SHORT COMMUNICATION

Mutants of *Escherichia coli* K12 Permeable to Haemin

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Mutants of *Escherichia coli* which require 5-aminolaevulinic acid (5-ALA), the first intermediate of haem biosynthesis, do not respond to haemin and porphyrins. The probable explanation of the lack of response is that *E. coli* may be impermeable to haemin and porphyrins. Mutants are described which responded to haemin and porphyrins as well as to 5-ALA. Indirect evidence is presented that the mutants were permeable to haemin. The mutants showed other phenotypic changes, and resembled some mutants which are known to have changes in the cell envelope.

INTRODUCTION

Escherichia coli K12 is impermeable to haemin and porphyrins under normal conditions (Säsärman *et al.*, 1968) and does not yield haemin-requiring mutants. Mutants are known which respond to 5-aminolaevulinic acid (5-ALA), the first intermediate of haem biosynthesis, and do not respond to haemin and porphyrins. Because of the impermeability of K12 to haemin, indirect methods have been used to detect genes concerned with haem biosynthesis. Thus Powell *et al.* (1973) studied mutants which accumulated various porphyrins. Genetical analysis was slow because the mutants grew on minimal medium and selective methods could not be used. The isolation of mutants which respond to haemin is described in this paper. They simplify the analysis of haem biosynthesis.

METHODS

Unless stated otherwise, the methods were those used by McConville & Charles (1979). Glucose minimal medium (GM) was medium E of Vogel & Bonner (1956) supplemented with thiamin (5 mg l⁻¹) and glucose (5 g l⁻¹). Glucose minimal agar (GMA) was GM containing Difco Bacto agar (13 g l⁻¹). Complete medium (CM) contained (g l⁻¹): Tryptone (Oxoid), 10; yeast extract (Difco), 5; K₂HPO₄, 3; KH₂PO₄, 1; glucose, 5. Stock solutions of haemin (type III, Sigma) and protoporphyrin IX (Calbiochem) were prepared by dissolving 0.2 g in 5 ml Tween 80 and slowly diluting to 50 ml with sterile distilled water; a few drops of alkali were added to give clear solutions. The procedure gave sterile solutions which were stored at -18 °C and added aseptically to give concentrations of 25 mg l⁻¹ in CM and 10 mg l⁻¹ in GM and GMA. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was used by the method of Adelberg *et al.* (1965). The following K12 strains were used: AB1515 F⁻ *leu-13 lacY5 proC14 purE27 trp-21 mal-7 rpsL xyl-11 mtl-5 thi-9* (strain 13-6 of Schwartz, 1964); AB1621 F⁻ *ara-14 tfrA lac-1 tsx-57 gal-2 rpsL xyl-5 mtl-1 thi-1* λ⁺ (Adelberg *et al.*, 1965); AB312 Hfr *thr leu rpsL* (Taylor & Adelberg, 1960). Strain MLM106 was a 5-ALA-requiring mutant of AB1621 carrying the new allele *hemA106*.

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RESULTS

Strain MLM106 was treated with MNNG, incubated with 5-ALA overnight, washed three times and resuspended in 10 ml GM containing haemin (20 mg l⁻¹), to give about 10⁶ bacteria ml⁻¹. The cultures were incubated with shaking for 36 h, washed and plated on the same medium solidified with agar. For every 500 bacteria plated about one large colony was obtained after 1 to 3 d. Bacteria from some large colonies responded to haemin and 5-ALA and will be referred to as permeable mutants. Six permeable mutants were studied; except when stated otherwise, they behaved alike. Streaked on GMA they gave tiny colonies, as did the parental strain MLM106; when 1 mg haemin l⁻¹ was present they gave larger colonies, and with 8 mg l⁻¹ they gave as good growth as with the optimal concentration of 5-ALA (1 mg l⁻¹), although the colonies took longer to grow. The stimulatory effect was not due to the Tween 80 used to dissolve the haemin. Strains AB1621 and MLM106 were not stimulated or inhibited by haemin.

Anderson & Ivánovics (1967) reported that haemin-requiring mutants of *Bacillus subtilis* required bovine plasma albumin and cysteine as well as haemin. Cysteine, cystine and thiosulphate proved to be strongly stimulatory to the permeable mutants in the presence of haemin. Henceforth, cysteine (40 mg l⁻¹) was added to haemin-supplemented GM and GMA.

Commercially available porphyrins and related compounds were tested by the auxanographic method in the presence of cysteine; deuteroporphyrin, mesoporphyrin, haematoporphyrin, protoporphyrin and haemin stimulated the permeable mutants but did not stimulate MLM106. The zones of stimulation were usually small, probably because of the low solubilities of the substances in water.

The permeable mutants resembled strain MLM106 in growing well on GMA supplemented with 5-ALA (1 mg l⁻¹), but they differed in that they did not grow as well on CM agar supplemented with 5-ALA (20 mg l⁻¹). One permeable mutant, MLM10613, did not grow at all on CM agar, whether supplemented with 5-ALA or haemin. Addition of magnesium salts, spermine or spermidine to the medium gave good growth of MLM10613; iron and calcium gave weaker stimulations. Henceforth, MgSO₄ (0.01 M) was included in CM.

On GMA (plus 5-ALA) the permeable mutants were inhibited by actinomycin D (10 mg l⁻¹) and methylene blue (8 mg l⁻¹); concentrations greater than 30 mg l⁻¹ and 50 mg l⁻¹, respectively, were required to inhibit the parental strains. Morphologically, when compared with MLM106 in CM (with MgSO₄), the permeable mutants exhibited many more pairs of rods and short chains, rather than separate rods and dividing pairs, and filaments 10 to 12 µm long were present. In CM without MgSO₄, the chains contained more rods, filaments were more frequent and the bacteria grew more slowly.

Strain MLM106 was used as recipient in a time-of-transfer experiment with Hfr AB312; phage T6 was used to stop chromosome transfer. The *xyl*⁺, *gal*⁺ and *hemA*⁺ alleles were transferred after 9, 45 and 62 min. Transconjugants resistant to actinomycin were selected on GMA containing 5-ALA plus actinomycin D (10 mg l⁻¹); a gene for resistance was transferred after 55 min. Fifty resistant transconjugants were tested; all required 5-ALA, none responded to haemin, all grew on CM without added MgSO₄, and they showed normal morphology. The evidence suggests that the characters were pleiotropic effects of a gene mapping near min 20 on the linkage map. Transduction experiments with MLM10613 failed because the bacteria gave lysates containing less than 10⁷ phage ml⁻¹.

Beljanski & Beljanski (1957) showed that a haemin-deficient mutant of *E. coli* 'Monod' lacked catalase when grown without haemin; catalase formed when haemin was added to extracts, showing that the apoenzyme was synthesized in haem-deficient bacteria. Haddock & Schairer (1973) similarly showed that apoproteins of cytochromes were present in *E. coli* bacteria which were haem- and porphyrin-deficient. Mutant MLM106 grew without 5-ALA and haemin when incubated anaerobically. If the apoprotein of catalase was synthesized under anaerobic conditions in the absence of haem, it seemed possible that

Table 1. *Effect of haemin on catalase activity of whole bacteria (and extracts) grown anaerobically without haemin and 5-aminolaevulinate*

Catalase activity was assayed immediately before, and 10 min after, addition of haemin solution (final concentration 10 mg l^{-1}) or Tween 80 (final concentration 0.025 %, v/v) to bacteria or extract; specific activities (K) are expressed in $\text{l (g dry wt bacteria, or protein)}^{-1} \text{ s}^{-1}$. Strain MLM106 was haemin-impermeable; MLM10613 was haemin-permeable.

	$10^3 \times \text{Catalase activity}$					
	Whole bacteria preincubated with glucose		Whole bacteria preincubated without glucose		Bacterial extract	
	MLM106	MLM10613	MLM106	MLM10613	MLM106	MLM10613
Before addition of haemin solution	0.41	0.76	0.26	0.24	0.39	0.58
After addition of haemin solution	0.59	13.78	0.28	0.32	14.53	18.70
Haemin solution added after bacteria or extract had been heated at 100°C for 5 min	0.20	0.24	0.22	0.18	0.10	0.10
After addition of Tween 80 solution	0.39	0.56	0.30	0.32	0.40	0.56

haemin-permeable bacteria might form catalase more quickly than impermeable bacteria when haemin was provided. Bacteria were grown anaerobically (H_2/CO_2 , 95:5, v/v) in GM containing cysteine. After 18 h they were resuspended in minimal medium to about $200 \text{ mg wet wt ml}^{-1}$ and 25 ml volumes were added to two 250 ml flasks. Glucose (5 g l^{-1}) was added to one of the flasks because Jensen (1957) showed that preincubation with glucose was necessary for *Staphylococcus aureus* to form catalase on addition of haemin. The flasks were incubated at 37°C for 30 min. Samples were then assayed for catalase, before and after addition of haemin, following the method of Herbert (1955). Table 1 shows that addition of haemin to live permeable bacteria incubated with glucose caused an 18-fold increase in catalase activity within 10 min. Addition of haemin to impermeable bacteria, and to permeable bacteria incubated without glucose, did not enhance catalase activity. Extracts of all the bacteria formed catalase on addition of haemin. Catalase activity reached its maximum about 3 min after addition of haemin to whole permeable bacteria.

DISCUSSION

The ease with which the mutants were obtained suggests that it may be profitable to look for permeable mutants in other kinds of investigations in which impermeability is a problem. Because of impermeability to haemin, analysis of the biochemistry and genetics of haem biosynthesis in *E. coli* has been slow and difficult, and several genes remain to be identified. Isolation of haemin-requiring mutants from the haemin-permeable mutants is described by McConville & Charles (1979).

The response of the mutants to haemin, and the experiments on catalase formation, gave reasonable evidence, but not proof, that the changes in the mutants were changes of permeability. Direct tests of the uptake of radioactive haemin into permeable and impermeable bacteria may not give clear evidence of increased permeability because K12 bacteria avidly adsorb haemin and become coated with the pigment which is not easily washed away. The mutants did not show indiscriminate increase in permeability; they did not, for example, grow on citrate as a carbon and energy source. It is possible that they may have lost surface materials such as lipopolysaccharide, which may normally restrict ingress

of foreign molecules. Another possibility is that a transport system may have changed such that it became able to transport haemin and porphyrins.

The change to haemin-permeability was associated with other phenotypic changes, and the same or similar genes may have been identified in other ways. Examples of possible genes are *envA* and *envB* at min 2 and 70 (Normark, 1969, 1970), *envC* at min 80 (Rodalakis *et al.*, 1973) and *acrA* at min 10 (Imae, 1968).

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REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEIN CHING CHEN, G. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788–795.
- ANDERSON, T. J. & IVÁNOVICS, G. (1967). Isolation and some characteristics of haemin dependent mutants of *Bacillus subtilis*. *Journal of General Microbiology* **49**, 31–40.
- BELJANSKI, M. & BELJANSKI, M. (1957). Sur la formation d'enzymes respiratoires chez un mutant d'*Escherichia coli* streptomycine-résistant et auxotrophe pour l'hémine. *Annales de l'Institut Pasteur* **92**, 396–412.
- HADDOCK, B. A. & SCHAIRER, H. U. (1973). Electron-transport chains of *E. coli*. Reconstitution of respiration in a 5-aminolaevulinic acid-requiring mutant. *European Journal of Biochemistry* **35**, 34–45.
- HERBERT, D. (1955). Catalase from bacteria. *Methods in Enzymology* **2**, 784–788.
- IMAE, Y. (1968). Mitomycin C-sensitive mutant of *Escherichia coli* K12. *Journal of Bacteriology* **95**, 1191–1192.
- JENSEN, J. (1957). Biosynthesis of haematin compounds in a haemin-requiring strain of *Micrococcus pyogenes* var. *aureus*. I. The significance of coenzyme A for the terminal synthesis of catalase. *Journal of Bacteriology* **73**, 324–333.
- MCCONVILLE, M. L. & CHARLES, H. P. (1979). Isolation of haemin-requiring mutants in *Escherichia coli* K12. *Journal of General Microbiology* **113**, 155–164.
- NORMARK, S. (1969). Mutation in *Escherichia coli* mediating sphere-like envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. *Journal of Bacteriology* **97**, 1334–1342.
- NORMARK, S. (1970). Genetics of a chain-forming mutant of *Escherichia coli*. Transduction and dominance of the *envA* gene mediating increased penetration to some antibacterial agents. *Genetical Research* **16**, 63–78.
- POWELL, K. A., COX, R., MCCONVILLE, M. L. & CHARLES, H. P. (1973). Mutations affecting porphyrin biosynthesis in *Escherichia coli*. *Enzyme* **16**, 65–73.
- RODALAKIS, A., THOMAS, P. & STARKA, J. (1973). Morphological mutants of *Escherichia coli*. Isolation and ultrastructure of a chain-forming *envC* mutant. *Journal of General Microbiology* **75**, 409–416.
- SCHWARTZ, N. (1964). Suppression of a *lacO* mutation in *Escherichia coli*. *Journal of Bacteriology* **88**, 996–999.
- SÁSÁRMAN, A., SURDEANU, M., SZÉGLI, G., HORODNICEANU, T., GRECEANU, V. & DUMITRESCU, A. (1968). Hemin-deficient mutants of *Escherichia coli* K12. *Journal of Bacteriology* **96**, 570–572.
- TAYLOR, A. L. & ADELBERG, E. A. (1960). Linkage analysis with very high frequency males of *Escherichia coli*. *Genetics* **45**, 1233–1243.
- VOGEL, H. J. & BONNER, D. M. (1956). A convenient growth medium for *Escherichia coli* and some other micro-organisms (medium E). *Microbial Genetics Bulletin* **13**, 43–44.