Further studies of clinical isolates of cysteine-requiring *Escherichia coli* and *Klebsiella* and possible mechanisms for their selection *in vivo*

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Summary. Cysteine-dependent (*cys*[−]) *Escherichia coli* and *Klebsiella* spp., defective in sulphate assimilation, were isolated from urine and stool samples of infected patients. These isolates reverted to prototrophy under conditions of cysteine deprivation but the revertant strains and a prototrophic wild-type *E. coli* strain became auxotrophic for cysteine in a cysteine-enriched medium. This suggested that excess cysteine acts as a repressor of the *cys* *HII* operon known to control aspects of cysteine biosynthesis. A group of mostly elderly patients infected with *cys*[−] strains suffered a disproportionate amount of renal impairment as compared with a control group. In renal impairment, sulphur compounds, including cysteine, are retained. This raises the possibility that these raised levels of cysteine and related compounds may enhance the selection of *cys*[−] strains *in vivo*.

Introduction

About 1.5% of *Escherichia coli* and *Klebsiella* isolates from urinary tract infections (UTI) are auxotrophic for cysteine (*cys*[−]).^{1,2} Such strains may also occasionally cause septicaemia and other forms of sepsis, for which the urinary tract is usually the source.³ *Cys*[−] *E. coli* and *Klebsiella* strains have an altered pathway of sulphate assimilation.^{2,3} Their requirement for cysteine, and other characteristics relating to identification and antibiotic susceptibility testing, have been investigated previously.^{4–6} Patients infected with these strains are usually elderly, with a systemic or local predisposition to UTI.^{2,3,7}

Since most UTI are due to bowel bacteria, stool samples from patients with *cys*[−] *E. coli* UTI were examined for the presence of such organisms. An *in-vitro* method, with conditions of cysteine excess, was devised to derive a *cys*[−] *E. coli* variant from a prototrophic strain originally cultured from a patient whose faeces also contained a *cys*[−] isolate. The previously observed ability of *cys*[−] strains to revert to prototrophy under conditions of cysteine deprivation⁶ was also further investigated. It has been noted previously that *cys*[−] strains may produce colonies of differing sizes on a single culture plate, giving the appearance of a mixed growth.⁷ Therefore, the sulphur assimilation pathway of these different colonial types was investigated to determine whether reversion to prototrophy is a single or a multiple step process.

Analysis of the clinical findings of patients infected with *cys*[−] strains suggested that renal impairment was

a common feature in this group. Since sulphur-containing compounds are known to be retained in patients with renal impairment,^{8–10} this and other observations were used to advance a hypothesis to explain the possible origin of *cys*[−] strains *in vivo*.

Materials and methods

Faeces samples

Stool samples were collected from 11 patients in whose blood or urine *cys*[−] *E. coli* or *Klebsiella* strains had been detected by methods described previously.^{3,5} Four of these specimens were collected before antibiotic treatment had been started. Samples of faeces from 100 patients aged ≥ 60 years with diarrhoeal disease, but without UTI, were also collected and examined for the presence of *cys*[−] strains.

Isolation of cys[−] strains

Cys[−] strains were isolated from faeces samples by a direct or an antibiotic-selection method. For the direct method a small amount faeces was inoculated on to MacConkey Agar (Oxoid) which was incubated at 37°C for 18 h. The plates were examined for dwarf colonies characteristic of *cys*[−] auxotrophs. These were stained by Gram's method and their cysteine requirement was determined.

For the antibiotic-selection method, faeces were seeded into a cysteine-free, penicillin-supplemented broth in which *cys*[−] strains will not grow and should, therefore, be indifferent to the action of penicillin, while actively dividing prototrophs will be greatly

reduced in numbers.¹¹ Faeces (0.5 g) were suspended in Davis and Mingioli (DM) medium¹² supplemented to 0.63 mM with L-cysteine HCl. After overnight incubation, the broth was filtered through sterile gauze to remove solid material and then centrifuged at 1600 g for 10 min. The deposit was washed three times with saline and a 3-ml suspension of this was used to inoculate 20 ml of cysteine-free DM broth at 37°C to an initial absorbance of 0.15–0.25 at 640 nm (Spectronic-R 20 series spectrophotometer; Milton Roy, Rochester, NY, USA). After incubation for 2 h in a shaking water bath, penicillin was added to 10000 IU/ml and incubation continued for a further 2 h. The growth was then harvested as above and 10 serial 10-fold dilutions were made in saline. Each dilution was used to flood MacConkey agar plates which were incubated for 48 h and examined for the presence of *cys*[−] strains as described above.

Growth response of cys[−] *colonial variants to intermediates of the sulphate assimilatory pathway*

Colonial variants of two *cys*[−] *E. coli* strains (ECC-1 and 13) and one *cys*[−] *Klebsiella* strain (KC-6) of the class *cys ACDH* of Jones-Mortimer^{2,3,13} were selected on the basis of their colony size after growth for 24 h on MacConkey agar. Dwarf colonies (0.5–1.0 mm in diameter), intermediate colonies (partial revertants; 1.5–2.0 mm in diameter) and larger colonies (complete revertants; ≥ 2.0 mm in diameter) were selected and inoculated into DM broth containing 0.63 mM L-cysteine HCl. After incubation for 24 h at 37°C, the growth was harvested, washed three times in saline, inoculated at an initial absorbance of 0.1 at 640 nm into 2.5-ml volumes of Pasternak's¹⁴ sulphur-free basal medium (PM) with or without 10 mM Na₂SO₄, 0.5 mM L-cysteine sulphinic acid (CSH), 0.42 mM Na₂S or 0.63 mM L-cysteine HCl and incubated for 8 h at 37°C in a shaking water bath. The growth of each strain in each medium was determined by measuring absorbance at 30–40-min intervals. Prototrophic *E. coli* NCTC 10418 and *K. pneumoniae* ATCC 12657 were used as controls. These experiments were performed in duplicate and repeated once.

Mutants deficient in sulphate permease, controlled by *cys A*, may be identified by their failure to assimilate relatively low levels of thiosulphate.^{15,16} Therefore, the above strains were included in a batch of 34 *cys*[−] strains of class *cys ACDH* (32 *E. coli* and two *K. pneumoniae*), isolated and phenotyped in this laboratory. They were examined for their capacity to grow on PM containing 0.2 mM thiosulphate. Only dwarf variants of *cys*[−] phenotype were examined in this experiment, which also tested their ability to grow on sulphur-free PM and on PM containing 10 mM Na₂SO₄.

Rate of reversion of cys[−] *strains to complete and "partial" prototrophy*

Five *cys*[−] strains isolated in this laboratory—four *E.*

coli (ECC-1, 5, 13 and 21) and *K. pneumoniae* KC-6—were used. Dwarf colonies of these strains, grown on MacConkey agar overnight, were suspended in 2.5 ml of sterile saline to an absorbance of *c.* 0.2 at 640 nm. Each suspension was serially diluted 100-fold three times and 0.2 ml of each dilution was inoculated on to separate plates of PM supplemented with 10 mM Na₂SO₄, 0.5 mM CSH or 0.63 mM cysteine. Plates were incubated for 6 days in air at 37°C. Total counts were made of the revertant colonies appearing on plates supplemented with sulphate and CSH and the count on the latter plate corrected for the presence of complete revertants (prototrophs) on the sulphate plate. A ratio of counts of partial (corrected CSH growth) and complete (growth in the presence of sulphate) revertants to the viable count of the initial inoculum (determined by surface viable counts on cysteine-supplemented PM) was calculated. A mean of the rates of partial and complete reversion to prototrophy was determined after repeating the experiment four times.

Derivation of cys[−] *strains from prototrophic strains*

A single colony of the strain under examination was taken from an overnight growth on MacConkey agar and suspended in 20 ml of DM broth containing 0.63 mM L-cysteine HCl. After overnight incubation, the broth was centrifuged, the deposit was washed three times in saline and resuspended in saline before inoculation into 20 ml of unsupplemented DM medium and then the above penicillin selection process was applied. The process was repeated with the unused portion of the deposit. The presence of *cys*[−] strains was determined according to colonial morphology, subculture and auxanography as described above. The organisms used in these experiments were: a prototrophic *E. coli* isolated from a stool sample from which a *cys*[−] *E. coli* was also isolated; *E. coli* NCTC 10418 as a prototrophic control; and partial and complete revertants to prototrophy (described above) of *cys*[−] *E. coli* ECC-1 and ECC-13.

Source of patient data and cys[−] *strains*

A prospective study over 12 months was undertaken to confirm the incidence of *cys*[−] UTI and to assess certain features of the patients from whom these strains were isolated. Significant growths of Enterobacteriaceae from patients with UTI were recorded and such patients were placed into two groups, < and ≥ 50 years of age. Plasma creatinine determinations (performed as part of standard patient investigations by means of an Olympus AU 500 autoanalyser, Tokyo, Japan) were recorded for these and other unselected patients with UTI due to *cys*[−] strains. A control group comprised patients aged ≥ 60 years admitted for elective surgery but without evidence of UTI, uropathy or other serious illness. The reference range for the

creatinine determinations was 0.06–0.11 mmol/l. Tests for significance were performed with the χ^2 test and Yate's correction for continuity.

Source and preparation of chemicals

Sodium sulphate was obtained from Merck and CSH, used as a sulphite analogue,¹⁷ and all other chemicals were from Sigma. Cysteine was prepared from L-cysteine HCl immediately before use.¹⁸

Results

Isolation of *cys*⁻ strains from faecal samples

Four *cys*⁻ strains (three *E. coli*, one *Klebsiella* sp.) were isolated from a faeces sample from each of four patients whose blood or urine, or both, was also infected with *cys*⁻ isolates, but who had not received antibiotic therapy. In each case, the faecal and blood or urine strains were phenotypically identical. *Cys*⁻

strains were not isolated from the stools of another seven patients also with *cys*⁻ UTI. However, these patients had received an antibiotic, to which their *cys*⁻ isolate was sensitive, before collection of the stool sample. Only one *cys*⁻ *E. coli* strain was isolated from the 100 stool samples from patients without evidence of UTI. It was from a 75-year-old female patient readmitted for an unrelated illness after recent sigmoid colectomy.

The five *cys*⁻ strains were detected by both the direct and selective techniques. Although a significant reduction in prototrophic growth was obtained with the selective technique, and auxotrophs were much easier to distinguish, additional *cys*⁻ strains were not detected by the use of this technique.

Growth response of *cys*⁻ strains to intermediates of the sulphate assimilatory pathway

The growth responses in liquid medium of the phenotypic variants of *E. coli* *cys*⁻ ECC-1 and of a prototrophic control are shown in figs. 1 and 2. All

Fig. 1. Growth response of (a) *E. coli* NCTC 10418 and (b) a dwarf variant of *E. coli* ECC-1 in Pasternak's sulphur-free medium and the same medium supplemented with intermediates of the sulphate assimilatory pathway: —□—, blank; —●—, sulphate; —▲—, CSH; —○—, sulphide; —■—, cysteine.

Fig. 2. Growth response of (a) partial and (b) complete revertants of *E. coli* ECC-1 in Pasternak's sulphur-free medium and the same medium supplemented with intermediates of the sulphate assimilatory pathway: —□—, blank; —●—, sulphate; —▲—, CSH; —○—, sulphide; —■—, cysteine.

cys⁻ variants were able to use cysteine and sodium sulphide. The dwarf and partial revertants were unable to use sulphate as a sulphur source, whereas the growth response to sulphate of the complete revertant closely resembled that of the prototrophic control. It can be seen also that the partial revertant differed from the auxotrophic "dwarf" variant in an increased capacity to use the sulphite analogue CSH as a sulphur source, but this was less than that of the complete revertant or the prototrophic control strain. It would seem that the partial revertants represent an intermediate stage between auxotrophy and prototrophy in which the metabolic defect demonstrated in the dwarf variant has been partially corrected. The results obtained with *E. coli* ECC-13 and *K. pneumoniae* KC-6 were similar.

All 34 *cys ACDH* strains grew on PM with 0.2 mM thiosulphate, but not on sulphur-free PM or on PM containing Na₂SO₄. Prototrophic control strains grew on all media except sulphur-free PM.

Rate of partial and complete reversion to prototrophy of cys⁻ *strains*

The reversion rates of four *cys*⁻ *E. coli* and one *cys*⁻ *Klebsiella* strains to partial and complete prototrophy are shown in table I. The *Klebsiella* strains showed the highest reversion rate. The auxotrophic *E. coli* strain formed partial revertant colonies at a rate of 10⁻⁷–10⁻⁸ and the rate of complete reversion was 100-fold lower for two strains, but was not precisely determined for the remaining two isolates.

Derivation of cys⁻ *strains from prototrophic strains*

Dwarf colonies were obtained from partial and complete revertants grown in a cysteine broth. The cysteine requirement of these dwarf variants was confirmed auxanographically. Their detection was facilitated by the penicillin selection process, with *cys*⁻ dwarf colonies appearing after one cycle when partial revertants were used. However, distinction between *cys*⁻ auxotrophs and complete revertants was achieved only after repeated culture in cysteine broth followed by penicillin selection in cysteine-free medium. The *cys*⁻ strains derived in this way were able to revert to

Table I. Reversion rates to partial and complete prototrophy of four *cys*⁻ *E. coli* and one *cys*⁻ *K. pneumoniae* strains

Cysteine auxotroph	Rate of reversion (cells/ml)	
	Partial	Complete
<i>E. coli</i> ECC-1	2.2 × 10 ⁻⁷	3.9 × 10 ⁻⁹
<i>E. coli</i> ECC-5	3.1 × 10 ⁻⁸	> 10 ⁻¹⁰
<i>E. coli</i> ECC-13	4.6 × 10 ⁻⁸	> 10 ⁻¹¹
<i>E. coli</i> ECC-21	1.5 × 10 ⁻⁸	1.4 × 10 ⁻¹⁰
<i>K. pneumoniae</i> KC-6	1.8 × 10 ⁻⁶	4.4 × 10 ⁻⁸

Table II. Comparison between the plasma creatinine concentration in patients with UTI due to *cys*⁻ *E. coli* and a control group aged > 60 years

Age (years)	Number of patients		Number with raised plasma creatinine (%)
	infected	not infected	
> 50	27	...	15 (55.5)
< 50	10	...	3 (30)
> 60	...	50	10 (20)

prototrophy when cultured again on cysteine-deficient MacConkey medium. *Cys*⁻ strains could not be derived from *E. coli* NCTC 10418. However, prototrophic wild-type *E. coli*, from the stool of a patient from whom a *cys*⁻ *E. coli* was cultured simultaneously, yielded *cys*⁻ auxotrophs after two cycles of growth and selection. This experiment was repeated on three separate occasions and each time a *cys*⁻ strain was obtained. Again, the *cys*⁻ strain reverted to prototrophy after subculture on cysteine-deficient media.

Incidence of UTI with cys⁻ *strains*

A total of 1637 enterobacterial strains was isolated from patients with UTI in a 12-month period. Of these, 1106 (67.6%) were *E. coli*, 226 (13.8%) *Klebsiella* spp., 189 (11.5%) *Proteus*—*Providencia*—*Morganella* spp. and the remaining 116 (7.1%) *Enterobacter*, *Citrobacter* or *Serratia* spp. Of the *E. coli* strains, 18 (1.6%) were *cys*⁻, but only one (0.4%) of the *Klebsiella* strains was *cys*⁻. Most (15 of 18) of the *cys*⁻ *E. coli* strains were isolated from patients aged > 50 years and represented 2.7% of the 551 *E. coli* cultured from this age group. In patients < 50 years of age, three (0.5%) of 555 of *E. coli* were *cys*⁻.

Plasma creatinine concentrations in infected and uninfected patients

Plasma creatinine concentrations were available for 37 patients with UTI due to *cys*⁻ strains. They were compared in patients < and ≥ 50 years of age and in a control group of uninfected patients aged > 60 years (table II). The proportion of patients in the group infected with *cys*⁻ strains and with a raised plasma creatinine level (18 of 37) was significantly higher than in the control group (0.001 < p < 0.01) and this difference was greater in the infected subgroup ≥ 50 years of age.

Discussion

This study confirmed that *cys*⁻ *E. coli* strains represent a small percentage of all *E. coli* strains associated with UTI but that this is much higher in elderly patients. Although *cys*⁻ *Klebsiella* strains were less

common than the 1.5% previously reported,² the smaller sample examined on this occasion may be responsible for the apparent discrepancy. It was also shown that, in common with other urinary pathogens, *cys*⁻ strains involved in UTI are derived from the bowel flora.

The effect of exogenous cysteine on the metabolic status of *cys*⁻ strains was studied. In the absence of cysteine, *cys*⁻ strains reverted to prototrophy but remained or became auxotrophic in its presence. This was shown with *cys*⁻ strains, their colonial variants in different stages of reversion to prototrophy, and with a prototrophic wild-type *E. coli* isolated from the stool of a patient in whom a *cys*⁻ strain was also present.

Cys⁻ strains examined in these and earlier studies were of the class *cys ACDH* of Jones-Mortimer.¹³ This includes mutants deficient in the sulphate-utilising enzymes sulphate permease (*cys A*), adenosine 5'-phosphosulphate kinase (*cys C*), ATP sulphurylase (*cys D*) and 3'-phosphoadenosine 5'-phosphosulphate sulphotransferase (*cys H*). In this study, the capacity of *cys ACDH* strains to utilise 0.2 mM thiosulphate was also demonstrated. This compound is metabolised at the level of sulphite by mutants deficient in the enzymes for sulphate reduction and activation.¹⁶ However, as the *cys A* gene controls the uptake of both sulphate and thiosulphate,¹⁹ it may be inferred from the growth response of these strains to thiosulphate that the permease system is operable and that the metabolic anomaly in these auxotrophs is related to the loss of functions controlled by *cys C*, *cys D* or *cys H* rather than *cys A*.

The genes *cys C*, *cys D* and *cys H* are located at 59 min on the *E. coli* chromosome and share the same location as *cys I* and *cys J* that code for the apo-haemoprotein and apoflavoprotein components of sulphite reductase, respectively.²⁰ The genes *cys H*, *cys I* and *cys J* form an operon (*cys HIJ*) which is separated from *cys C* and *cys D* by a "silent region" of c. 25 kb.¹⁶ The findings described here in relation to the effects of cysteine on these clinically derived *cys*⁻ variants and the manner in which they utilise the sulphite analogue CSH, are consistent with the involvement of an operon model in the regulation of cysteine synthesis described for laboratory-derived

mutants.^{16,21} The improved growth response of the partial revertants in the presence of CSH may be explained by the gradual derepression of the *cys HIJ* operon in the absence of the product of the sulphate assimilatory pathway. Cysteine can be postulated as playing the role of repressor in this model. As a consequence, there is an energy saving for the cell, since the assimilatory pathway requires two mols of ATP to activate sulphate.

The postulated operon mechanism in *cys*⁻ strains provides the basis of a possible explanation for their selection *in vivo*, if levels of cysteine over and above those present from dietary sources occur in the bowel. This possibility led to a consideration of some patient parameters to ascertain whether this is indeed the case. Patients with impaired renal function retain sulphur-containing as well as nitrogen-containing compounds, and the plasma concentrations of taurine, cystine and cysteine-homocysteine are consistently raised in patients with a raised plasma creatinine.⁸⁻¹⁰ A preliminary examination of data from patients infected with *cys*⁻ strains confirmed that they comprise a mostly older age group and that there is an association between the isolation of *cys*⁻ strains and reduced renal function. However, considerable difficulties are associated with the proper assay of cystine and cysteine in plasma²² and these, together with the contentious nature of the methodology involved,^{23,24} have prevented confirmation of this inferred accumulation of cysteine-related compounds in the plasma or bowel lumen of these patients. Furthermore, there is no information about the relationship between plasma and bowel concentrations of these compounds, but at least some of these substances are excreted in the bile and their concentration in the plasma and bowel lumen are known to equilibrate to some extent. However, if raised concentrations of sulphur-containing compounds, that can be assimilated by *cys*⁻ strains, actually appear in the bowel through this or other mechanisms, the selection of *cys*⁻ strains may also take place in the bowel. This would be in the type of patient in whom these infections predominantly occur and it should be recalled that the bowel is the source of most uropathogens. This hypothesis awaits further clarification.

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