water, resuspended in water at the same volume and used to inoculate the test cultures (0.01 ml. per 2 ml. in test tubes with metal caps). Growth was measured in terms of the extinction at 670 nm.

Minimum inhibitory concentrations of antibiotics (MIC) were determined after 18 h. growth in minimal medium + succinate + lysine to which sterile dilutions of the antibiotics had been added.

To study the effects of antibiotics during growth, antibiotics (in 1 ml. water) were added to cultures in complex medium (100 ml./l. flask) which had been inoculated with an overnight subculture (10 ml. complex medium).

Enzyme assays. The products of diaminopimelate decarboxylase (EC 4.1.1.20) and diaminopimelate epimerase activity were detected chromatographically (Work, 1963) in the bacteria after 18 h. growth in minimal medium + succinate + lysine. Quantitative assays were carried out by Dr P. J. White. DAP decarboxylase was measured by the evolution of carbon dioxide from *meso*-DAP in the Warburg apparatus at 37° in an atmosphere of nitrogen (White, Kelly, Suffling & Work, 1964). DAP epimerase activity was measured manometrically by following the evolution of carbon dioxide from LL-DAP in the presence of excess CAP decarboxylase. The activity of each enzyme was expressed in manometric units (White, Lejeune & Work, 1969).

Accumulation of DAP and ultraviolet-absorbing material. After 18 h. growth in minimal medium + succinate + lysine (40 $\mu\text{g./ml.}$) the cultures were harvested and cell-free extracts prepared in a French pressure cell (12,000 lb/in.²). The amounts (Work, 1957) and the isomers of DAP (Hoare & Work, 1955) were determined in the extracts and in the culture-filtrates concentrated 100-fold by rotary evaporation. Extinction of the concentrated culture-filtrate was measured at 260 nm, and expressed in terms of UTP.

Preparation and extraction of walls. After overnight growth in complex medium (1 l./5 l. flask) the bacteria were harvested and washed with saline-citrate solution pH 7 (0.15 M-NaCl + 0.15 M-trisodium citrate). They were resuspended in 20 ml. saline-citrate and broken in a French pressure cell (12,000 lb/in.²). The broken suspension was diluted tenfold with saline-citrate before centrifuging at 3000 *g* for 10 min. to remove unbroken bacteria. The supernatant fluid was centrifuged at 14,000 *g* for 20 min. to collect the walls. After washing

the packed walls once more with saline-citrate and six times with water, they were resuspended in water and dialysed against water for 18 h. at 4° to remove any adhering DAP. The walls were lyophilized in an Edwards High Vacuum freeze drier model 10 P and stored at 4°. Lipopolysaccharide was prepared from the walls by phenol extraction (Fensom & Gray, 1969).

Analytical methods. Amino acid composition of samples hydrolysed with 6 N-HCl at 110° for 18 h. were determined in a Technicon amino acid autoanalyser. Phospholipids and fatty acids were extracted and analysed by thin-layer chromatography and gas-liquid chromatography (Hancock & Meadow, 1959). Amino sugar and sugar compositions were determined as previously described (Fensom & Meadow, 1970). Free amino groups in lyophilized walls were measured by the method used by Ghuysen, Tipper & Strominger (1966) except that the dinitrophenylated derivatives were not separated by thin-layer chromatography but by electrophoresis in pyridine-acetate buffer (pyridine, 10 % (v/v) + glacial acetic acid, 0.3 % (v/v)) pH 6.4 for 30 min. at 100 mA and 2 kV. The mono-dinitrophenyl DAP separated well in this system and was eluted from the paper with 0.01 M-NH₄OH-methanol (1:1, v/v). The extinction was measured at 360 nm. in a Unicam SP-800 spectrophotometer in a 1 cm. cell and results expressed as nmoles mono-DNP-DAP/mg. dry wt of wall.

Isolation and characterization of diaminopimelic acid from walls. Walls were prepared from 100 l. of bacteria grown in minimal medium + glucose + lysine in a fermentation vessel. The freeze-dried walls were hydrolysed under a reflux condenser in 6 N-HCl for 18 h. The hydrolysate was filtered, decolorized with activated charcoal and evaporated to dryness in a rotary evaporator. The residue was washed three times with water, dissolved in 2 ml. water, applied to a column (30 × 5 cm.) of Dowex-50 (×2) and then eluted with 1.25 N-HCl (Meadow & Work, 1959). The fractions containing DAP were heavily contaminated with cystine, which was removed by peroxide oxidation before separating the DAP by electrophoresis (90 mA/h., 2 kV) in pyridine-acetate buffer followed by chromatography in methanol-water-pyridine-HCl (Hoare & Work, 1955). Di-DNP- derivatives of the isolated DAP and of pure *meso*- and *LL*-isomers were prepared (Ghuysen *et al.* 1966) and their optical rotatory dispersion measured in a Bendix Polarimatic 62 machine in a silica cell (1 cm. path length).

Chemicals. *meso*-DAP was separated by fractional crystallization from a mixture of *meso*- and *LL*-isomers (Koch-Light Laboratories, Colnbrook, Buckinghamshire) as described by White *et al.* (1964). The *LL*-isomer was isolated from the mother liquor by crystallization of its salt with naphthalene-2-sulphonic acid (White *et al.* 1969). Carbenicillin was obtained from Beecham Research Laboratories. Vancomycin and cycloserine were provided by Eli Lilly & Co. Ltd, Basingstoke, Hampshire.

RESULTS

Growth requirements. Four stable lysine auxotrophs designated *lys-1*, *lys-2*, *lys-3*, *lys-4* were isolated and their growth requirements studied. None of the mutants grew in less than 6 days in minimal medium with any of the carbon sources tested; addition of 1 mM-lysine gave optimal growth of all four mutants (Fig. 1*a*). The rate of growth of the mutants in minimal medium + glucose + lysine was the same as that of the parent strain. For mutants *lys-2*, *lys-3* and *lys-4* lysine could not be replaced by any of the other amino acids tested, but *lys-1* grew slowly with added *meso*-DAP (1 mM), maximum growth being reached after 3 to 4 days as compared with growth in 8 to 10 h. on lysine. When higher concentrations of DAP were used, maximum growth could be achieved in 48 h. (Fig. 1*b*). *LL*-DAP was ineffective.

DAP metabolism. DAP decarboxylase was detected in the parent strain and in the *lys-1* mutant but *lys-2*, *3*, and *4* apparently lack this enzyme (Table 1). This was to be expected from the lysine requirements of these three mutants. DAP epimerase, however, was detected only in *lys-2*, *3* and *4* and the parent strain, and not in mutant *lys-1*. Since this mutant was capable of growing with lysine as sole added amino acid it seemed likely that it had a low level of epimerase activity which could not be detected by the chromatographic method. By using manometric methods Dr P. J. White assayed the enzyme and found that when the

Fig. 1. Response of *lys-1* to L-lysine (a) and meso-DAP (b). Bacteria were grown in minimal medium with glucose as carbon source for 18 h. (a) and 48 h. (b).

Table 1. *DAP metabolism of mutants and parent strain of Pseudomonas aeruginosa* 8602

DAP decarboxylase and epimerase were detected chromatographically as described in Methods.

	Mutant				Parent strain
	<i>lys-1</i>	<i>lys-2</i>	<i>lys-3</i>	<i>lys-4</i>	
DAP decarboxylase	+	—	—	—	+
DAP epimerase	—	+	+	+	+
Isomer of DAP accumulated in culture filtrate	LL	meso + LL	meso + LL	meso + LL	—
Amount of DAP accumulated (mg./100 ml.)	10.0	0.6	0.98	0.53	0
Ultraviolet-absorbing material in culture filtrate (as μ moles UTP/100 ml.)	12.2	14.7	18.0	16.1	0

+ = present; — = not detectable. DAP and u.v.-absorbing material in the culture filtrate after growth were detected and estimated as described in Methods.

mutant and wild-type were grown in minimal medium + citrate + lysine there was epimerase activity detectable in both the parent strain (0.010 unit/mg. protein) and the mutant (0.001 unit/mg. protein). The activity of DAP decarboxylase in *lys-1* (about 0.005 unit/mg. protein) was comparable to that in the parent strain (0.01 unit/mg. protein).

In some species lysine-requiring mutants accumulate DAP and extracellular wall precursors when grown in limiting lysine (Lilly, Clarke & Meadow, 1963; Knox, Cullen & Work, 1967). When the pseudomonad mutants were grown under these conditions, DAP and u.v.-absorbing material were found to accumulate in the growth medium, but no amino sugar compounds could be detected either in the growth medium or intracellularly (Table 1).

Fig. 2. Effect of carbenicillin on logarithmically growing cultures of *lys-1* (a) and the parent strain (b). Bacteria were grown in complex medium at 37° with shaking. At the time indicated by the arrow carbenicillin was added to four of the five flasks. Carbenicillin (final concentration, µg./ml.): △, 300; ●, 200; □, 100; ▲, 50; ○, 0.

Antibiotic sensitivities. The sensitivities of the mutants to various antibiotics known to interfere with wall synthesis were compared with those of the parent strain. There was no detectable difference between the MIC of vancomycin (150 µg./ml.) and cycloserine (500 µg./ml.) for the mutant or the parent strain. However, there was a difference in the MIC of carbenicillin. The MIC apparently depended on inoculum size and a sharp end-point was difficult to determine because of lysis, but the *lys-1* mutant appeared consistently to be more sensitive (MIC 10 µg./ml.) than the parent strain and the other lysine auxotrophs (MIC 20 µg./ml.). This differential effect of carbenicillin was investigated more thoroughly by adding it to logarithmically growing bacteria (Fig. 2a, b). *Lys-1* lysed in the presence of carbenicillin (50 µg./ml.), but even with 300 µg./ml. the parent strain and the other lysine-requiring mutants showed no lysis although their growth rate was slowed. Sucrose (10%, w/v) protected the mutant against lysis by carbenicillin. The growth rate in the presence of sucrose was less than in its absence, but even with 200 µg. carbenicillin/ml. there was no lysis although growth was inhibited. Addition of vancomycin to logarithmically growing cultures caused lysis, but the amount of vancomycin required to produce this effect (500 µg./ml.) was the same for all the mutants and the parent strain.

Wall composition. The greater sensitivity of *lys-1* to carbenicillin suggested that its wall composition might differ from that of the other mutants and its parent strain. The amino acid composition of walls of *lys-1* was compared with that of walls of the parent strain

before and after phenol extraction which removes most of the lipopolysaccharide and lipoprotein components (Table 2). No significant differences could be detected. It was possible that the low DAP epimerase activity might cause the mutant to use a different isomer of DAP in its mucopeptide. Paper chromatography showed that both the parent and mutant contained no LL-DAP in their walls, but this method does not distinguish between the *meso*- and DD-isomers. The di-dinitrophenylated derivatives of the DAP isolated from both batches of walls showed no optical rotation and hence were shown to be derived from pure *meso*-DAP.

The degree of cross-linking of the DAP in the walls was measured by isolating mono-dinitrophenyl DAP from walls prepared from the mutant and parent strain at various stages of growth. The number of free amino groups in the logarithmic phase was higher (4.3 nmoles DNP-DAP/mg. dry wt walls) than in the stationary phase (3.1 nmoles DNP-DAP/mg. dry wt walls) and addition of carbenicillin (100 µg./ml. 1 h. before harvesting) increased this further (to 6.4 nmoles/mg. dry wt), but there was no detectable difference between the mutant and parent strain.

Table 2. *Amino acid composition of acid hydrolysed walls of lys-1 and the parent strain before and after phenol extraction*

Walls were prepared and analysed from 8 l. of culture grown in complex medium for 18 h. Results are expressed as relative molar proportions with Glu = 100.

Amino acid	Isolated walls		Phenol-extracted walls	
	<i>lys-1</i>	Parent strain	<i>lys-1</i>	Parent strain
Cysteic acid	0	4.6	0	0
Asp	66.2	82.1	69.7	68.2
Thr	*	*	*	*
Ser	*	*	36.6	41.5
Muramic acid	+	+	+	+
Glu	100	100	100	100
Pro	34.6	36.5	34.8	41.7
Gly	75.7	71.5	67.7	67.4
Ala	101.2	115.5	101.6	100.4
Glucosamine	+	+	23.4	25.0
Val	47.4	53.4	59.3	59.1
Cys	4.7	+	5.1	4.8
Met	15.6	0	16.3	16.8
DAP	5.4	6.2	7.8	8.4
Ileu	34.3	34.7	32.0	48.6
Leu	75.9	65.5	76.7	72.9
Tyr	21.9	14.5	19.3	19.3
Phe	27.4	26.5	28.9	30.2
Unknown X	15.0	12.0	+	+
Orn	1.8	4.1	+	+
Lys	44.4	45.8	45.3	45.8
His	14.3	13.8	15.2	15.0
Arg	42.9	46.5	49.7	56.1

+ = present but not sufficient to estimate; * = present but not separated well enough to estimate; 0 = absent.

Lipid composition. The total amount of extractable fatty acids in the mutant (9.6%) was similar to that in the parent strain (10.4%) and analysis by gas-liquid chromatography showed that the individual fatty acids present were comparable both qualitatively and quantitatively to those found in the parent strain, 85% of the fatty acids being 16:0, 18:1 and 19 cyclopropane acids (Hancock & Meadow, 1969). The major phospholipid component

in the mutant was phosphatidyl ethanolamine (70 to 80 %) with smaller amounts of phosphatidyl glycerol (10 to 15 %) and bis-phosphatidyl glycerol (5 to 9 %). This too was indistinguishable from the phospholipid composition of the parent strain.

Lipopolysaccharide composition. Lipopolysaccharide extracted by 45 % phenol, as described in Methods, comprised approximately 30 % of the dry weight of both the parent strain and mutant. Analysis of the isolated lipid A components showed that 70 % was made up of 2-OH 12:0 and 3-OH 12:0 in approximately equal amounts. Although the isolated mutant polysaccharides contained less of the side-chain components glucosamine and mannose than did the parent strain, the major side-chain component rhamnose was unchanged (Table 3).

Table 3. *Carbohydrate composition of the isolated lipopolysaccharides from lys-1 and the parent strain*

Lipopolysaccharide was prepared from 10 l. bacterial cultures grown for 18 h. in minimal medium + citrate. For the mutant *lys-1* lysine was also present in the medium. The lipopolysaccharide was isolated and analysed as described by Fensom & Gray (1969).

Component	Composition (% w/w) lipopolysaccharide from	
	<i>lys-1</i>	Parent strain
Rhamnose	8.7	8.1
Glucosamine	3.6	7.9
Mannose	0.2	1.0
Glucose	6.4	5.4
Heptose	6.4	5.4
2-Keto-3-deoxyoctonate	3.4	2.4

DISCUSSION

The properties of the lysine auxotrophs *lys-2*, *3* and *4* are consistent with their identification as mutants with a single defect in the gene determining DAP decarboxylase. The only differences detected between these mutants and the parent strain were the absence of DAP decarboxylase activity, their obligate requirement for lysine, which was not replaced by DAP, and their accumulation of DAP in the growth medium and intracellularly when grown in lysine-deficient media. The accumulation of DAP by organisms deficient in or lacking DAP decarboxylase has been shown in *Escherichia coli* (Lilly *et al.* 1963) and more recently in *Staphylococcus aureus* (Barnes, Bondi & Moat, 1969). This accumulation probably results from the mode of control of DAP and lysine metabolism in these organisms. L-Lysine represses the formation of DAP decarboxylase in *E. coli* and in some but not all strains of *S. aureus* (White *et al.* 1964), and it seems likely that it has the same effect in the pseudomonad (P. J. White, unpublished). Lysine auxotrophs of *E. coli* grown in lysine-deficient media also accumulate lipopolysaccharides related to the bacterial cell wall and, to some extent, the UDP-amino sugar precursors involved in mucopeptide synthesis (Strominger & Threnn, 1959; Lilly *et al.* 1963; Knox *et al.* 1967) as well as DAP. No such compounds accumulated in the pseudomonad mutants deprived of lysine.

The DAP epimerase-deficient mutant *lys-1* poses some interesting problems. In part its behaviour is similar to that of the only other DAP epimerase-deficient mutant so far described. Barnes *et al.* (1969) isolated a lysine auxotroph of *Staphylococcus aureus* designated *lys-F*, in which they could detect no DAP-epimerase activity and which accumulated LL-DAP when grown in lysine-deficient media. DAP (145 μ M) permitted a low level of growth

in the absence of lysine, but the authors do not report whether DAP could replace lysine completely at higher concentrations or which isomer was used. The slow growth of the pseudomonad mutant *lys-1* on *meso*-DAP (mM) compared with its growth rate on equimolar lysine and its increased growth rate on higher concentrations of *meso*-DAP suggest that there may be difficulty in transporting this amino acid into the bacterium. In this respect this mutant resembles *Escherichia coli*, which contains a DAP-permease of activity so low that DAP auxotrophs require the addition of supplementary lysine for optimal growth rate (Davis, 1952; Leive & Davis, 1965). In pseudomonads the DAP-transport system seems to be even less efficient, since the growth rate of *lys-1* in lysine-limited media was unchanged by added DAP (mM).

The surprising feature of the epimerase-deficient mutant is its sensitivity to carbenicillin. This α -carboxy benzyl penicillin was developed specifically against the Gram-negative bacteria (Knudsen, Rolinson & Sutherland, 1967). The minimum inhibitory concentration (20 to 30 μ g./ml.) for the parent strain was similar to that reported for other sensitive pseudomonad strains (25 to 50 μ g./ml., Knudsen *et al.* 1967; Bodey & Terrell, 1968). The mutant was, however, about ten times more sensitive by the tube dilution technique, and addition of carbenicillin to logarithmically growing cultures caused rapid lysis unless the organisms were protected by sucrose. We could not detect any major differences between the isolated walls of the parent strain and the epimerase mutant, either in amino acid, carbohydrate or lipid composition. It is unlikely therefore that the greater sensitivity of the mutant is due to greater accessibility of the antibiotic to its site of action, as is the case in a mutant lacking the side chain of the lipopolysaccharide (Fensom & Meadow, 1970). Nor is there any evidence that the antibiotic causes any greater inhibition of cross-linking in the mutant walls as compared with those of the parent strain. One possible explanation of the rapid lysis of the mutant lies in its accumulation of large amounts of DAP. Mutant *lys-1* accumulated ten times more DAP than the other lysine auxotrophs and none was accumulated by the parent strain. If the DAP transport system is equally slow in both directions it is possible that the accumulation of these amounts of LL-DAP by the mutant increases the internal osmotic pressure until it bursts a wall already weakened by carbenicillin inhibition.

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