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A factor produced by *Escherichia coli* K-12 inhibits the growth of *E. coli* mutants defective in the cytochrome *bd* quinol oxidase complex: enterochelin rediscovered

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***Escherichia coli* produces an extracellular factor that inhibits the aerobic growth of Cyd⁻ mutants, defective in the synthesis or assembly of the cytochrome *bd*-type quinol oxidase. This paper shows that such a factor is the iron-chelating siderophore enterochelin. Mutants in *entA* or *aroB*, defective in the production of enterochelin, did not produce the factor that inhibits the growth of *cydAB* and *cydDC* mutants; purified enterochelin inhibited the growth of Cyd⁻ mutants, but not that of wild-type cells. Other iron-chelating agents, particularly ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA), whose complex with Fe(III) has a large stability constant (log K = 33·9), also inhibited the growth of Cyd⁻ mutants at micromolar concentrations, but not that of wild-type cells. Supplementation of agar plates with Fe(III) or boiled catalase prevented the inhibition of Cyd⁻ mutants by the extracellular factor. Spontaneous mutants isolated by being able to grow in the presence of the extracellular factor on plates also showed increased resistance to iron chelators. The reducing agent ascorbate, ascorbate plus In(III), ascorbate plus Ga(III), or Ga(III) alone, also alleviated inhibition by the extracellular factor, presumably by reducing iron to Fe(II) and complexing of the siderophore with alternative trivalent metal cations. The preferential inhibition of Cyd⁻ mutants by the extracellular factor and other iron chelators is not due to decrease in expression, activity or assembly of cytochrome *bo′*, the major alternative oxidase mediating quinol oxidation. Cyd⁻ mutants overproduce siderophores, presumably reflecting intracellular iron deprivation.**

Keywords: cytochrome *bd*, oxidase, *Escherichia coli*, enterochelin, iron

INTRODUCTION

Like almost all bacteria, the enteric bacterium *Escherichia coli* has to cope with constantly changing conditions in the environment, such as oxygen supply. *E. coli* consumes oxygen using two well-characterized membrane-bound terminal oxidases, cytochromes *bo′* and *bd* (Poole, 1994). Cytochrome *bo′* is a member of the haem–copper superfamily of terminal oxidases and has a moderately high affinity for oxygen, with a K_m in the submicromolar range (D'mello *et al.*, 1995). In contrast, cytochrome *bd* uses a haem–haem binuclear centre to bind oxygen as a surprisingly stable oxygenated form and reduce oxygen to water (for a review, see Poole, 1994). Cytochrome *bd* is believed not to be a proton pump, but it has an extraordinarily high apparent affinity for oxygen, with a K_m *in vivo* as low as 5 nM (D'mello *et al.*, 1996). The distinct properties of these oxidases, and thus their suitability for growth

The authors wish to dedicate this paper to Professor Frank Gibson, Australian National University, on the occasion of his 75th birthday and in recognition of his pioneering contributions to the biochemistry of siderophores.

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Abbreviations: CAS, Chrome Azurol S; EDDHA, ethylenediamine-di(o-hydroxyphenylacetic acid).

under different aerobic conditions, requires that they be differentially regulated. Cytochromes *bo'* and *bd* are maximally synthesized during growth with high (Cotter *et al.*, 1990) or limited (Fu *et al.*, 1991) aeration, respectively. Expression of operons comprising genes for both oxidases (*cyoABCDE* and *cydAB*, respectively) is affected by Fnr and ArcA/ArcB.

The *cydDC* operon in *E. coli*, encoding a heterodimeric ATP-binding cassette (ABC-type) transporter is also required for a functional cytochrome *bd* in *E. coli* (Poole *et al.*, 1993; Cook *et al.*, 1997). Mutations in *cydDC* do not influence the transcription of *cydAB* or the assembly of CydA or CydB polypeptides into the membrane (Bebbington & Williams, 1993). In *cydDC* mutants, the CydA and CydB polypeptides appear to be assembled, but lack the spectrally distinctive chlorin haem *d* and presumably haems b_{595} and b_{558}. Assembly of periplasmic cytochromes *c* and b_{562} is also defective (Poole *et al.*, 1994; Goldman *et al.*, 1996a). At present, neither the function of, nor the substrate for, the CydDC ABC-type transporter is known.

The cytochrome *bd*-type quinol oxidase is highly conserved in many bacterial species, but has been best studied in *E. coli* and *Azotobacter vinelandii* (Poole, 1983, 1994; Jünemann, 1997). A growing number of studies reveal that cytochrome *bd* is required under conditions of environmental stress and may have crucial roles in cellular physiology other than acting as an oxidase. For example, cytochrome *bd* is induced when *E. coli* is grown under unfavourable conditions (Cotter *et al.*, 1990; Avetisyan *et al.*, 1991; Bogachev *et al.*, 1995). Mutants that cannot synthesize cytochrome *bd* have a pleiotropic phenotype: they are sensitive to H_2O_2, temperature sensitive (Delaney *et al.*, 1992, 1993; Wall *et al.*, 1992), and are unable to exit from stationary phase and resume aerobic growth at 37 °C (Siegele & Kolter, 1993; Siegele *et al.*, 1996).

Macinga & Rather (1996) have shown that the growth of Cyd^- mutants (*cydAB* or *cydD*) is inhibited by an extracellular factor excreted by wild-type cells or Cyd^- mutants. This inhibition was abolished by growth under anaerobic conditions. Goldman *et al.* (1996b) hypothesized that this factor is hydrogen peroxide or a similar oxygen radical species, but no direct evidence for this hypothesis was provided. The results presented in this paper demonstrate that this factor is not peroxide, but enterochelin, an iron-chelating siderophore produced by *E. coli* in response to iron starvation conditions.

METHODS

Strains, plasmid and phage. *E. coli* K-12 strains and the plasmid used in this study are described in Table 1. The absence of cytochrome *d* in Cyd^- mutants was checked regularly by dual-wavelength scanning spectrophotometry (Poole *et al.*, 1989). Genetic crosses were performed using bacteriophage P1*vir*-mediated transduction (Miller, 1972). Plasmid DNA was isolated using the 'Wizard' column system from Promega. Transformation of bacteria with plasmid DNA was done using the single-step method of Chung *et al.* (1989).

Culture media, growth conditions and inhibition studies. Unless otherwise indicated, cells were grown in Luria broth, initial pH 7·0 (Miller, 1972), supplemented as described. All additions were at the concentrations listed in the text. Tetracycline, chloramphenicol, kanamycin and ampicillin were used at final concentrations of 15, 25, 50 and 150 μg ml^{-1}, respectively. Agar and other dehydrated media were from Difco or Oxoid. Other components were from Sigma. Enterochelin was generously donated by Dr Henry J. Rogers and Lyndall Hatch and was dissolved in acetone before application to agar plates. Other iron chelators were from Sigma.

Culture optical density was generally measured with a Pye-Unicam SP6-550 spectrophotometer at 600 nm, using culture samples diluted with medium to bring OD_{600} to below 0·7 when measured in cells of 1 cm pathlength. Aerated cultures were grown with shaking (200 r.p.m.). Anaerobic cultures were grown in screw-capped glass 200 ml bottles filled to the brim. An anaerobic growth atmosphere was generated using a BBL GasPak anaerobic system in an anaerobic jar. Where indicated, growth was monitored with a Klett colorimeter using 10 ml cultures shaken (200 r.p.m.) in 250 ml flasks fitted with side arms (Søballe & Poole, 1998).

To test for growth inhibition on nutrient agar plates, two procedures were followed. When the effect of an inhibitory compound, chelator or metal ion was being tested, the compound was added directly to the molten nutrient agar (45 °C) prior to pouring of the plates or added as a stock solution and spread evenly on the agar surface to achieve the stated concentration, assuming even distribution through 20 ml agar. Test strains were streaked directly onto the surface of the plate and growth was determined after 12 h. To test for growth inhibition by the extracellular factor, the factor-producing strain was streaked across one-third of an agar plate to achieve dense cell growth. The plate was incubated overnight (12 h). The test strain was grown overnight in Luria broth and 5 μl was spotted to the plate and streaked, using a sterile tooth pick, at 90° to the edge of growth of the producer strain. The streak of the test strain extended to within 4 mm of the producer strain. Suspensions of all test strains were adjusted to the same OD_{600} before cross-streaking. Aerobic and anaerobic growth was on nutrient agar plates and incubations were for 12 h. Growth inhibition was determined by measuring the extent of growth of the perpendicular streak. For example, cross-streaks were approx. 4 cm in length and *cydAB* mutants failed to grow in the final 1·5 cm of the streak (i.e. adjacent to the producer strain). The growth inhibition was calculated by dividing 1·5 cm by the control 4 cm to give an inhibition of 38%. Such measurements were highly reproducible and replicates did not differ by more than 15%. All cultures were grown at 37 °C.

Siderophore assays. Chrome Azurol S (CAS) plates were prepared as described by Schwyn & Neilands (1987). For quantitative assays of siderophores produced in liquid cultures, cells were grown in a medium based on MM9 with 0·1 M Tris buffer (Schwyn & Neilands, 1987), supplemented with Casamino acids (final concn 0·3%), glucose (final concn 0·4%) and a trace element solution (pH 7·0) which, at 100× strength, contained (l^{-1}) 50 mg ZnO, 10 mg $CuCl.2H_2O$, 10 mg $CoCl_2.6H_2O$, 10 mg H_3BO_3, 17 mg $MnSO_4.7H_2O$ and 24·6 g $MgSO_4.7H_2O$. Cells were removed by centrifugation at an OD_{600} of about 1 (in 1 cm cells in a Beckman DU650 spectrophotometer), or at the maximum yields attainable in

Table 1. Strains and plasmid used in this study

Strain or plasmid	Relevant genotype	Reference or source
***E. coli* strains**		
AN2342	F⁻; referred to as wild-type	Poole *et al.* (1989)
AN2343	F⁻ *cydD1*	Poole *et al.* (1989)
AN2847	*aroB*351	A. J. Pittard
AN346	*entA*403	Gibson *et al.* (1977)
BW831	*soxS3*::Tn*10* Δ(*argF–lac*) *rpsL*⁺sup(am)	Tsaneva & Weiss (1990)
DH5α	F⁻ φ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA–argF*)U169	Bethesda Research Laboratories
CG05	Φ(*cydC–lacZ*) Km^R	Georgiou *et al.* (1987)
ECL585	*arcA*1*zjj*::Tn*10*	Iuchi & Lin (1988)
ECL933	Φ(*cyo–lacZ*) *bla*⁺ *cyo*⁺	Iuchi *et al.* (1990)
ECL942	Φ(*cyd–lacZ*) *bla*⁺ *cyd*⁺	Iuchi *et al.* (1990)
GK100	Δ*cyo*::kan Δ*cyd*::Cm^R	Lemieux *et al.* (1992)
GL101	*cyo sdhC*::kan	Lemieux *et al.* (1992)
GS015	*rpoS*::Tn*10*	Altuvia *et al.* (1994)
GS09	*oxyR*::kan	Altuvia *et al.* (1994)
JD215	*cydD*::mini-Tn*10*	Delaney *et al.* (1992)
JM2267	*gorA*::Mu*cts*	Davis *et al.* (1982)
MG1655	F⁻; referred to as wild-type	Laboratory collection
QC1732	*fur*::Tn*5*	Touati *et al.* (1995)
RKP3524	As AN2342 but *cydD*::mini-Tn*10*	P1 (JD215) × AN2342
RKP3527	As AN2342 but *cydC*	P1 (CG05) × AN2342
RKP3528	As RKP3532 but spontaneous revertant resistant to extracellular factor	This work
RKP3532	As AN2342 but *cydAB*	P1 (UNF3502) × AN2342
RKP3622	AN2343 *cydD1*/pRG110	This work
RKP3623	RKP3532 *cydAB*/pRG110	This work
RKP3663	As AN2342 but *oxyR*	P1 (GS09) × AN2342
RKP3664	As AN2342 but *katEG*	P1 (UM255) × AN2342
RKP4152	Δ*ubiCA*::kan	Søballe & Poole (1998)
TA4315	*ahp*Δ*5*	Storz *et al.* (1989)
UM262	*katG2*::Tn*10*	P. Loewen
UNF3502	Δ*cydAB*::kan	D'mello *et al.* (1995)
Plasmid		
pRG110	*cyo*⁺ in pBR322	Au & Gennis (1987)

this medium in the case of the *entA* mutant and the *ubiCA* mutant. The CAS assay solution was prepared as described by Schwyn & Neilands (1987). Glassware was treated with 4 M HCl overnight and afterwards rinsed in distilled water. Siderophore production was determined by mixing 0·5 ml culture supernatant and 0·5 ml CAS assay solution and measuring the absorbance at 630 nm at equilibrium, which was reached after approximately 2 h. A reference was prepared containing uninoculated MM9 medium and CAS assay solution. Siderophore concentrations were determined from a standard curve obtained using purified enterochelin (0·2–12 μM).

Cytochrome assays. For identification and quantification of cytochrome *o*, cells were grown in LB medium (Miller, 1972), supplemented where indicated with EDDHA, to an OD_{600} of 0·9–1·0 (measured after correction for dilution where necessary) in 1 cm cells in a Jenway 6100 spectrophotometer. Harvested cells were washed in 0·1 M potassium phosphate buffer (pH 7·0) and used to record reduced *minus* oxidized and CO difference (CO-reduced *minus* reduced) spectra at room temperature in a custom-built SDB4 dual-wavelength scanning spectrophotometer (University of Pennsylvania School of Medicine Biomedical Instrumentation Group/Current Designs) as described briefly by Eaves *et al.* (1998). Spectra were scanned at about 4·25 nm s^{-1} and a spectral bandpass of 2 nm, and were stored and manipulated on a Macintosh computer. They were analysed using *Soft*SDB (Current Designs) and CA-Cricket Graph III. For quantification of cytochrome *o*, an absorption coefficient of 145 mM^{-1} cm^{-1} was used (Kita *et al.*, 1984) in the Soret region of the CO difference spectrum, measuring between the peak at about 416 nm and the trough near 433 nm (see Results). Cytochrome *d* was quantified in suspensions of intact cells as described by Poole *et al.* (1989). Photodissociation spectra at −100 °C were recorded as described by D'mello *et al.* (1997) at a scan rate of 1·43 nm s^{-1}. In all dual-wavelength scans, the reference wavelength was 500 nm.

Respiration rates. Cells were harvested from cells grown in LB

medium, supplemented where indicated with EDDHA, washed in 0·1 M potassium phosphate buffer (pH 7·0) and suspended to give protein concentrations of about 21 mg protein ml^{-1}. Respiration rates were measured in a Rank Bros Clark-type oxygen electrode at 37 °C. Where indicated, EDDHA was added to respiring cell suspensions in the electrode chamber to give final chelator concentrations of 10 or 50 μM. Inhibition was expressed as a percentage of the rate immediately before chelator addition.

β-Galactosidase and protein assays. Assays were carried out at room temperature, around 21 °C. For β-galactosidase, cell pellets were suspended in 2·5–4 ml Z buffer (Miller, 1972) and stored on ice. Activity was measured in $CHCl_3$- and SDS-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside. Activities are expressed in terms of the OD_{600} of cell suspensions, using the formula of Miller (1972). Each culture was assayed in triplicate; results were confirmed in at least two independent experiments. The protein content of cell suspensions was estimated by the method of Markwell *et al.* (1978).

RESULTS AND DISCUSSION

Effect of the self-produced extracellular factor on the growth of cytochrome *bo′*- and *bd*-defective mutants

We confirmed the report of Macinga & Rather (1996) that *E. coli* produces an extracellular factor that inhibits the aerobic growth of Cyd⁻ (*cydAB* and *cydD*) mutants and also showed that *cydC* mutants and two different *cydD* alleles are sensitive to the extracellular factor (Table 2). Mutants defective in cytochrome *bo′* (strain GL101) were unaffected by this factor (Table 2, legend). Macinga & Rather (1996) demonstrated that cytochrome *bd*-deficient mutants produced the factor that inhibited their own growth. Indeed, strain GK100, defective in both oxidases, also produced the factor (data not shown) but was itself sensitive to the factor (Table 2). Cyd⁻ mutants initially failed to grow in the vicinity of a dense growth of other strains, such as the wild-type strain AN2342 (Fig. 1a) but, after 2–3 d, small colonies appeared near the inhibitory streak. Such spontaneous mutants or variants were isolated in both *cydAB*::kan and *cydD*::Tn*10* genetic backgrounds, and on purification such strains (e.g. RKP3528) proved to have significantly greater resistance in cross-streaking experiments (Table 2). When assayed for cytochrome *d* content, they were still Cyd⁻, spectrally detectable cytochrome *d* being about 0·01% of wild-type levels (Table 2; strain RKP3528).

Mutants defective in cytochrome *bd* are sensitive to H_2O_2 and unable to exit from stationary phase and resume aerobic growth at high temperatures (Delaney *et al.*, 1992, 1993; Siegele & Kolter, 1993; Wall *et al.*, 1992). Goldman *et al.* (1996b) and Siegele *et al.* (1996) have shown that these phenotypes can be corrected by mutations in *arcB*, by overexpression of *cyo* on a multicopy plasmid or by addition of exogenous catalase and reducing agents (ascorbate). None of these restores cytochrome *bd* and it has been hypothesized that derepression of *cyo* or overexpression of cytochrome *bo′* allows this oxidase to substitute functionally for cytochrome *bd* (Wall *et al.*, 1992). However, when *cydD* or *cydAB* mutants were transformed with a multicopy plasmid containing the *cyo* operon (pRG110; strains RKP3622 and RKP3623), inhibition was only partially

Table 2. Effect of an extracellular factor produced by *E. coli* on the growth of various Cyd⁻ mutants of *E. coli*

To test for growth inhibition by the extracellular factor, the method of Macinga & Rather (1996) was used. The factor-producing strain AN2342 was streaked across the top third of an agar plate to achieve dense cell growth after incubation overnight. The test strain was grown overnight in Luria broth and 5 μl was spotted to the plate and streaked at 90 ° to the edge of growth of the factor-producing parental strain. After further overnight incubation, growth inhibition was determined as described in Methods and is given ± SD. The following strains were able to grow in the presence of the extracellular factor excreted by wild-type *E. coli*: GS015 (*rpoS*), BW831 (*soxS*), RKP3663 (*oxyR*), RKP3664 (*katEG*), GL101 (*cyo*), ECL585 (*arcA1*), UM262 (*katG*), TA4315 (*ahpΔ5*), and JM2267 (*gorA*). ND, Not determined.

Test strain	Relevant genotype	Growth inhibition by AN2342 (%)	10^6 × Cytochrome *d* concn [nmol (mg protein)$^{-1}$]
AN2342	Wild-type	5±0·3	44000–70000
AN2343	*cydD1*	44±5·3	2·9
RKP3524	*cydD*::mini-Tn*10*	33±2·8	3·0
RKP3532	*cydAB*	38±4·0	3·0
RKP3527	*cydC*	38±3·3	2·8
GK100	*cyo cyd*	31±5·6	5·3
RKP3622	*cydD*/pRG110	23±1·9	ND
RKP3623	*cydAB*/pRG110	15±0·9	ND
RKP3528	*cydAB* (resistant to factor)	11±1·2	2·9

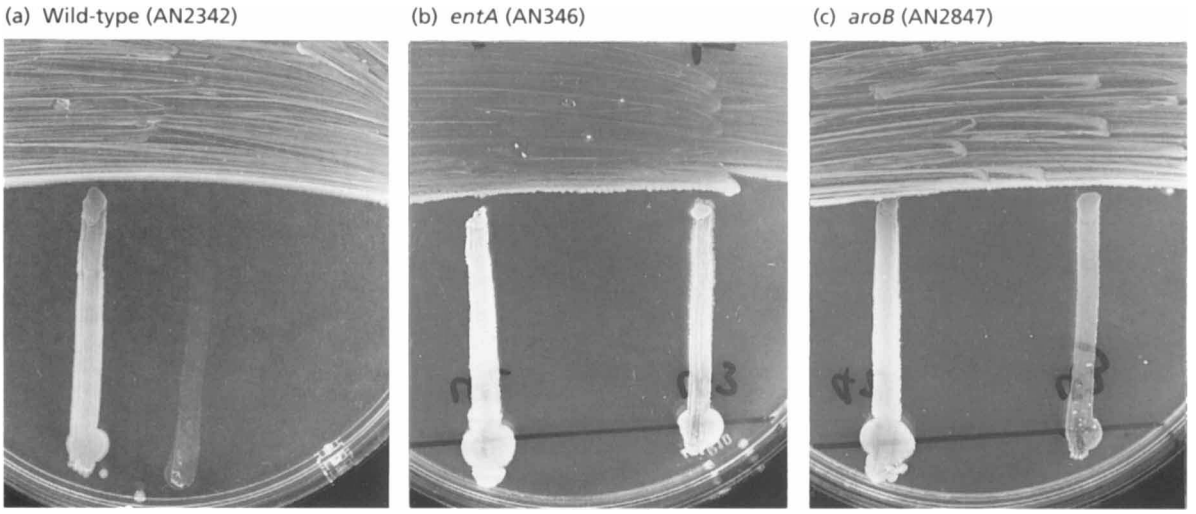

Fig. 1. Effect of a self-produced extracellular factor on the growth of wild-type and Cyd⁻ mutants. Samples containing 10^6 cells of either AN2342 (wild-type, left vertical streak in each panel) or AN2343 (*cydD*, right vertical streak in each panel) were cross-streaked against the following strains, each pre-grown for 12 h: (a) parental strain AN2342, (b) AN346 (*entA*), (c) AN2847 (*aroB*).

relieved (Table 2). Transduction of a mutant *arcA* allele into strain AN2343 (*cydD*) or RKP3532 (*cydAB*::kan) still gave significant growth inhibition (data not shown). Siegele *et al.* (1996) used *arcA* and *arcB* mutations to 'derepress expression of... cytochrome *o* oxidase' but such derepression is only observed in anaerobic growth (Iuchi *et al.*, 1990); therefore, suppression of the stationary-phase defect of *cyd* mutants by such mutations (Siegele *et al.*, 1996) might not be attributable to changes in cytochrome *o* levels, which were not checked. An *arcA1*::Tn*10* strain was not sensitive to the extracellular factor (Table 2, legend).

The extracellular factor is not hydrogen peroxide

Previous work (Georgiou *et al.*, 1987; Delaney *et al.*, 1992; Wall *et al.*, 1992) has shown that Cyd⁻ mutants lose viability in the presence of H_2O_2 in liquid media and therefore it is tempting to speculate, as have Goldman *et al.* (1996b), that the extracellular factor produced on plates is H_2O_2 or some 'oxidative species'. To test this hypothesis, H_2O_2 was added directly to agar plates, in an attempt to mimic the effect of a factor produced in the agar. Cyd⁻ mutants and wild-type strains were all inhibited at about 1 mM H_2O_2 and there was no difference in the sensitivity to H_2O_2 over a wide range of concentrations using our assay methods. Alkyl hydroperoxides (cumene hydroperoxide or tert-butyl hydroperoxide) also inhibited wild-type and Cyd⁻ strains at concentrations of 1·0 mM and above. Thus, neither H_2O_2 nor organic peroxides mimic the effect of the extracellular factor. Strains defective in the oxidative stress regulon, OxyR, or in genes activated by OxyR (*katG*, *katEG*, *ahp*, *gorA*) were tested for their ability to grow in the presence of the extracellular factor; the growth of these strains was unaffected (Table 2, legend). However, both *oxyR* and *katEG* strains were inhibited by very low concentrations (<0·25 mM) of H_2O_2, tert-butyl and cumene hydroperoxides, as expected. Sigma S (σ^S), a stationary-phase-specific sigma factor encoded by *rpoS*, regulates *katG* expression (Ivanova *et al.*, 1994), but an *rpoS* mutant (Table 2, legend) was also resistant to the extracellular factor, supporting the view that H_2O_2 was not the inhibitory factor. In all cases, when resistant strains were transduced with the mutant *cydAB*::kan allele, they became sensitive to the extracellular factor, demonstrating that the Cyd⁻ phenotype confers sensitivity.

Oxidative stress can also be brought about by the addition of superoxide radical generators such as paraquat, menadione or plumbagin (Farr & Kogoma, 1991). Strains defective in *soxS*, required for the activation of the SoxRS superoxide response regulon, were also resistant to the extracellular factor. When either paraquat (0·1 mM) or menadione (2 mM) was added to agar plates, both Cyd⁻ and Cyd⁺ strains were inhibited at the same concentrations (data not shown). Wild-type and Cyd⁻ mutants were also equally sensitive to the respiratory inhibitors juglone and 2-heptyl-4-hydroxyquinoline.

Ascorbate and catalase alleviate growth inhibition by the extracellular factor

Goldman *et al.* (1996b) have shown that exogenous reducing agents (i.e. glutathione and cysteine) and catalase overcome the temperature-sensitive phenotype of Cyd⁻ mutants; these compounds might overcome the inhibition through detoxification of the factor or by suppressing the Cyd⁻ phenotype. When the antioxidant ascorbate was added at 10 mM to plates prior to streaking with the inhibitory (factor-producing) strain,

Table 3. Effect of catalase, superoxide dismutase, peroxidase and ascorbate on the inhibition of growth of Cyd⁻ mutants in the presence of the extracellular factor

Compound or enzyme added*	Growth inhibition by AN2342 (%)†		
	AN2342 (wild-type)	AN2343 (*cydD*)	RKP3532 (*cydAB*)
No addition	4±0·3	42±5·0	38±4·1
Ascorbate	8±1·2	12±4·0	10±3·0
Catalase	3±0·6	10±2·0	5±1·2
Boiled catalase	4+0·3	10±1·0	10±1·1
Superoxide dismutase	6±0·9	35±4·3	28±3·2
Peroxidase	7±1·2	40±6·0	25±4·0

* All additions were made to nutrient agar plates prior to streaking with factor-producing strain. The final concentrations of additions were as follows: ascorbate, 10 mM; catalase, 0·3 mg ml^{-1}; superoxide dismutase, 3·6 μg ml^{-1}; peroxidase, 34 μg ml^{-1}.

† Growth inhibition tests were performed as described for Table 2.

Table 4. Effect of iron-chelating agents on the growth of wild-type and Cyd⁻ mutants

The test strains were grown overnight in Luria broth and 5 μl was spotted to the plate and streaked using a sterile tooth pick. All test strains were corrected to the same optical density before addition and grown for 12 h on nutrient agar plates aerobically. Compounds were dissolved in water and 50 μl spread onto the surface of the plate; concentrations shown assume even distribution through 20 ml agar, except for EDDHA, which was added as a filter-sterilized solution immediately before pouring plates. Inhibition was assessed by estimating lawn density by eye.

Chelator*	log stability constant†		Chelator concn (mM) required to inhibit growth by approx. 50% for strain:			
	Fe(III)	Fe(II)	AN2342	AN2343 (*cydD*)	RKP3532 (*cydA*)	RKP3528 (*cydAB*; resistant)
EDDHA	33·9	14·3	2	0·03	ND	ND
DTPA	28·6	–	0·3	0·05	0·05	0·25
EDTA	25·1	14·3	0·1	0·1	0·1	0·1
SSA	14·1	–	>0·3	0·2	0·2	>0·3
2,2′-Dipyridyl	–	17·6	0·25	0·05	0·05	0·20

* DTPA, diethylenetriaminepentanoic acid; SSA, 5-sulfosalicylic acid

† Values from Pirt (1975) and Dawson *et al.* (1986).

inhibition by the extracellular factor was reduced fourfold (Table 3). The same result was observed in spent liquid medium in which sensitive cells were suspended (not shown).

Because mutants defective in OxyR or SoxS were resistant to the extracellular factor (Table 2), it did not seem likely that the factor was H_2O_2 or superoxide. However, the addition of catalase (bovine liver, Sigma C9322) at high concentrations did reduce the growth inhibition. Remarkably, the same result was observed if the catalase was boiled (Table 3). Superoxide dismutase (Sigma C8254) and horseradish peroxidase (Sigma P6140) slightly reduced the level of inhibition in a *cydAB* mutant, but not in a *cydD* mutant (Table 3).

Iron-chelating agents mimic the effect of the extracellular factor

Because boiling catalase releases haem and iron, we investigated the ability of iron to overcome the inhibition by the extracellular factor. When $FeCl_3$ (0·05–1·0 mM) was incorporated into agar plates prior to streaking the factor-producing strain, there was a corresponding increase in the growth of Cyd⁻ mutants. At 0·2 mM $FeCl_3$, there was a 90% decrease in the inhibition by the extracellular factor (not shown), suggesting that the inhibition of growth could be due to iron availability, and that the excreted factor may affect the bioavailability of iron in the agar plates prior to growth of the test strain.

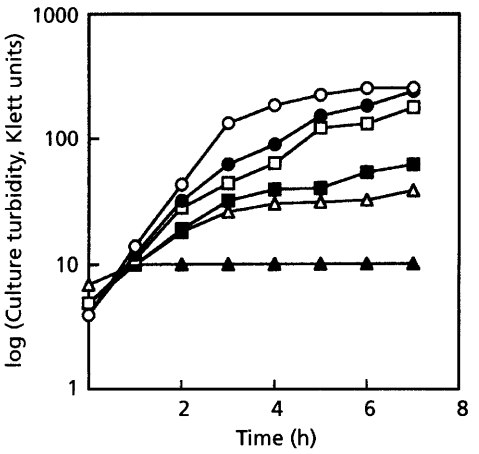

Fig. 2. Effect of EDDHA on aerobic growth of a *cydD* mutant. Strain AN2343 was grown in LB medium in 10 ml portions in side-arm flasks and growth measured using a Klett colorimeter. EDDHA concentrations were: none (control, ○), 2 μM (●) 3 μM (□), 4 μM (■), 5 μM (△), 10 μM (▲). Such concentrations were without effect on the wild-type strain AN2342 (not shown).

We therefore tested the ability of various iron-chelating agents to mimic the extracellular factor (Table 4). The most effective chelator of those tested was EDDHA, which has the highest stability constant for the Fe(III) complex; the stability constant for the Fe(II) complex is similar to that of EDTA. EDDHA inhibited the growth of Cyd⁻ mutants at about 3–5 μM in liquid medium (Fig. 2) and 30 μM in agar (Table 4). The higher concentrations required in agar may reflect metal contaminants in the agar. Concentrations almost 100-fold greater were required to inhibit growth of Cyd⁺ strains. Inclusion of 100 μM EDDHA in nutrient agar plates provides a clear-cut, useful test for the Cyd⁻ phenotype. Diethylenetriaminepentanoic acid (DTPA), another Fe(III) chelating agent, inhibited the growth of Cyd⁻ mutants at 0·05 mM but the wild-type was unaffected by >0·2 mM. 5-Sulfosalicylic acid (SSA) also chelates Fe(III), but with much lower affinity (Dawson *et al.*, 1986). This chelating agent did not totally inhibit the growth of Cyd⁻ mutants, but there was 50% inhibition at 0·2 mM, and Cyd⁺ cells were relatively resistant to this compound. A factor-resistant isolate (RKP3528) showed enhanced resistance to iron chelators (Table 4).

2,2′-Dipyridyl, an effective and widely used Fe(II)-chelating agent, partially inhibited the growth of Cyd⁻ mutants in a concentration-dependent manner. Both RKP3532 (*cydAB*) and AN2343 (*cydD*) mutants were inhibited by low concentrations (approx. 0·05 mM) of 2,2′-dipyridyl. Inhibition of wild-type cells was not observed until the concentration was greater than 0·2 mM (Table 4). The spontaneous mutant (strain RKP3528) isolated in the presence of the extracellular factor was also relatively resistant to low concentrations of 2,2′-dipyridyl and inhibited only at about 0·2 mM. Other metal-chelating agents, i.e. sodium citrate and EDTA (Table 4), did not show a differential inhibition of growth between wild-type cells and Cyd⁻ mutants.

Macinga & Rather (1996) showed that the inhibition of Cyd⁻ mutants by the extracellular factor was abolished by anaerobiosis. This was also true for growth inhibition by EDDHA and 2,2′-dipyridyl (not shown).

Enterochelin inhibits the growth of cytochrome *bd*-deficient mutants

Many bacteria, including *E. coli*, produce siderophores in response to low iron availability (Earhart, 1996). Enterochelin is a catecholate siderophore that is produced by *E. coli* at levels up to 100–200 mg (l medium)$^{-1}$ (Earhart, 1996). We therefore tested the ability of pure enterochelin to inhibit the growth of Cyd⁻ mutants. When enterochelin was added to absorbent discs that were overlaid on a lawn of strain AN2342 cells, no growth inhibition of these wild-type cells was observed (Fig. 3). However, on a lawn of AN2343 (*cydD*) (Fig. 3) or RKP3532 (*cydAB*) (not shown), there was a zone of clearing around the disc containing enterochelin, demonstrating that the growth of Cyd⁻ mutants was inhibited. This effect was concentration dependent, and control discs (having a volume of acetone equal to the amounts used for the enterochelin) produced no growth inhibition (Fig. 3).

These results demonstrate that enterochelin inhibits the growth of Cyd⁻ mutants and so we tested whether strains defective in the production of enterochelin were also able to produce the inhibitory factor. Mutants in *entA*, encoding 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (strain AN346), or *aroB*, encoding 3-dehydroquinate synthase (strain AN2847), both gene products being required for the production of enterochelin, were tested for their ability to produce the factor (Fig. 1). Cyd⁻ mutants cross-streaked against AN346 grew right up to the factor-producing strain and no growth inhibition was noted (Fig. 1b). Identical results were observed when Cyd⁻ mutants were cross-streaked against an *aroB* mutant (strain AN2847), i.e. no growth inhibition was observed (Fig. 1c) in marked contrast to Fig. 1(a).

Because a mutation in *entA* will cause a block in the metabolic pathway leading to enterochelin biosynthesis, we tested the ability of various intermediates in this pathway to inhibit the growth of Cyd⁻ mutants. Neither 2,3-dihydroxybenzoic acid, chorismic acid nor 2,3-dihydro-2,3-dihydroxybenzoic acid inhibited the growth of Cyd⁻ mutants (data not shown).

Siderophores are low-molecular-mass (approx. 1000 Da), non-proteinaceous, heat-stable compounds (Neilands, 1984; Schwyn & Neilands, 1987). Preliminary characterization of the extracellular factor from spent medium indicated that it was dialysable against distilled water using a molecular mass cut-off of 1000 Da, and resistant to boiling (100 °C for 20 min, pH 7·0) and protease treatment. Thus, the factor had properties consistent with those of a siderophore.

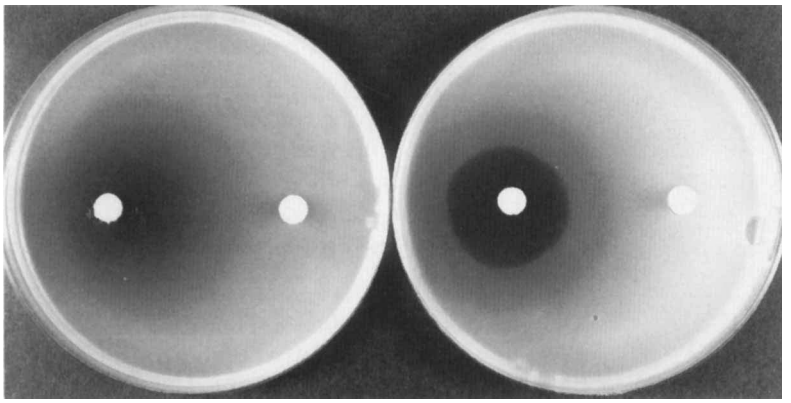

Fig. 3. Effect of purified enterochelin on the growth of the wild-type strain AN2342 (left plate) and Cyd⁻ mutant AN2343 (*cydD*, right plate). An enterochelin stock solution (10 mM; 50 µl) was applied directly onto a pre-sterilized antibiotic disc (left side of each plate) that was overlaid on a lawn of cells, and the plates were incubated overnight. Enterochelin is a coloured compound and therefore a diffuse darkness is observed around the discs. An equal volume of acetone was added to the control disc that is shown on the right side of each plate.

Table 5. Effects of ascorbate and trivalent cations on the inhibition of Cyd⁺ and Cyd⁻ strains by the extracellular factor

Addition to plates (final concn, mM)			Inhibition (%) by strain AN2342 of growth of:	
Ascorbate	In(III)	Ga(III)	AN2342 (Cyd⁺)	AN2343 (Cyd⁻)
–	–	–	0	47
1	–	–	0	46
3	–	–	0	42
–	–	1	0	38
–	–	3	0	0
1	–	1	0	14
3	–	1	0	0*
–	1	–	0	50
–	3	–	NG	NG
1	1†	–	0	15
3	1	–	0	0

NG, No growth.

* Growth inhibition was also completely relieved by 3 mM Ga(III) plus 3 mM ascorbate.

† Inclusion of 3 mM In(III) with either 1 mM or 3 mM ascorbate did not permit growth of either strain.

Gallium, indium and ascorbate alleviate the growth-inhibitory properties of the extracellular factor

Further evidence that a siderophore was responsible for the inhibition of Cyd⁻ mutants was provided by the use of gallium [Ga(III)], which forms a complex with siderophores and therefore is able to compete with Fe(III) (Ecker & Emery, 1983; Hubbard *et al.*, 1986; Emery, 1987; Rogers, 1987). Touati (1988) used 0·1 mM Ga(III) to amplify the effects of Fe(II) chelators on *E. coli*. Gallium nitrate was added to agar plates at concentrations ranging from 0·5 mM to 5·0 mM and the plates then streaked with the factor-producing cells and grown overnight. Cyd⁻ mutants were cross-streaked and again grown overnight. With increasing concentrations of Ga(III), there was a decrease in the amount of growth inhibition of the Cyd⁻ mutants (Table 5). At 1 mM Ga(III), inhibition of Cyd⁻ mutants in the presence was measurably reduced and at 3 mM Ga(III), no inhibition of Cyd⁻ mutants by the factor remained.

Since siderophores are markedly more effective at binding Fe(III) than Fe(II) (Emery, 1987), we reasoned that Ga(III) would compete more effectively with iron for excreted siderophores if Fe(III) were reduced to Fe(II). Indeed, displacement of Fe(III) from trihydroxamate ligands by Ga(III) can be pushed to completion with ascorbate as reductant and ferrozine as Fe(II)-trapping agent (Emery, 1986). In the experiments to be described, cellular uptake of Fe(II) may suffice as an Fe(II) trap. Inclusion of ascorbate and Ga(III) together, each at 1 mM, almost completely alleviated inhibition of the *cydD* mutant (Table 5) and were significantly more effective than either of these compounds alone at these concentrations. When the concentration of ascorbate was increased to 3 mM in the presence of 1 mM Ga(III), total abolition of inhibition was obtained, whereas 3 mM ascorbate alone had no measurable effect (Table 5).

Indium [In(III)] has also been reported to bind to siderophores, and the indium chelate, when added to exponential cultures of *E. coli*, induces a large increase in mean generation time (Rogers *et al.*, 1980). At 1 mM, In(III) was without effect on the inhibition of the *cydD* mutant; at 3 mM it inhibited both the mutant and the wild-type (Table 5). However, in the additional presence of 3 mM ascorbate, 1 mM In(III) was highly effective at alleviating inhibition by the extracellular factor. These experiments strongly support the evidence that the factor is a siderophore: trivalent metal cations compete with Fe(III) for binding, particularly when Fe(III) is reduced by ascorbate and thus made unavailable for binding by siderophores.

Are the effects of iron and iron-chelating agents due to altered expression and assembly of cytochrome *bo′*?

We tested whether the aerobic growth deficiency of Cyd⁻ mutants might be a consequence of depression by low iron levels of cytochrome *bo′* synthesis. Growth of

Table 6. Respiration and cytochrome *o* content of strain AN2343 (*cydD*) grown in the presence of EDDHA

EDDHA (final concn, μM)	O_2 uptake rate [nmol O_2 min^{-1} (mg protein)$^{-1}$]	Cytochrome *o* concn [nmol (mg protein)$^{-1}$]	Absorbance maxima, minima in CO spectra (nm)	Cytochrome *o* in photodissociation spectra
0	129	0·082	417, 433	+
3	123	0·075	416·5, 432	NA
4	122	0·064	415, 435	NA
50	108	0·074	415, 434·5	+

NA, Not assayed.

Table 7. Assay of siderophore production in wild-type *E. coli*, *cyd* and other mutant strains

Strain	Relevant genotype	OD_{600} of culture at harvest	Siderophore concn (μM)	Siderophore (μM per OD unit)	Siderophore concn (% of relevant wild-type)
AN2342	Wild-type	0·96	1·8	1·9	100
RKP3532	*cydAB*	0·96	2·8	2·9	156
AN2343	*cydD*	0·96	3·4	3·5	189
MG1655	Wild-type	0·96	4·3	4·5	100
RKP4152	*ubiCA*	0·49	1·1	2·3	50
QC1732	*fur*	0·96	7·8	8·1	–
AN346	*entA*	0·04	0	0	–

–, Values not expressed as percentage of F^- wild-type.

the *cydD* mutant in the presence of increasing concentrations of EDDHA (Fig. 2) resulted in dose-dependent inhibition of growth. We examined respiration of cells harvested from such cultures to determine if the activity of the residual oxidase was inhibited by the chelator. Table 6 shows that even in cells whose growth was severely slowed by 4 or 5 μM EDDHA, respiration was little affected. Furthermore, addition of much higher growth-inhibitory concentrations of EDDHA to non-proliferating cell suspensions in the oxygen electrode chamber produced no immediate inhibition of respiration. For example, 10 μM EDDHA inhibited respiration by 4·5% (SD 3·7, four determinations) and 50 μM EDDHA inhibited respiration by 4·7% (mean of two determinations). Thus the inhibition of growth of mutant cells by the chelator cannot be attributed to loss of oxidase activity.

Similar cell suspensions were also examined for their cytochrome content. Table 6 shows the results of cytochrome *o* quantification by CO difference spectroscopy; in all cases the spectra revealed a component with the characteristic band of the CO adduct at 415–417 nm (Poole, 1983) and a trough of variable width but at positions close to those anticipated for the reduced, unligated form (432–435 nm). Even at the highest EDDHA concentrations, cytochrome *o* synthesis was unaffected. Because CO difference spectra could conceivably include contributions from non-oxidase, but CO-binding, haem proteins, we performed photodissociation spectra at subzero temperatures. Photodissociation spectra, i.e. photolysed (reduced) *minus* unphotolysed (CO-ligated) revealed, in both control cells (no EDDHA) and cells grown with 5 μM EDDHA, a trough at 413–414 nm and a peak at 432–433 nm. These features are absolutely characteristic of cytochrome *o* in *E. coli* (Poole, 1983). Although such low-temperature spectra are not well suited to precise quantification, no obvious differences in the cytochrome *o* content of control or EDDHA-grown cells were evident.

Similar results were obtained with 2,2′-dipyridyl. CO difference spectra of strain AN2343 (*cydD*) confirmed that the levels of cytochrome *bo′* were also similar in the presence and absence of 2,2′-dipyridyl (data not shown). 2,2′-Dipyridyl had no effect on the oxygen consumption rate of Cyd⁻ mutants, the expression of Φ(*cydA–lacZ*)

(in strain ECL942), or the level of Φ(*cyo–lacZ*) expression in strain ECL933 (data not shown). When a *cydAB*::kan allele was transduced into this strain, there was still no effect on the expression of Φ(*cyo–lacZ*) in the presence of 2,2′-dipyridyl. Based on these observations, neither the expression nor activity of cytochrome *bo* is affected by low iron.

Do Cyd⁻ mutants make siderophores?

Preliminary experiments on CAS plates indicated that various *cyd* mutants make a siderophore, so we performed quantitative assays on culture supernatants to test the hypothesis that the inability of *cyd* mutants to grow in the presence of siderophore or other iron chelators was due to the inability of such mutants to excrete their own siderophores and compete with exogenous chelators. Siderophore concentrations, determined according to the method of Schwyn & Neilands (1987) were expressed per unit of culture OD at the time of harvest (Table 7); the results showed that both *cydD* and *cydAB* mutants not only synthesized siderophore but excreted it at levels in excess of those detected in supernatants from wild-type cultures.

We also examined the ability of a *ubi* mutant to make the factor, since the factor is not made or is ineffective anaerobically and such mutants grow very poorly under these conditions. A knockout *ubiCA* mutant produced about half the level of siderophore as the appropriate wild-type control strain, but comparisons are difficult because of the poor growth of the mutant. In marked contrast, and as controls, a *fur* mutant greatly overproduced siderophore and an *entA* mutant produced barely detectable levels.

Are extracellular iron chelators involved in quorum sensing?

While this work was in progress, Surette & Bassler (1998) described a small (< 1000 Da), heat-labile organic molecule capable of causing luminescence in a quorum-sensing reporter strain of *Vibrio harveyi*. Whilst certain aspects of that extracellular factor (e.g. its lack of production in LB medium plus glucose, and its destruction in stationary phase) suggest that this molecule is not enterochelin, we took advantage of the observation that *E. coli* strain DH5α does not make this soluble factor. We argued that if the factor reported by Surette & Bassler (1998) is enterochelin, strain DH5α should not be able to grow in the presence of EDDHA. However, strain DH5α did grow on nutrient agar plates supplemented with 100 μM EDDHA (not shown).

Conclusions

This work reveals another facet of the surprisingly complex phenotype of cytochrome *bd* mutants, namely sensitivity to low iron concentrations. The consequences of iron deficiency on microbial growth and metabolism are diverse, and include alterations of metabolism, particularly involving iron-containing enzymes, effects on toxin production, enhancement of secondary metabolic production and restriction of growth of pathogenic micro-organisms in serum (Hughes & Poole, 1989). There is much evidence for the preferential loss of iron-containing enzymes in micro-organisms provided with an inadequate iron supply. In aerobic and facultative bacteria, levels of catalase, peroxidase and cytochromes are reduced markedly and aerobic pathways are suppressed, with a shift to anaerobic glycolysis to meet energy requirements (Hubbard *et al.*, 1986). In the present work, the degree of iron deprivation experienced by Cyd⁻ mutants was insufficient to prevent haem protein synthesis.

Enterochelin, EDDHA and, to a lesser extent, other iron chelators are effective inhibitors of the growth of Cyd⁻ mutants. The molecular basis of this is not understood but the present data suggest that it is not loss of the quinol oxidase activity of cytochrome *bd per se* that is responsible for the growth phenotype. In chelator-inhibited cultures, respiration rates and cytochrome levels are virtually unaffected and EDDHA concentrations that totally inhibit growth do not inhibit respiration. It should be noted that even double oxidase mutants (*cyo cyd*) having neither oxidase and almost unmeasurable respiration rates will grow to give small colonies on the rich media used here (T. M. Stevanin & R. K. Poole, unpublished).

The physiological basis of this effect is not understood. Although the inhibition of Cyd⁻ mutants by an extracellular factor from *E. coli* is mimicked by enterochelin and, less dramatically, by other iron chelators, we cannot rule out the possibility that other factors contribute to the growth inhibition. Since the availability of iron to bacterial pathogens has a decisive role in the outcome of infectious diseases (Byers & Arceneaux, 1998), we anticipate that elucidation of the molecular basis of growth inhibition at low iron concentrations will have important implications for *E. coli* and perhaps *Salmonella* pathogenesis. These results and the recent discovery of quorum-sensing molecules in enterobacteria (Surette & Bassler, 1998, and references therein) emphasize the important role of small, hitherto-unrecognized or 'rediscovered' extracellular molecules in interactions between bacteria.

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